Enzymological studies on bacterial aldehyde dehydrogenases
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Enzymological studies on bacterial aldehyde dehydrogenases

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LUYKX

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One love
One blood
One life
You got to do what you should

One life
With each other
Sisters
Brothers

One life
But we’re not the same
We get to carry each other
Carry each other

One

("One" - U2)
Aan mijn ouders & Pauline
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General introduction

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Abbreviations. AIDH, aldehyde dehydrogenase from Comamonas testosteroni; AIDHs, aldehyde dehydrogenases; ALOR, aldehyde oxidoreductase; AMP, adenosine 5’-phosphate; AO, aldehyde oxidase; AOR, aldehyde ferredoxin oxidoreductase; ATP, adenosine 5’-triphosphate; CMP, cytidine 5’-phosphate; CoA, coenzyme A; DL-AIDH, (dye-linked) aldehyde dehydrogenase from Amycolatopsis methanolicola; DMS, dimethylsulphide; DMSOR, dimethylsulfoxide reductase; DNA, deoxyribonucleic acid; ECAO, aromatic amine oxidase from Escherichia coli; EPR, electron paramagnetic resonance spectroscopy; ESR, electron spin resonance spectroscopy; EXAFS, extended X-ray absorption fine structure; FAD, flavin-adenine dinucleotide; FADH’, flavosemiquinone; FAID, formaldehyde dismutase; FAIDH, formaldehyde dehydrogenase; FEDH, formate ester dehydrogenase from Amycolatopsis methanolicola; FOR, formaldehyde ferredoxin oxidoreductase; GAPOR, glyceraldehyde-3-phosphate ferredoxin oxidoreductase; GMP, guanosine 5’-phosphate; GSH, glutathione; GTP, guanosine 5’-triphosphate; HMP, hypoxanthine monophosphate; HPLC, high-performance liquid chromatography; IEF, isoelectric focussing; ISFET, ion-sensitive field-effect transistor; MCD, molybdopterin cytosine dinucleotide; MGD, molybdopterin guanine dinucleotide; MNO, methanol:NDMA oxidoreductase; Moco, pterin molybdenum cofactor; MPT, molybdopterin; MySH, mycothiol; NAD-AIDH(s), NAD(P)-dependent aldehyde dehydrogenase(s); NAD(P), nicotinamide-adenine dinucleotide (phosphate) and its oxidized and reduced forms; NDMA, p-nitroso-N,N-dimethylaniline; NMR, nuclear magnetic resonance spectroscopy; PAD, phenylacetaldehyde dehydrogenase from Escherichia coli; PAGE, polyacrylamide gel electrophoresis; PMS, phenazine methosulfate; PMSF, phenylmethanesulfonylfluoride; PQQ, pyrroloquinoline quinone; SDS, sodium dodecyl sulfate; SO, sulfite oxidase; TPQ, topaquinoine; TTQ, tryptophyl-tryptophanquinone; UV/Vis, ultraviolet/visible; Wco, pterin tungsten cofactor; XDH, xanthine dehydrogenase; XO, xanthine oxidase.
1. Aldehydes in the environment

In our environment many different aldehydes are present. Formaldehyde, acetaldehyde and acrolein are products of combustion and are present in smog and cigarette smoke [1]. Concentrations of acetaldehyde in smoky atmospheres may reach 0.1 ppm [2]. Acetaldehyde is also an important industrial chemical. Industrial emissions of acetaldehyde in 1993, during the production of several chemicals, were estimated to exceed 4,500,000 kg [3]. Furthermore, many foods, especially fruits and vegetables, are sources of aldehydes, including a range of aliphatic and aromatic ones [4]. These aldehydes are responsible for the flavors and odors of foods and beverages.

Aldehydes can function as communication molecules [4]. They may act as information-transmitting molecules, either between species or within a species. These aldehydes derive from the metabolism of other compounds. They can be generated from a large number of endogenous and exogenous sources [4,5]. Endogenous sources include amino acids, biogenic amines, carbohydrates, vitamins, steroids, and lipids (Table 1). The major exogenous source of aldehydes are the xenobiotics. The biotransformation of a large number of drugs and of other xenobiotics generates aldehydes (Table 2). Some of the direct reactions that can produce aldehydes include oxidative deaminations and dealkylations, as well as the oxidation of primary alcohols [4,6-19].

Table 1. Generation of aldehydes in metabolic/anabolic pathways.

<table>
<thead>
<tr>
<th>Source</th>
<th>Aldehyde</th>
</tr>
</thead>
<tbody>
<tr>
<td>Threonine catabolism</td>
<td>acetaldehyde</td>
</tr>
<tr>
<td>Putrescine catabolism</td>
<td>γ-aminobutyraldehyde</td>
</tr>
<tr>
<td>Choline catabolism</td>
<td>betaine aldehyde</td>
</tr>
<tr>
<td>Corticosteroid catabolism</td>
<td>21-dehydrocorticosteroids</td>
</tr>
<tr>
<td>Dopamine catabolism</td>
<td>3,4-dihydroxyphenylacetaldehyde</td>
</tr>
<tr>
<td>Proline biosynthesis</td>
<td>glutamic-γ-semialdehyde</td>
</tr>
<tr>
<td>Lipid peroxidation</td>
<td>hexanal, 4-hydroxynonenal, malondialdehyde</td>
</tr>
<tr>
<td>Serotonin catabolism</td>
<td>5-hydroxyindoleacetaldehyde</td>
</tr>
<tr>
<td>Vitamin A metabolism</td>
<td>retinal</td>
</tr>
<tr>
<td>GABA shunt</td>
<td>succinic semialdehyde</td>
</tr>
</tbody>
</table>
Aldehydes may have a variety of effects on biological systems. They can diffuse or be transported from their site of generation to other sites, for example, to another cell compartment, another cell, or even another tissue. The high reactivity of the aldehydes is due to the electrophilic nature of their carbonyl group causing reactions with cellular nucleophiles, including nucleic acids and proteins [4,20]. Therefore, most aldehydes exhibit important biological effects such as cytotoxicity, mutagenicity, genotoxicity, and carcinogenicity [1,4,5].

Table 2. Examples of exogenous sources of aldehydes.

<table>
<thead>
<tr>
<th>Source</th>
<th>Aldehyde</th>
</tr>
</thead>
<tbody>
<tr>
<td>Combustion (smog, cigarette smoke)</td>
<td>formaldehyde, acetaldehyde, acrolein</td>
</tr>
<tr>
<td>Ethanol</td>
<td>acetaldehyde</td>
</tr>
<tr>
<td>2-Butoxyethanol</td>
<td>butoxyaldehyde</td>
</tr>
<tr>
<td>Diethylnitrosamine</td>
<td>acetaldehyde</td>
</tr>
<tr>
<td>N-Butyl-N-(4-hydroxy-butyl)nitrosamine</td>
<td>N-butyl-N-(propyl-3-aldehyde)nitrosamine</td>
</tr>
<tr>
<td>Succinylcholine</td>
<td>betaine aldehyde</td>
</tr>
<tr>
<td>Benzene</td>
<td>trans, trans-muconaldehyde</td>
</tr>
<tr>
<td>Toluene</td>
<td>benzaldehyde</td>
</tr>
<tr>
<td>Xylene</td>
<td>tolualdehyde</td>
</tr>
<tr>
<td>Laetrile</td>
<td>benzaldehyde</td>
</tr>
<tr>
<td>Codeine</td>
<td>formaldehyde</td>
</tr>
<tr>
<td>Phenacetin</td>
<td>acetaldehyde</td>
</tr>
<tr>
<td>Cyclophosphamide</td>
<td>aldophosphamide</td>
</tr>
<tr>
<td>Nicotine</td>
<td>γ-3-pyridyl-γ-methylaminobutyraldehyde</td>
</tr>
</tbody>
</table>

Although aldehydes are highly reactive, not all of their interactions with biological systems should be considered deleterious. For example, retinoic acid, the oxidation product of retinal, is involved in embryonic differentiation, and retinal itself is required for vision [21]. In addition, certain aldehydes generated by membrane lipid peroxidation may be chemotactic, recruiting cells to sites of injury or inflammation [22].

A variety of enzymes have evolved to convert aldehydes to less reactive forms. Among the most effective pathways for aldehyde metabolism is their oxidation or reduction to the corresponding carboxylic acid or alcohol, respectively, by aldehyde oxidoreductases [20].
2. Different types of aldehyde oxidoreductases

The last two decades have been exciting decades for researchers studying the family of aldehyde dehydrogenases (AIDHs). Until that time AIDHs were known mainly for their role in acetaldehyde oxidation. Although several different mammalian AIDHs were known, there was little understanding of the physiological roles played by the various enzymes. Nowadays, we know that the role in ethanol metabolism is only one of several important physiological functions for these AIDHs. It is clear that AIDHs are responsible for the metabolism of a wide variety of aldehydes [23].

In mammals, aldehydes can be metabolized via three different enzyme types: aldehyde oxidases (AOs), aldo-keto reductases [24,25] and NAD(P)-dependent AIDHs (NAD-AIDHs) (Table 3). All three types are widespread and representatives can be found in virtually each tissue. In addition, they have broad and sometimes overlapping substrate preferences [20].

The variety of types of aldehyde converting enzymes in microbes is much larger than in mammals, although the latter have many isoenzymes which have not been found so far in microbes [26]. Microbial aldehyde oxidation proceeds via NAD(P)-independent AIDHs, such as haemoprotein/quinoprotein AIDHs, molybdoprotein AIDHs and tungstoprotein AIDHs, and via NAD-AIDHs and aldehyde dismutases (nicotinoprotein alcohol/aldehyde oxidoreductases) (Table 3). Most bacterial AIDHs are so-called ‘soluble’ enzymes and are located in the cytoplasm, but some have been shown to be membrane-bound.

2.1 Aldehyde oxidases

Aldehyde oxidase (AO) is a cytosolic molybdoprotein which occurs in eukaryotes. Its molecular mass is approximately 300 kDa and it consists of two identical subunits each containing molybdenum in the form of a pterin molybdenum cofactor (Moco) (see also Fig. 1B), flavin adenine dinucleotide (FAD), and a pair of iron-sulphur clusters. It converts the aldehyde to the corresponding acid and hydrogen peroxide. Under certain conditions AO can catalyze the formation of superoxide radicals. According to its cofactor content, molecular mass, amino acid sequence and substrate specificity the enzyme is very similar to xanthine oxidase (XO) [27] (see section 3.5) although some differences exist. AO, for example, does not use NAD or NADP as
Table 3. An overview of the different aldehyde oxidoreductases existing in nature. AAIDH, acetaldehyde dehydrogenase; ALOR, aldehyde oxidoreductase; AOR, aldehyde ferredoxin oxidoreductase; BAIDH, betaine aldehyde dehydrogenase; BV-AIDH, benzylviologen-linked aldehyde dehydrogenase; DL-AIDH, (dye-linked) aldehyde dehydrogenase; FAID, formaldehyde dismutase; FAIDH, formaldehyde dehydrogenase; FD-FAIDH, NAD/ factor(MySH) dependent formaldehyde dehydrogenase; FEDH, formate ester dehydrogenase; FOR, formaldehyde ferredoxin oxidoreductase; GAPOR, glyceraldehyde-3-phosphate ferredoxin oxidoreductase; GD- FAIDH, NAD/GSH dependent formaldehyde dehydrogenase; MNO, methanol:NDMA oxidoreductase; NDMA- ADH, NDMA-dependent alcohol dehydrogenase; PAD, phenylacetaldehyde dehydrogenase; RBAO, rabbit liver aldehyde oxidase; TAO1, tomato aldehyde oxidase.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Coenzyme</th>
<th>Cofactor</th>
<th>Source</th>
<th>Ref.</th>
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<td></td>
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</tr>
<tr>
<td>RBAO</td>
<td>Mo, Fe/S, FAD, Moco</td>
<td>Rabbit</td>
<td>245</td>
<td></td>
</tr>
<tr>
<td>TAO1</td>
<td>Mo, Fe/S, FAD, Moco</td>
<td>Tomato</td>
<td>246</td>
<td></td>
</tr>
<tr>
<td><strong>NAD(P)-independent</strong></td>
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<td></td>
<td></td>
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<tr>
<td><strong>AIDHs</strong></td>
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<tr>
<td><strong>Haemoproteins/ quinoproteins</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AIDH</td>
<td>Haem c</td>
<td>Methylosinus trichosporium</td>
<td>33</td>
<td></td>
</tr>
<tr>
<td>AAIDH</td>
<td>Haem b, c, Fe/S, Moco</td>
<td>Acetobacter europaicus</td>
<td>34</td>
<td></td>
</tr>
<tr>
<td>FAIDH</td>
<td>quinone</td>
<td>Hyphomicrobium zavarzinii</td>
<td>36</td>
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<tr>
<td><strong>Molybdoproteins</strong></td>
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<tr>
<td>AIDH</td>
<td>Mo, Fe/S, FAD, Moco</td>
<td>Comamonas testosterone</td>
<td>Chap.2</td>
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</tr>
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<td>FEDH</td>
<td>Mo, Fe/S, FAD, Moco</td>
<td>Amycolatopsis methanolica</td>
<td>Chap.2</td>
<td></td>
</tr>
<tr>
<td>DL-AIDH</td>
<td>Mo, Fe/S, FAD, Moco</td>
<td>Amycolatopsis methanolica</td>
<td>Chap.3</td>
<td></td>
</tr>
<tr>
<td>ALOR</td>
<td>Mo, Fe/S, Moco</td>
<td>Desulfovibrio gigas</td>
<td>73</td>
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<tr>
<td><strong>Tungstoproteins</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AOR</td>
<td>W, Fe/S, Fe, Wco</td>
<td>Pyrococcus furiosus</td>
<td>38</td>
<td></td>
</tr>
<tr>
<td>FOR</td>
<td>W, Fe/S, Wco</td>
<td>Pyrococcus furiosus</td>
<td>39</td>
<td></td>
</tr>
<tr>
<td>GAPOR</td>
<td>W, Fe, Wco</td>
<td>Pyrococcus furiosus</td>
<td>42</td>
<td></td>
</tr>
<tr>
<td>BV-AIDH</td>
<td>W, Fe/S, Wco</td>
<td>Desulfovibrio gigas</td>
<td>247</td>
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<tr>
<td><strong>NAD(P)-dependent</strong></td>
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<tr>
<td><strong>AIDHs</strong></td>
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<td></td>
</tr>
<tr>
<td>AIDH</td>
<td>NAD(P)</td>
<td>Human</td>
<td>132</td>
<td></td>
</tr>
<tr>
<td>BAIDH</td>
<td>NAD(P)</td>
<td>Spinach</td>
<td>248</td>
<td></td>
</tr>
<tr>
<td>AIDH</td>
<td>NAD(P)</td>
<td>Bakers' yeast</td>
<td>249</td>
<td></td>
</tr>
<tr>
<td>PAD</td>
<td>NAD(P)</td>
<td>E. coli</td>
<td>Chap.5</td>
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<td>GD-FAIDH</td>
<td>NAD/GSH</td>
<td>Pseudomonas putida</td>
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<tr>
<td>FD-FAIDH</td>
<td>NAD/MySH</td>
<td>Amycolatopsis methanolica</td>
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</tr>
<tr>
<td>FAIDH</td>
<td>NAD(P)</td>
<td>Pseudomonas putida</td>
<td>252</td>
<td></td>
</tr>
<tr>
<td><strong>Aldehyde dismutases (Nicotinoproteins)</strong></td>
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<tr>
<td>FAID</td>
<td>NAD</td>
<td>Pseudomonas putida F61</td>
<td>49</td>
<td></td>
</tr>
<tr>
<td>MNO</td>
<td>NADP</td>
<td>Amycolatopsis methanolica</td>
<td>51</td>
<td></td>
</tr>
<tr>
<td>NDMA-ADH</td>
<td>NADP</td>
<td>Methanosarcina barkeri</td>
<td>53</td>
<td></td>
</tr>
</tbody>
</table>
electron acceptor, and functions as a true oxidase with oxygen as its physiological electron acceptor. Furthermore, although the substrate specificities of AO and XO overlap they are distinct. AO, for example, is not reactive toward xanthine. Finally, several stretches of amino acids in XO thought to be involved in NAD binding and substrate specificity are absent from AO.

The name AO fails to do justice to the wide range of substrates oxidized by the enzyme. Although AO can oxidize a variety of aldehydes in vitro, a physiological function of the enzyme is believed to be the oxidation of purines, pyrimidines, and other nitrogen-containing heterocycles [28]. In humans, AO has also been implicated in hepatotoxicity of alcohol [29] and familial amyotrophic lateral sclerosis [30]. In the latter case defects in the oxygen radical metabolism contribute to the pathogenesis of this disease. These defects are suggested to be caused by mutations in enzymes, such as AO, but the way in which AO is involved in the disease remains unclear. In plants, AO activities are involved in the biosynthesis of two plant hormones, abscisic acid and indole acetic acid [31]. AO is thought to catalyze the conversion of abscisic acid aldehyde to abscisic acid, which is considered to be the last step in the biosynthesis of abscisic acid. The biosynthetic pathway of indole acetic acid remains to be elucidated. However, an AO activity was shown to efficiently oxidize indole-3-acetaldehyde, a putative precursor of indole acetic acid. Structural and mechanistic aspects of AO are discussed in section 3.5.

2.2 NAD(P)-independent aldehyde dehydrogenases

2.2.1 Haemoprotein/quinoprotein aldehyde dehydrogenases

Dye-linked (form)aldehyde dehydrogenases are, for example, haemoprotein dehydrogenases and quinoprotein dehydrogenases.

From *Methylomonas methylovora*, a soluble, phenazine methosulfate (PMS)-linked, haem-containing AIDH was isolated [32]. This enzyme is a homodimer with a molecular mass of approximately 45 kDa and catalyzes the oxidation of straight chain aldehydes (C$_1$-C$_{10}$ tested), aromatic aldehydes (benzaldehyde, salicylaldehyde), glyoxylate, and glyceraldehyde. It appears that crude extracts of various methylotrophic bacteria contain such a soluble PMS-linked AIDH [33].

Acetaldehyde dehydrogenase from *Acetobacter europaeus* is also a haemoprotein [34].
It contains besides haems $c$ and $b$ also a Moco and an [2Fe-2S] cluster. So, in fact it is a molybdohaemoprotein. The enzyme consists of three different subunits with molecular masses of 79, 46, and 17 kDa and acts on a wide range of aliphatic aldehydes except for formaldehyde. It is localized on the outer surface of cytoplasmic membrane of the organisms, and the oxidation of aldehyde is linked to the respiratory chain. The enzyme is involved in vinegar production in acetic acid bacteria by oxidizing acetaldehyde which is produced by an alcohol dehydrogenase from ethanol. According to its structure, cofactor content, molecular mass, and amino acid sequence the enzyme is similar to molybdoprotein aldehyde dehydrogenases [Chapter 2].

Quinoproteins are enzymes containing the detachable pyrroloquinoline quinone (PQQ) or the protein-integrated quinone cofactors topaquinone (TPQ) or tryptophyl-tryptophanquinone (TTQ) [35]. Methylamine-grown *Hyphomicrobium zavarzinii* ZV 580 contains a dye-linked formaldehyde dehydrogenase (FAIDH) which has a covalently bound quinone as cofactor, as established with electron paramagnetic resonance (EPR)-spectroscopy, a positive result in a quinone assay and the fact that this compound could not be detached by denaturing the enzyme [36]. This enzyme is a tetramer of 210 kDa with subunits of 54 kDa and shows optimal affinity for, and activity with, formaldehyde.

### 2.2.2 Molybdoprotein aldehyde dehydrogenases

These NAD-independent AldHs are found in anaerobic as well as in aerobic bacteria. The first type of bacteria contain an aldehyde oxidoreductase that, in case of from *Desulfovibrio gigas* (ALOR) [37], oxidizes aldehydes to carboxylic acids with little specificity for the nature of the side group. It was shown that ALOR is the first component of an interesting *in vitro* reconstituted electron transfer chain, consisting of four proteins from *D. gigas*, flavodoxin, cytochrome, and hydrogenase such that oxidation of aldehydes is linked to the generation of hydrogen. The AldHs from *Comamonas testosteroni* and *Amycolatopsis methanolica* belong to the aerobic bacteria [Chapter 2-4]. For these enzymes the natural electron acceptor and physiological role are unknown.

The molybdoprotein AldHs contain molybdenum, FAD (not in ALOR), iron-sulphur clusters and a Moco like in AO or XO. Although these AldHs are less well known than the NAD-AldHs, much progress has been made during the last few years especially with respect to
unravelling the Moco and enzyme structure. An extended discussion about the molybdoprotein aldehyde oxidoreductases is given in section 3 (including the AIDHs from *Comamonas testosteroni* and *Amycolatopsis methanolica* (section 3.9, [Chapter 2-4])).

### 2.2.3 Tungstoprotein aldehyde dehydrogenases

These enzymes have so far only been found in anaerobic prokaryotes. Three types of tungsten-containing aldehyde oxidizing enzymes have been purified from hyperthermophilic archaea: Aldehyde ferredoxin oxidoreductase (AOR) from *Pyrococcus furiosus* [38], *Pyrococcus* strain ES-4 [39] and *Thermococcus* strain ES-1 [40]; Formaldehyde ferredoxin oxidoreductase (FOR) from *T. litoralis* [41] and *P. furiosus* [39]; and Glyceraldehyde-3-phosphate ferredoxin oxidoreductase (GAPOR) from *P. furiosus* [42]. All three types of enzymes catalyze the oxidation of various aldehydes with ferredoxin (Fd) as the electron acceptor but differ in their substrate specificity. Whereas GAPOR has been proposed to be involved in the sugar fermentation, AOR and FOR have been proposed to be involved in the peptide fermentation in *P. furiosus* [43]. The enzymes consist of one single type of subunit of approximately 67 kDa but differ in their quaternary structures: GAPOR is monomeric, AOR is dimeric, and FOR is a tetramer. Similarity in the N-terminal sequences of the three enzymes from different sources indicated that they are structurally related. In addition, they were found to have a similar cofactor content per subunit, namely, one tungsten atom and four to six iron atoms [43].

The isolation of a benzylviologen-linked aldehyde dehydrogenase from *D. gigas* (BV/AIDH) showed that tungsten-containing AIDHs are also present in mesophilic bacteria. Crystallographic analysis of *P. furiosus* AOR provided more information about the pterin tungsten cofactor (Wco) [44] which is identical, with respect to its pterin moiety, to the Moco present in molybdenum-containing proteins. This explains that in literature the term Moco is also used to indicate the pterin containing cofactor of tungstoproteins (Table 4) (see section 3.8).

### 2.3 NAD(P)-dependent aldehyde dehydrogenases

These dehydrogenases have been isolated from many different organisms and catalyze the oxidation of a wide variety of aldehydes to their corresponding acids. Except for the NAD-
AIDHs which use only NAD(P) as coenzyme, there are also NAD-AIDHs which use, beside the NAD(P), a second coenzyme, like coenzyme A (CoA) [45], glutathione (GSH) [46] or mycothiol (MySH) [47]. Some NAD-AIDHs are constitutive, others are inducible, and both tetrameric and dimeric enzymes are known. Some of them display a broad substrate specificity, oxidizing a variety of aliphatic and aromatic aldehydes, whereas others possess narrower substrate specificities. The NAD-AIDHs are discussed in more detail in section 4 (including phenylacetaldelyde dehydrogenase from E. coli (section 4.6, [Chapter 5])).

2.4 Aldehyde dismutases

In recent years a limited number of so-called nicotinoprotein alcohol/aldehyde oxidoreductases, containing tightly but noncovalently bound NAD(P) [48], have become recognized. Analogous to for instance FAD in flavoproteins and PQQ in quinoproteins, NAD(P)(H) acts as a cofactor in nicotinoproteins and remains bound to the enzyme during catalysis. An external electron donor or acceptor subsequently may reduce or oxidize the cofactor in situ. The first nicotinoprotein alcohol/aldehyde oxidoreductase was purified from Pseudomonas putida F61 grown in the presence of formaldehyde [49]. The enzyme is called formaldehyde dismutase (FAID) as it catalyzes formaldehyde dismutation into methanol and formate and serves to strongly increase formaldehyde resistance of the cells. The cofactor, NAD, is reduced and reoxidized again when formaldehyde is converted into formate and methanol, respectively. FAID can also be assayed as alcohol dehydrogenase when p-nitroso-N,N-dimethylaniline (NDMA) is used as artificial electron acceptor. It oxidizes primary alcohols, except methanol, and some secondary alcohols. Because of similar sensitivity of both formaldehyde dismutation and alcohol dehydrogenase activities to pyrazole (a class I alcohol dehydrogenase specific inhibitor), it was concluded that both activities occur at the same active site [49,50].

Studies on methanol-utilizing Gram-positive bacteria have resulted in the identification of several nicotinoproteins in recent years. In this context, methanol:NDMA oxidoreductase from A. methanolic (MNO) is of interest. MNO is able to oxidize alcohols with NDMA as electron acceptor and contains NADP as cofactor [51]. The enzyme is proposed to be involved in the oxidation of methanol in a multienzyme complex upon growth on methanol as sole carbon source
[52]. In the multienzyme complex, MNO is the first enzyme in the oxidation of methanol in *A. methanolica*. MNO is also an effective formaldehyde dismutase in the absence of NDMA and, in addition, it can reduce aldehydes with NADH [51]. In this way the physiological role of MNO could also include (form)aldehyde detoxification. The *in vivo* electron acceptor is known but its identity remains to be elucidated [52].

Cell-free extracts of *Methanosarcina barkeri* DSM 804 contain a NDMA-dependent alcohol dehydrogenase (NDMA-ADH) which is most probably a homodimeric enzyme consisting of subunits of 45 kDa [53]. Each subunit contains 1 mol of tightly but noncovalently bound NADP(H)/mol, about 2 mol Zn^{2+}/mol and significant amounts of magnesium. This enzyme preferably oxidizes primary alcohols but also catalyzes the stoichiometric dismutation of aldehydes, especially higher aliphatic aldehydes, to form equimolar amounts of the corresponding alcohol and acid. The enzyme does not catalyze the dehydrogenation of methanol or the dismutation of formaldehyde indicating that it is not directly involved in methanogenesis. The natural electron acceptor is still unknown.

3. Molybdoprotein aldehyde oxidoreductases

3.1 Molybdoproteins

Molybdenum is an essential trace element in life and occurs in organisms ranging from bacteria to man. The metal is found associated with a diverse range of redox active enzymes that catalyze basic metabolic reactions in the nitrogen, sulphur and carbon cycles. There are two types of molybdoenzymes - the nitrogenase type, the enzyme carrying a molybdenum-iron cofactor and the other type, encomprising all other molybdoenzymes, carrying Moco [54]. This thesis focuses on the latter type of molybdoenzyme since the bacterial molybdoprotein ALDHs (see section 3.9, [Chapters 2-4]) contain a Moco.

Much progress has been made during the past few years especially with respect to unravelling the nature of the Moco and enzyme structure. Based on sequence alignments and spectroscopic properties, four families of Moco containing enzymes have been identified. These families are the XO (including the bacterial molybdoprotein ALDHs, see section 3.9, [Chapters 2-4]), the sulfite oxidase (SO), the dimethylsulfoxide reductase (DMSOR) and the aldehyde
ferredoxin oxidoreductase (AOR) family (Table 4) [55,56]. Moco-containing enzymes may be further subdivided into two categories that are characterized by the types of reactions being catalyzed [55,57]. The first class contains enzymes such as XO and AOR that catalyze oxidative hydroxylation reactions of aldehydes and aromatic heterocyclic compounds. The second class, containing DMSOR and SO, catalyzes oxygen atom transfer to or from an available electron lone pair atom of a substrate.

Table 4. Representatives of Moco-containing enzyme families.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Source</th>
<th>Subunit Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Xanthine oxidase family</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Xanthine oxidase</td>
<td>Bovine milk</td>
<td>α₂</td>
</tr>
<tr>
<td>Xanthine dehydrogenase</td>
<td>Homo sapiens</td>
<td>α₂</td>
</tr>
<tr>
<td>Aldehyde oxidase</td>
<td>Homo sapiens</td>
<td>α₂</td>
</tr>
<tr>
<td>Aldehyde oxidoreductase</td>
<td>Desulfovibrio gigas</td>
<td>α₂</td>
</tr>
<tr>
<td><strong>Sulfite oxidase family</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sulfite oxidase</td>
<td>Rattus norvegicus</td>
<td>α₂</td>
</tr>
<tr>
<td>Nitrate reductase (assimilatory)</td>
<td>Spinacea oleracea</td>
<td>α₂</td>
</tr>
<tr>
<td><strong>DMSO reductase family</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DMSO reductase</td>
<td>Rhodobacter sphaeroides</td>
<td>a</td>
</tr>
<tr>
<td>Nitrate reductase (dissimilatory)</td>
<td>E. coli</td>
<td>aβγ</td>
</tr>
<tr>
<td>Formate dehydrogenase</td>
<td>E. coli</td>
<td>aβγ</td>
</tr>
<tr>
<td><strong>Aldehyde ferredoxin oxidoreductase family</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aldehyde ferredoxin oxidoreductase</td>
<td>Pyrococcus furiosus</td>
<td>α₂</td>
</tr>
<tr>
<td>Formaldehyde ferredoxin oxidoreductase</td>
<td>Pyrococcus furiosus</td>
<td>α₄</td>
</tr>
<tr>
<td>Glyceraldehyde-3-phosphate ferredoxin oxidoreductase</td>
<td>Pyrococcus furiosus</td>
<td>a</td>
</tr>
</tbody>
</table>

Common to both types of reactions is the cycling between the oxidized Mo(VI) and the reduced Mo(IV) states of the enzyme. Because these enzymes catalyze a two-electron redox reaction to or from the substrate, it is likely that the molybdenum also directly undergoes a two-electron change in oxidation state during the half-reaction (see also Chapter 4). Completion of the catalytic cycle must involve either addition or removal of electrons at the molybdenum site by means of electron transfer involving a second redox centre. Because the second centre is typically a one-electron redox group such as a haem or iron-sulphur cluster, the redox state of molybdenum is restored to the resting state through a sequence of two one-electron transfer steps with the Mo(V) state as intermediate. In most cases, the second redox centre is contained within the same enzyme molecule as the Moco [58].
3.2 The nature of the pterin molybdenum cofactor (Moco)

The common component of all forms of Moco is molybdopterin (MPT). A first proposal for the structure of MPT was given by Rajagopalan and coworkers [59]. It consists of a pterin derivative (Fig. 1A) with the pterin ring substituted at position 6 with a phosphorylated dihydroxybutyl side chain containing a cis-dithiolene group which coordinates the molybdenum. This structure has been deduced from the analysis of four derivatives (Fig. 1A) of the cofactor as the cofactor itself is labile upon release from the enzyme. Form A and form B, the first two derivatives, are formed by in vitro oxidation of MPT [60]. Dicarboxamidomethylmolybdopterin, a third derivative, is produced by iodoacetamide treatment of MPT [61], and urothione, the fourth derivative, is a metabolic by-product of MPT identified in human urine [59].

All eukaryotic molybdoenzymes contain an unmodified form of MPT as Moco. The possible existence of a variant form of Moco was initially proposed by Krüger and Meyer [62-64]. This was shown to be a molybdopterin cytosine dinucleotide (MCD) (see also Fig. 4B). Later it appeared that prokaryotic molybdoenzymes usually contain the Moco with either an AMP [65], CMP [62,66], GMP [65,67-69] or HMP [65] nucleotide attached to the MPT via a pyrophosphate linkage. However, as an apparent exception, xanthine dehydrogenase (XDH) from prokaryotic organisms [66,70] has Moco without a nucleotide.

The 3 D-structure of AOR from *P. furiosus* [71], containing a Wco, established the general validity of Rajagopalan's proposal for the structure of MPT, with the additional feature that the dihydroxybutyl sidechain actually forms a pyran ring (Fig. 1B). The tetrahydropterin state for the tricyclic structure is equivalent to a dihydropterin (either the 5,6-dihydropterin or the 7,8-dihydropterin) in the ring-opened form of the bicyclic pterin (see also Chapter 4). Ring closure has been proposed to occur upon incorporation of the cofactor into the apo-enzyme and the final structure is presumed to be stabilized in the environment provided by the enzyme [58]. As demonstrated in the structures of AOR [71], DMSOR [72], ALOR [73] and formate dehydrogenase H from *E. coli* [74], the tricyclic MPT system is clearly nonplanar. From the stereochemistry at positions 6 and 7, the MPT seems to be in the fully reduced tetrahydropterin oxidation state. Additional support for this conclusion comes from the observation that both N5 and N8 are likely to be protonated, because they are involved in hydrogen bonding in the crystal structures analysed so far. This behaviour argues against the existence of less reduced states such
Fig. 1. Structure of MPT. (A) Proposed structure of MPT according to Rajagopalan and coworkers [90] deduced from four derivatives (form A, form B, dicarboxamidomethylmolybdopterin, urothione). The atom numbering scheme of MPT is included. (B) Structure of MPT as first observed in P. furiosus AOR.
as 5,6-dihydropterin and 7,8-dihydropterin, as well as the various quinonoid forms of the
dihydropterin, because these latter structures are not compatible with the observed pattern of
hydrogen bonding.

The role of the MPT, except for binding the molybdenum, is unclear. Changes in the
MPT conformation due to the redox state of the enzyme and interaction with the molybdenum
have been observed for DMSOR and ALOR [72,73] (see also Chapter 4). It is possible that
pterins can participate in the reaction and electron transfer mechanism of these latter enzymes,
although this has not been experimentally demonstrated [75]. Variations in the conformations
and number of pterins may help to modulate the redox potential of the metal centre to an optimal
value.

The role of the attached nucleotide is also not clear, although two possibilities have been
suggested by Chan et al [71]. They postulated that Mocos containing such a nucleotide do have
a structure similar to that observed in AOR, with the only difference being replacement of the
second pterin, which is present in AOR, by a nucleotide, and metal ligation by the sugar hydroxyl
oxygens (see also Figs. 4B and 9). There is a precedent for metal coordination by sugar hydroxyl
groups in this fashion [76,77]. Such binding may help to explain why the sugar ring is only
present among the different Mocos including a dinucleotide attached to the MPT, and why
differences appear to exist in the ligation of the molybdenum in different molybdoenzymes. An
alternative possibility is that the nucleotide-containing end of the Moco could adopt a somewhat
different conformation that would permit metal coordination by groups on the nucleotide ring
or, perhaps by amino acid side chains.

3.3 Biosynthesis of the pterin molybdenum cofactor (Moco)

Three different biosynthetic pathways, in which pterins are formed either as end products
or as intermediates, are known [78]. Two of these, the de novo formations of the pterin ring of
folate and of the lumazine ring during the biosynthesis of riboflavin, occur in plants and
microorganisms but not in animals. The third known route presumably occurs only in animals
and leads to the synthesis of tetrahydrobiopterin. All three pathways utilize GTP as the source
of the pterin ring and involve the replacement of C-8 of guanine with two carbons to generate
C-6 and C-7 of the pterin ring by unknown reactions.
Information on the biosynthesis of the cofactor has been obtained from a study of Moco mutants of *E. coli*, as a group termed chlorate-resistant mutants (*moa*), and from a Moco mutant of *Neurospora crassa*, *nit-1* [79]. These mutants carry mutations which map to five distinct regions of the *E. coli* chromosome: *moa*, *mob*, *mod*, *moe* and *mog*. Two extra mutants, *mom* and *mon*, have been mapped to the *moa* and *moe* regions of the genome, respectively. Each of the loci has been cloned and almost all have been sequenced [80-84]. The overall functions encoded by most of the loci have been deduced (Fig. 2).

![Diagram of Moco biosynthesis]

**Converting Factor**

[Large Subunit (*moa* gene 5)]

\[ \downarrow \] (moa genes 1-3)

\[ \rightarrow \] **Precursor Z**

**Active Converting Factor**

\[ \rightarrow \] \[ \downarrow \] **Converting Factor-MPT Complex**

**Converting Factor**

[Small Subunit (sulfo)]

\[ \uparrow \] (mon gene product)

\[ \rightarrow \] **Molybdopterin (MPT)**

\[ \uparrow \] (mod, g gene products)

\[ \downarrow \] **Source of GMP**

\[ \rightarrow \] **Molybdate**

\[ \downarrow \] (mob gene product)

**Converting Factor**

[Small Subunit (*moa* gene 4)]

\[ \rightarrow \] **Pterin Molybdenum Cofactor (Moco)**

[Molybdopterin Guanine Dinucleotide]

Fig. 2. Summary of the roles of *mo* gene products in the biosynthesis of the Moco in *E. coli*.

The *moa* and *moe* loci encode five (*moa1* representing the first reading frame) and two (*moe5* and *mon*) proteins, respectively, which assemble the MPT portion of the cofactor [85,86]. The extracts from the *moa1*, *mom*, *moe5* and *mon* mutants exhibited precursor activity. Studies on the structure of the precursor molecule, termed precursor Z (Fig. 3), indicate that it contains a dihydropterin ring and lacks sulphur. The activity in *moa1*, required for the conversion of the
precursor to MPT, termed the converting factor [87], has been shown to consist of two different dissociable subunits, both of which are essential for activity. Addition of purified converting factor to purified precursor leads to the formation of stoichiometric amounts of MPT. The smaller of the two subunits of the converting factor is essential for the sulphur addition reaction and appears to sequester an active sulphur atom from the mon protein sufficient for a single turnover activation reaction. The complete formation of MPT requires the ligation of the metal to the dithiolene sulphurs. Not much is known about the proteins or the form of molybdenum required for these reactions, other than the implication of the mod and mog gene products in the uptake and further processing of molydate [88]. In prokaryotic organisms the appropriate dinucleotide forms of the cofactor also have to be synthesized. In E. coli it has been established that the mob gene product is essential for the attachment of the GMP to the MPT to form a molybdopterin guanine dinucleotide (MGD) as Moco [89].

3.4 Human molybdoenzyme deficiencies

Children born with a genetic deficiency of SO display a syndrome of seizures, mental retardation, and dislocated ocular lenses [90]. The urine contains elevated amounts of sulfite, thiosulfate, and S-sulfocysteine which are abnormal metabolic products of sulphur-amino acid degradation. In contrast, virtually no inorganic sulfate is excreted. Rapid neurological degeneration is the common outcome of the disease and most often leads to death in infancy. Examination of autopsy tissue of the first reported patient demonstrated a deficiency of SO in liver, brain and kidney.

Individuals with a genetic deficiency in XO and XDH, which are two forms of the same enzyme in mammals, appear to be free of any cellular dysfunctions [91]. Even a combined
deficiency of XO and AO appears to be benign [92], except for the absence of urate, due to XO deficiency, and N-methyl nicotinamide pyridone, due to AO deficiency, in the urine.

All three known human molybdoenzymes (SO, XO/XDH, AO) contain a Moco. In the last few years, more than 40 patients with Moco deficiency have been identified [93]. The severe pathophysiology of the disease is quite similar to that seen in patients with simple SO deficiency and consists of neurological abnormalities, dislocated ocular lenses, and mental retardation in surviving patients.

3.5 The xanthine oxidase family

Members of this family (Table 4) are, e.g., XO, XDH, AO and ALOR. Whereas XO and AO are only found in eukaryotes and ALOR only in prokaryotes, XDH is found in both kind of organisms. The enzymes are α2 homodimers and the molecular mass of the monomer is approximately 150 kDa. In general these enzymes consist of four domains starting at the N-terminus with two small domains each containing a single [2Fe-2S] cluster, followed by a FAD domain (not present in ALOR), and completed by the Moco domain. The molybdenum is coordinated by two sulphur ligands from the pterin cofactor, a cyanide-labile sulphido group, and another oxygen or nitrogen ligand. XO and XDH catalyze the conversion of xanthine to uric acid, a step in the catabolic metabolism of purine bases. All enzymes in this family have broad substrate specificities which are for some enzymes partially overlapping [94,95].

The first 3 D-structure of a XO-related enzyme was that of ALOR (solved at 2.2 Å resolution) [73]. ALOR is a dimer composed of two 907-residue subunits that each contain a molybdenum coordinated by a single MCD, and two different [2Fe-2S] clusters. The first cluster-binding domain adopts a fold analogous to that of plant type [2Fe-2S] ferredoxins, whereas the second cluster-binding domain folds into a four-helical bundle that had not been previously observed to coordinate [2Fe-2S] clusters. Whereas one [2Fe-2S] cluster is not accessible for the solvent, the other cluster is close to the protein surface. The remaining two molybdenum domains are responsible for binding the Moco which is deeply buried in the protein. A 15 Å-long channel provides access to the active site. The strong similarities between the amino acid sequences of ALOR and XO suggest also similar structures. XO contains, however, also a FAD domain which may be relatively close to the [2Fe-2S] cluster and Moco binding domains because of the rapid
Fig. 4. (A) Structure of the active site of ALOR, with the pterin, oxygen, and sulphido ligands to the molybdenum indicated. (B) Structure of Moco in ALOR and schematic representation (---) of hydrogen bonded contacts between protein and Moco in ALOR.

internal electron transfer [96].

The pentacordinate Mo(VI) centre of ALOR exhibits an approximately square pyramidal coordination geometry. The equatorial plane consists of the two dithiolene sulphurs and two oxygen ligands, whereas the remaining ligand, which is sulphido in the active form [97], occupies the apical site (Fig. 4A). No ligands to the metal are provided by the protein, although the sidechain of Glu-869, which forms a hydrogen bond to one of the oxygen ligands of the molybdenum, is sufficiently close (3.5 Å) that a slight rotation could allow weak binding. The structure of ALOR showed also the presence of a covalently attached nucleotide, a cytosine. This nucleotide and the pterin interact with the protein through multiple hydrogen bonding interactions (Fig. 4B). Changes in molybdenum coordination by the pterin ligand occur with alterations in oxidation state of the enzyme [98]. The metal centres are approximately linearly arranged. Whereas the molybdenum and second [2Fe-2S] cluster are ~ 15 Å separated from each other, the two [2Fe-2S] clusters are separated by ~ 12 Å. The pterin interacts directly with the second [2Fe-2S] cluster through a hydrogen bond formed between the N2 atom and the Sγ of the cluster ligand Cys-139. The two [2Fe-2S] clusters are further linked through a series of covalent
Fig. 5. Proposed reaction mechanism of ALOR, based on 3-D structure information.

and hydrogen bond interactions connecting the two cluster ligands Cys-45 and Cys-137. It is possible that these interactions facilitate electron transfer between these [2Fe-2S] clusters, resulting in the final transfer to an external electron acceptor [73].

The proposed reaction mechanism of ALOR has been based on crystallographic studies [73,97]. The chemical reaction at the molybdenum-site is proposed to proceed by transfer of a molybdenum-bound water molecule as OH, obtained after proton transfer to the sidechain of Glu-869, to the carbonyl carbon of the substrate in concert with hydride transfer to the sulphido group linked to the molybdenum (Fig. 5). This results in reduction of the metal centre. After completion of the reductive half-cycle, the Mo(VI) state would be regenerated through a series of one-electron transfers to the [2Fe-2S] clusters of ALOR.

3.6 The sulfite oxidase family

These enzymes have a dioxo molybdenum-centre at the active site (Fig. 6), with most likely one Moco coordinated to the molybdenum through the dithiolene sulphur atoms. They catalyze oxygen atom transfer to or from an electron lone pair of a sulphur or nitrogen atom of the substrate [58]. SO is mainly found in eukaryotes and is located in the mitochondrial intermembrane space where it catalyzes the oxidation of sulfite to sulfate. This is a terminal
reaction in the oxidative degradation of sulphur-containing amino acids like cysteine and methionine. The enzyme is a homodimer with a molecular mass of ~ 110 kDa [99] and each monomer exists of two domains. The smaller domain (~ 10 kDa) contains a $b_3$ cytochrome whereas the larger domain (~ 42 kDa) contains the Moco. Assimilatory nitrate reductase catalyzes the reduction of nitrate to nitrite, which is then converted to NH$_4^+$ by nitrite reductase. This enzyme is also a homodimer but with a molecular mass of ~ 220 kDa and each monomer consists of three domains. The N-terminal domain (~ 59 kDa) binds the Moco, the central domain (~ 14 kDa) a $b$-type cytochrome, and the C-terminal domain (~ 24 kDa) FAD and has also a NAD(P)$^+$ binding site [100]. High sequence similarities (~ 35 %) have been observed between the sequences of SO and nitrate reductase [101-103]. Although the crystal structure of the FAD domain of nitrate reductase has been solved by X-ray crystallography [104,105], there is no 3 D-structure of the Moco domain of this enzyme family yet.

Structural information on these enzymes has been provided by extended X-ray absorption fine structure (EXAFS)- and EPR-spectroscopies. EXAFS studies [106] with the oxidized SO show that two oxygen atoms coordinate the molybdenum at a distance of 1.71 Å. In addition, the molybdenum was also found to be coordinated by three thiolates at a distance of 2.42 Å, suggesting one pterin cofactor coordinating the molybdenum. Two of the three thiolates would be contributed from the pterin cofactor and the third most likely be contributed from a cysteine [107]. The coordination environment of the molybdenum seems to be sensitive to the oxidation state of the molybdenum and to the pH of the solution [94,106]. As determined by EXAFS, at
pH 9.0 all three oxidation states of the molybdenum possess five ligands. At pH 6.0, an additional ligand, most probably chloride, is observed in the Mo(V) and Mo(IV) states.

Based on the results from spectroscopic data of chicken liver SO and studies of model compounds, a catalytic mechanism has been proposed [57] (Fig. 6). The initial binding of sulfite at the active site could either be a direct coordination of the substrate to the molybdenum or more likely, a nucleophilic attack of the lone pair of sulfite, which is a good oxo-group acceptor, on one of the Mo=O bonds. The molybdenum would then be reduced from the (VI) to the (IV) state and sulfate would be released by replacement with a hydroxide ion from the solvent [108,109]. The catalytic cycle would be completed by two single-electron transfer steps to the haem $b_2$ of the enzyme and concomitant deprotonation leading to the resting dioxo molybdenum centre. It seems very likely that only one of the two oxygen ligands is the catalytically labile oxygen, whereas the other oxygen cannot be attacked owing to steric hindrance in the active site of the enzyme [58].

3.7 The DMSO reductase family

Members of this family are exclusively found in eubacteria and include DMSOR, dissimilatory nitrate reductase, formate dehydrogenase, trimethylamine-N-oxide reductase, and biotin sulfoxide reductase. These enzymes all contain MGD as Moco. In the absence of oxygen and in the presence of their respective substrates, these enzymes serve as terminal oxidoreductases to provide the cell with more ATP than available by fermentation alone. DMSOR is found in several bacteria [110-112] and varies in structure. DMSOR from *Rhodobacter sphaeroides*, for example, is a water-soluble monomeric protein (85 kDa), containing only the Moco, whereas the *E. coli* enzyme is an integral membrane protein, consisting of three different subunits, containing four [4Fe-4S] clusters (present in the β-subunit) besides the Moco (present in the α-subunit). The latter enzyme has the same architecture as the dissimilatory nitrate reductase. Additional interest in DMSOR arose from the fact that dimethylsulphide (DMS), the volatile reaction product, is the major component of reduced sulphur in the atmosphere and has been implicated in global climate control [113-115]. DMS is produced during zooplankton grazing on phytoplankton [116] and is subsequently released into the atmosphere. However, DMSOR plays only a minor role in the production of DMS in global
Fig. 7. Structure of Moco and schematic representation (-----) of hydrogen bonded contacts between protein and cofactor in *R. sphaeroides* DMSOR. Contacts to water molecules have been omitted for clarity.

terms.

The polypeptide chain of DMSOR from *R. sphaeroides*, which was the first enzyme of the DMSOR family to be characterized by X-ray crystallography, folds into four domains that form a slightly elongated molecule [72]. The spatial arrangement of domains I to III creates a large depression on one side of the molecule resembling a funnel, with the active site located at the bottom of the funnel. Domain I is the only domain that forms no direct interactions with the Moco. Sequence similarities indicate that the enzymes of the DMSOR family share the same basic architecture, including domains II, III, and IV.

The active site of DMSOR from *R. sphaeroides* was found to contain two MGDs (designated as P- and Q-pterin as they coordinate the molybdenum atom in an asymmetric fashion) that coordinate the molybdenum with an approximate twofold axis of symmetry passing through the molybdenum (Fig. 7) [58,72]. Numerous interactions between the protein and Moco exist, including 45 direct hydrogen bonds, and also a few hydrogen bonds to water molecules, which are not located near the molybdenum atom. The DMSOR family is also characterized by
Fig. 8. Proposed reaction scheme of DMSOR. Coordination in the Mo(IV) and Mo(VI) states is as observed in the crystal structures of these forms.

A protein ligand to the molybdenum which may be either a serine (like in DMSOR), cysteine or selenocysteine (as in formate dehydrogenase H from E. coli). In the oxidized form of the enzyme the four dithiolene sulphur atoms of the two pterins coordinate the molybdenum atom in an asymmetric fashion. An oxo-group forms an additional ligand and the coordination sphere is completed by the protein ligand. The reduced enzyme did not reveal any major conformational changes in the protein structure. Significant changes were, however, observed at the active site including the expected loss of the oxo-ligand, and a different coordination of the molybdenum atom by the pterin sulphur atoms.

The reaction catalyzed by DMSOR can be subdivided into two half-cycles [58]. In the oxidative half-cycle, the reduced Mo(IV) form of the enzyme binds the substrate, and two electrons are transferred from molybdenum to the substrate, yielding the reaction product DMS
and the oxygen atom of the substrate bound to the metal as an oxo-ligand (Fig. 8). In the second, reductive half-cycle, two protons and two electrons are transferred to the molybdenum centre, yielding H₂O and regenerating the Mo(IV) state. In this step an oxygen atom is transferred from the substrate into water [117]. Since DMSOR from R. sphaeroides does not contain a second cofactor that could transfer electrons to Moco, an external electron donor, presumably a soluble cytochrome, is required for this step. Based on the observed differences of the coordination of the pterins to the molybdenum atom in the oxidized and the reduced forms of DMSOR, the Q-pterin would influence the electronic and accessibility properties of the metal, thereby providing some of the necessary requirements for catalysis. In the reductive half-cycle of the reaction, two cycles of binding of the physiological electron donor to the oxidized enzyme are required. In each cycle a single electron is transferred to the active site, finally restoring the Mo(IV) state of the enzyme. In case cytochrome is binding to the surface nearest the pterin moiety of the Q-pterin and electron transfer occurs via the Q-pterin to the molybdenum would involve a direct role for a pterin in facilitating electron transfer between the external donor and the oxidized molybdenum centre [58,72].

3.8 The aldehyde ferredoxin oxidoreductase family

Members of this family are tungsten proteins, including AOR, FOR and GAPOR, which convert aldehydes into carboxylates. In addition, a carboxylic acid reductase and an aldehyde dehydrogenase from D. gigas (not ALOR) [56] are also members of this family. Although not much is known about their functions, AOR and FOR probably play an important role in peptide fermentation, with the aldehydes generated by the transamination and subsequent decarboxylation of the keto acids.

The first Moco-type enzyme that was structurally characterized, thereby providing evidence for the structure of the metal-coordinating pterin, was the AOR from the hyperthermophile P. furiosus [71]. This homodimer with subunits of 66 kDa contains three different types of metal binding sites, including two copies of the tungsten centre, two copies of an [4Fe-4S] cluster, and that for a single metal atom (magnesium ion) located at the dimer interface (Fig. 9). The Wco and the [4Fe-4S] cluster are positioned in close proximity within each subunit (~ 8 Å). Each subunit folds into three domains, with the binding sites for the Wco
Fig. 9. Structure of Moco and schematic representation (-----) of hydrogen bonded contacts between protein and Moco in AOR.

and [4Fe-4S] cluster located at the interfaces of these domains. The overall polypeptide fold of AOR reveals no structural similarities to ALOR and DMSOR folds.

In AOR the tungsten is symmetrically coordinated by the four dithiolene sulphurs from two MPTs bound to each subunit (Fig. 9) [71]. No protein residues are involved as ligands for tungsten although the side chains of residues Glu-313 and His-448 are very close so that they could participate in proton transfer reactions coupled to electron transfers. The two MPTs are linked through their phosphate groups, which coordinate axial sites of the same magnesium ion. Furthermore, the MPT ligands are approximately related by a twofold rotation about an axis that passes through both the tungsten and magnesium sites. The magnesium ion exhibits octahedral geometry, with two coordination sites filled by phosphate oxygens, two sites provided by backbone carbonyl oxygens from residues Asn-93 and Ala-183, and two water molecules. Each water is hydrogen bonded to at least one phosphate oxygen and a N5 nitrogen of a pterin ring, with one of the two waters also within hydrogen-bond distance of the pyran oxygen of one of the MPT ligands. Between the protein and the MPT rings also multiple hydrogen bonding interactions exist (Fig. 9).

The [4Fe-4S] cluster is positioned approximately 10 Å from the tungsten atom and is
buried ~ 6 Å below the van der Waals surface of the protein [71]. This is consistent with the postulated role of the cluster as mediator for electron transfer between the Wco and ferredoxin, the physiological electron acceptor of AOR. The relationship between the cluster and MPT in AOR is very different from that observed in ALOR, where the [2Fe-2S] cluster directly interacts with the N2 nitrogen of the pterin. In both cases, however, interactions could provide electron transfer pathways between the metal and the iron-sulphur cluster. This arrangement suggests that the pterin ligand does not merely play a passive structural role but may be an active participant in the redox chemistry of Moco-type containing enzymes [Chapter 4]. The heterogeneous nature of the tungsten centre in as-isolated active and inactive forms of AOR [58], with W(IV), W(V), and W(VI) oxidation states, has so far been complicating the structural analysis of the environment and the relevant oxidation state of the tungsten [75].

Not much is known about the mechanism of action of AOR, especially concerning the possibility of oxo-group or hydride-transfer reaction mechanisms [58, 71]. Like in ALOR, the active site is buried within the protein, with substrate access provided by a channel leading to the protein surface. Unless substantial rearrangements in the tungsten coordination sphere occur during substrate oxidation, the presence of two MPT ligands enforces cis-coordination for the substrate, which would have stereochemical consequences for the oxygen transfer and oxidation-reduction reaction mechanism. It is clear that further spectroscopic, biochemical, and structural studies are needed to understand the reaction mechanism of AOR.

3.9 Bacterial molybdoprotein aldehyde dehydrogenases

In the past not much was known about molybdoprotein AIDHs from aerobic bacteria. An extended characterization of three AIDHs from C. testosteroni (AIDH) and A. methanolicum (FEDH and DL-AIDH) [Chapter 2.4], however, provided a strong contribution to information concerning this group of enzymes. Although the natural electron acceptors of the latter three enzymes are unknown, from the fact that artificial dyes but not O2 act as electron acceptor, they are called dehydrogenases. Further differentiation in names has been based on the observation that the enzymes convert aldehydes into acids and formate esters into pyrocarbonate esters (the name FEDH, formate ester dehydrogenase, was introduced when it was found that such a type of AIDH exhibited this activity, the property thought to be unique; the latter appears to be
incorrect now since all three AIDHs show it; however, the name is still used here for practical reasons) [Chapter 2]. With respect to their physiological role still not much is known.

Since these molybdoprotein AIDHs exhibit structural, cofactor, spectroscopic and catalytic properties that are in many respects similar to those of XO, these AIDHs are suggested to be members of the XO-family. The AIDHs each consist of three different subunits (αβγ in case of AIDH and FEDH and αβγ2 in case of DL-AIDH), have a total molecular mass of approximately 150 kDa and contain MCD [Chapter 2 and 3]. Several other bacterial molybdoproteins carrying a MCD, like quinoline oxidoreductase from *Pseudomonas putida* 86 (QOR) [117] and carbonmonoxide dehydrogenase from *Pseudomonas thermocarboxydovorans* (CODH) [118], have also been reported to be three-subunit complexes with subunit sizes similar to those of the molybdoprotein AIDHs. Although XO-related enzymes are homodimers, they have been reported to consist of three different domains per monomer corresponding to the three different subunits of the AIDHs [119-122]. In addition, similarity has been observed between the amino-terminal sequences of the subunits of the AIDHs and those of the corresponding domains of eukaryotic XDH (15-42 %) [Chapter 2]. The native molecular mass of XO is approximately equal to that of two times the (α+β+γ) structure of the AIDHs.

The molybdoprotein AIDHs contain FAD, iron, acid-labile sulphide, molybdenum and CMP in a molar ratio of 1:4:4:1:1 [Chapter 2 and 3]. The UV/Vis spectra of these enzymes confirm this cofactor composition as these spectra are typical for flavin-iron/sulphurmolybdoproteins. The N-terminal sequences of the subunits of the AIDHs show greatest similarity with other MCD-containing enzymes (32-55%), including QOR and CODH [Chapter 2]. Like XO, the AIDHs contain two nonidentical [2Fe-2S] clusters (although only one cluster has been detected for DL-AIDH [Chapter 3]) which show magnetic interaction with each other [Chapter 2]. Even the extent of line broadening in the EPR spectrum is similar to that found in XO and other (bacterial) molybdoproteins. For DL-AIDH a Mo(V) signal can be observed with EPR [Chapter 3]. The possible hyperfine interaction of this molybdenum with a proton in close proximity has also been observed for XO [27,123]. In addition, the magnetic coupling between the Mo(V) and [2Fe-2S]-1 cluster in DL-AIDH and XO is of the same order of magnitude.

EPR spectra of the three different AIDHs show in their oxidized state a $g = 2.004$ signal which has never been observed in a molybdoprotein before [Chapter 4]. Determination of the concentration of the signal yielded 12, 15 and 2% of the enzyme concentration for AIDH, FEDH
and DL-AIDH, respectively. Via isotope substitution experiments it was possible to elucidate the identity of the organic radical. The values of the various hyperfine coupling constants are consistent with the properties expected for a molybdenum(VI)-trihydropterin radical in which the N5-atom is engaged in two hydrogen bonding interactions with the protein. The majority of the electron (spin) density of the radical is located at and around the N5-atom and at the proton bound to the C6-atom of the pterin ring. The radical is not magnetically isolated as there is cross-relaxation with a nearby, rapidly relaxing, oxidized [2Fe-2S]-cluster involving its magnetic $S = 1$ excited state in this process.

Preliminary experiments on the function of the radical have not given definitive answers on its role in catalysis. It is well known that radicals present in purified enzyme preparations may simply be artifacts of the isolation procedure. We observed that addition of the substrate aldehyde did not affect the amount of radical present, whereas addition of dithionite did. The specific activity of dithionite-reduced enzyme, which was subsequently reoxidized with ferricyanide (no radical), was the same as that of the untreated - as isolated - enzyme [Chapter 4]. This finding indicates either that the enzyme molecules with a radical are inactive and cannot be reactivated by this treatment or that the radical is a true intermediate in catalysis. However, why the radical did not show up in the simple reoxidation experiment mentioned above needs to be investigated further. When the radical turns out to be a genuine catalytic intermediate, the pterin system might act as a one-electron carrier, shuttling between its trihydro- and tetrahydro-redox states, mediating electron transfer between the molybdenum ion and the nearest [2Fe-2S] cluster.

Sequence data of molybdoenzymes like carbonmonoxide dehydrogenase and XDH indicate that the $\gamma$-subunit or $\gamma$-domain, respectively, binds both [2Fe-2S] clusters [118,119]. The N-terminal sequences of the $\gamma$-subunits of the molybdoprotein AIDHs show similarity to those of the former molybdoenzymes suggesting that the two clusters of the AIDHs are also located in the $\gamma$-subunit [Chapter 2]. The purified $\gamma$-subunit of AIDH contains, however, only the plant-type [2Fe-2S] cluster suggesting that the second [2Fe-2S] cluster was lost during isolation. Since the reduced $\gamma$-subunit is unstable, it was not possible to decide whether a [2Fe-2S]-1 or [2Fe-2S]-2 type of cluster is present in the isolated subunit.

Deflavo-AIDH also showed the characteristics of the plant-type [2Fe-2S] cluster and lacked the same [2Fe-2S] cluster as the purified $\gamma$-subunit [Chapter 2]. An optical difference spectrum (deflavo-AIDH minus $\gamma$-subunit) suggests that in deflavo-AIDH the pterin-moieity is
in the fully oxidized state. Determination of the redox state of the Moco of native SO indicates a dihydropterin reduction level [124, 125]. This holds also for native ALOR, as suggested from the non-planarity of Moco. Assuming that native AIDH contains Moco in the same redox state, the calcium treatment, leading to loss of FAD and an [2Fe-2S] cluster, may result in oxidation of the pterin either by direct exposure to oxygen or by the [2Fe-2S] cluster during unfolding. Eukaryotic deflavo-XO/XDH do not catalyze reactions performed by the holo-enzymes [127]. Since they do when reconstituted with FAD [126], apparently the procedure applied for obtaining the deflavo-form does not affect other structural alterations other than FAD removal crucial for activity. Deflavo-XO shows XDH activity with artificial electron acceptors [127], suggesting that the presence of FAD is not essential for substrate oxidation but plays a role in electron transfer from reduced molybdenum to O₂. Since deflavo-AIDH did not show AIDH activity with a variety of artificial electron acceptors and reconstitution with FAD was not achieved, no clue was obtained for the role of FAD in AIDH [128]. However, since ALOR lacks FAD, FAD does not play an essential role in the aldehyde oxidation step by molybdoproteins. The absence of the [2Fe-2S] cluster and the higher oxidation state of the pterin moiety of Moco in deflavo-AIDH, as compared to the situation in holo-AIDH, indicates that this molybdoprotein is more vulnerable to structural damage caused by the deflavinylation procedure than eukaryotic XO and XDH.

Although the prokaryotic molybdoprotein AIDHs are able to convert a wide variety of aldehydes and formate esters, their substrate specificities are different, indicating variation in the nature of their active sites [Chapter 2 and 3]. Accepting the present view on the mechanism of XO-related enzymes, the AIDHs oxidize their substrate by catalyzing hydride transfer from the C1-atom of the substrate to the sulphido group linked to the molybdenum centre in concert with an attack of a hydroxyl group (derived from H₂O) on the C1-atom. In principle, special aldehydes like formaldehyde and benzaldehyde and aldehyde analogs like formate esters, could also be oxidized via such a mechanism. The AIDHs show a very low enantioselectivity towards racemic aldehydes [Chapter 2] which implies that the steric conformation of the residues attached to the asymmetric C-atom is irrelevant with respect to discrimination between the enantiomers in binding (Kₘ values) as well as in turnover rates (Vₘₐₓ values). The lack of discrimination could be explained by assuming that the site where the catalytic event takes place is at the outside of the enzyme molecule so that scarcely any interaction would occur between the enzyme molecule and the chain attached to the carbonyl group of the substrate. However, this is very unlikely since
the structure of ALOR indicates that the aldehyde has to pass a deep tunnel before conversion of it takes place. Assuming that the catalytic site in the AIDHs is buried in a similar way, the chain should make contact with the tunnel walls. The following observations indicate indeed that the nature of the chain is relevant for binding and/or turnover [Chapter 2]: benzaldehyde binds very well but is a poor substrate for AIDH (although it is a very good substrate for FEDH); similarly, although 3-phenylpropanal binds so strongly to AIDH that it cannot be replaced by propanal, it is a very poor substrate for the enzyme (although it is reasonably well for FEDH); benzaldehyde becomes a good substrate for AIDH when this compound is substituted with an electron withdrawing group at the $p$-position (although lower FEDH activity was obtained). These examples show that interaction occurs between the chain and enzyme, suggesting that the steric conformation of the substituents to the chain, should affect the strength of this interaction. Since this does not occur further investigations are required to explain poor enantioselectivity.

The redox potentials ($E_m$) of the redox centres present in AIDH differ from those of XO and XDH [Chapter 2]. In many molybdoproteins, the [2Fe-2S]-2 cluster has a more positive redox potential than [2Fe-2S]-1, however, the reverse is true for AIDH. In addition, compared to XO and XDH, all the potentials of the redox couples in AIDH are 0.1 to 0.2 V higher, except for the [2Fe-2S]-2 cluster. For AIDH a blue FADH$^+$, accounting maximally for 20% of the enzyme concentration, can be observed during redox titrations. No significant amount of Mo(IV) is observed. The potentials of both [2Fe-2S] clusters are pH dependent, which is surprising since at least one of the two clusters may be deeply buried in the protein and will probably be inaccessible to water (cf. ALOR). This pH dependency is most simply explained by the presence of an acid/base group in electrostatic contact and/or close to the [2Fe-2S] cluster. The nature of this proton acceptor and its possible function in electron transfer remain to be determined.

The distances between the various prosthetic groups are apparently well conserved in the molybdoproteins investigated given the similarities in strength of magnetic interaction between the redox centres and are probably not or no longer subject to evolutionary change. A variation in the values of $E_m$ of the redox centres in AIDH by 0.1-0.2 V uphill would slow down internal electron transfer a thousand fold, but the absolute rate may still be significantly higher than the overall turnover rate and so the values of $E_m$ of the various prosthetic groups are not subject to strong evolutionary pressure. Apparently, once the first molybdoprotein had evolved many variations on the overall structural theme were allowed - including the absence of FAD, the
presence of haems c and b instead of FAD, unionization of the three subunits to a single large
subunit, monomeric or dimeric enzymes, apparent unfavourable stabilities of a FADH' and
Mo(V), thermodynamically 'unfavourable' $E_m$'s, a short Mo=S or Mo=O bond - as long as the
rate of internal electron transfer was high relative to the overall rate of turnover. In time
differences in substrate binding domains (also of the electron acceptor) evolved widening the
spectrum of catalysis performed by this class of enzymes, while leaving intact the spatial
arrangement of its very core, Moco and two different [2Fe-2S] clusters.

4. NAD(P)-dependent aldehyde dehydrogenases

4.1 General aspects

NAD-AlDHs are found in every organism investigated [129-132]. Some of them are
capable of oxidizing both aliphatic and aromatic aldehydes to their corresponding acids. They
are involved in such diverse physiological processes as ethanol tolerance, neurotransmission,
carcinogenesis and immunosuppression [133-135], explaining why these enzymes are of
biomedical interest. Beside the NAD-AlDHs with a broad substrate range, a number of enzymes
use specific aldehydes as substrates. Examples for the latter include glyceraldehyde-3-phosphate
dehydrogenase [136], betaine aldehyde dehydrogenase [137], succinic semialdehyde
dehydrogenase [138], glutamic semialdehyde dehydrogenase [139], and formaldehyde
dehydrogenase [140]. NAD-AlDHs use NAD or, to a lesser extent, NADP as coenzyme. Most
enzymes exhibit a strong preference for either NAD or NADP, in agreement with the distinct
metabolic functions generally assigned to NAD (catabolic role) and NADP (anabolic role) [141].

The first report on a NAD-AlDH purification dates from 1949 [142] but the first NAD-
AlDH primary sequence was determined only in 1984 [143]. Now primary structures of more
than 30 NAD-AlDHs are known [144]. Subunit molecular masses range from 50 to 65 kDa [145-
149] and the subunits associate to homotetramers and sometimes homodimers. Instability of
these enzymes is frequently overcome by using a reducing agent during their purification [150].

During the last three decades much has been accomplished to understand the mechanism
of the NAD-AlDHs. Both a cysteine- and a glutamate residue are essential in catalysis, as was
concluded from a variety of inactivating chemical modifications and site directed mutagenesis
studies [151-155]. The cysteine functions as a nucleophile [156] whereas the glutamate acts as a general base to activate the nucleophile that leads to the initiation of the catalytic reaction [157]. A serine is believed to be involved in the enzyme-coenzyme interaction [158].

The typical glycine box (GXGXXG), a consensus sequence for the binding of the ADP moiety of NAD [159], is lacking in all of these NAD-AIDHs. However, all the glycine residues at the two regions postulated as putative NAD-binding sites in the human protein (positions 223-229 and 245-250) [143] are conserved for almost all NAD-AIDHs. The nucleotide binding domain is located to the amino-terminal half of these proteins as was concluded from limited proteolysis [160].

Many NAD-AIDHs show esterase activity as they are capable of hydrolyzing p-nitrophenyl acetate to p-nitrophenol and acetic acid. Nowadays resorufin acetate is also used to detect esterase activity [161]. Competitive behaviour between the aldehyde and ester and inhibition of the dehydrogenase as well as the esterase activity by bromoacetophenone indicate that an enzyme-thioester is an important intermediate in both reaction pathways [146,Chapter 5]. This has been confirmed by mutational analyses [153,158]. The characteristic stimulation of esterase activity by NAD(P)(H) has been reported for NAD-AIDHs from various sources [146,162-168]. The presence of NAD(P)(H) is proposed to increase the nucleophilicity of the active site cysteine [158,165] and hence to stimulate the attack of the nucleophile on the ester carbonyl-group to form a covalent intermediate.

4.2 Mammalian NAD(P)-dependent aldehyde dehydrogenases

Mammalian NAD-AIDHs are classified according to their primary sequences. Class 1 NAD-AIDHs include constitutive and inducible cytosolic enzymes, whereas class 2 NAD-AIDHs are localized in mitochondria. Both classes have been extensively studied, and are active in the oxidation of short-chain aliphatic and aromatic aldehydes [20]. Class 3 enzymes include tumor-specific and inducible cytoplasmic NAD-AIDHs along with constitutive microsomal NAD-AIDHs. NAD-AIDHs are found in a number of different locations within the body, including liver, stomach, kidney, eye and brain. Liver has been considered to possess the highest AIDH activity, and for certain classes of the enzyme this is indeed the case [20].

The different NAD-AIDHs classes have different substrate preferences. However, for
only a few NAD-AIDHs in a small number of tissues have putative physiological substrates been identified. In fact, it seems likely that a particular NAD-AIDH may be able to oxidize efficiently a number of aldehydes, the exact substrate varying with the tissue and/or physiological situation [20]. The cytoplasmic class 1 enzyme, however, appears to be involved in the conversion of retinaldehyde to retinoic acid, important in embryonic development and differentiation [169,170]. The enzyme has also been implicated in the metabolic inactivation of activated metabolites of the widely used anti-cancer and immunosuppressive agent cyclophosphamide and other members of the oxazaphosphorine class of DNA alkylating agents [16]. Class 2 liver mitochondrial NAD-AIDH is the major enzyme responsible for the oxidation of acetaldehyde to acetate [171,172]. Many Oriental people (about 50% of the population) produce an inactive form of this enzyme [173,174] due to mutation of one amino acid. They possess an enzyme with a lysine instead of a glutamate at position 487 [175-177]. These people accumulate toxic acetaldehyde after consumption of ethanol. This leads to alcohol-associated symptoms, such as facial flushing and nausea [178,179]. The class 2 enzyme may also be involved in the metabolism of monoamines (serotonin, dopamine, norepinephrine) after these amines have been converted into their corresponding aldehydes [180,181]. In contrast to class 1 and 2 enzymes, relatively little is known about the class 3 microsomal NAD-AIDHs. These enzymes have been found in rat liver [182-185], rabbit intestine [186,187], human liver [188] and human polymorphonuclear leukocytes [189]. Although their physiological role is not well defined, the substrate specificities of these enzymes suggest that they may be important in the detoxification of aldehydic products originating from lipid peroxidation [20,185,190], omega-oxidation of 20-CHO-leukotriene B4 [189], and oxidation of aldehydes formed during the fatty alcohol metabolism [186]. In the latter case a fatty AIDH is involved which shows high activity towards saturated and unsaturated aliphatic aldehydes ranging from 6 to 24 carbons in length. The class 3 cytoplasmic NAD-AIDH on the other hand is most likely involved in the metabolism of aminoaldehydes (aldehyde metabolites of diamines and polyamines) like γ-aminobutyraldehyde [191,192].

4.3 Structure

Recently, the first 3-D structure of a NAD-AIDH, the rat liver class 3 NAD-AIDH, has been determined by X-ray crystallography (at 2.6 Å resolution) [193]. This enzyme is a
homodimer and each subunit (452 residues) contains three domains, a NAD-binding domain, a catalytic domain and a bridging domain. A new mode of NAD binding, which differs substantially from the classic β-α-β binding mode associated with the ‘Rossmann fold’ [194], has been observed and is termed the β-α,β mode. The dinucleotide-binding fold is made up of 5 β-strands, connected by 4 α-helices, which is in contrast to the 6 β-strands usually found in the dinucleotide-binding fold of other NAD-dependent dehydrogenases [195]. Furthermore, the pyrophosphate group of NAD(P) and the GXTXXG binding motif are near the N-terminal end of helix αD instead of helix αA, as was found in other NAD-dependent dehydrogenases. The adenine ribose, however, forms two hydrogen bonds with Glu-140 from strand β2, which is similar to other NAD-dependent dehydrogenases [193].

4.4 Active site

The catalytic site is located at the bottom of a funnel-shaped passage and is composed of residues from all three domains mentioned above [193]. The cysteine residue (in class 3 NAD-AiDH Cys-243), the catalytic thiol, is positioned some 15 Å from the opening near the bottom of the passage, which is blocked by the nicotinamide ring. The side chain of Cys-243 extends into the centre of the channel with the catalytic thiol positioned 6.8 Å from the C4 of the nicotinamide ring of NAD. The surface of the channel between Cys-243 and the NAD-binding site contains a number of highly conserved residues suggesting similar catalytic environments for the class 1 and 2 enzymes [196]. Since conserved residues are found at key locations within the entire NAD-AiDH structure, the overall structural fold and the novel NAD-binding motif are likely also present in the class 1 and 2 members of the NAD-AiDH family.

4.5 Mechanism of action

The active site structure of rat liver class 3 NAD-AiDH suggests that the portion of the funnel, formed by residues from all three domains, provides the required specificity toward a particular aldehyde [193]. The structure of the lower portion of the funnel, formed by highly conserved residues from both the NAD-binding and the catalytic domains, as well as the presence of the catalytic thiol and nicotinamide C4, suggests that this is the catalytic site where hydride
transfer from aldehyde to NAD takes place. Since this mechanism is believed to be common for all aldehydes, a very similar catalytic site structure is anticipated for all NAD-AIDHs. First the cysteine (nucleophile) attacks the aldehyde to form a thiohemiacetal intermediate. The hydride transfer from the aldehyde to NAD requires the formation of a tetrahedral intermediate. The mechanism for the formation of such an intermediate complex is proposed in Fig. 10. In this simple model, the hydride is found to be almost coplanar with the NAD pyridine ring and little can be inferred about the specificity of hydride transfer [193]. After hydride transfer and formation of an acyl enzyme, the thioacyl intermediate will finally be hydrolyzed [146,197].

4.6 Phenylacetaldehyde dehydrogenase from Escherichia coli

The NAD-dependent phenylacetaldehyde dehydrogenase from E. coli (PAD) has been
studied extensively by us for several reasons [Chapter 5]; the gene for this enzyme is present in *E. coli* next to the gene of the aromatic quinoprotein amine oxidase (ECAO) [198] (transcribed in the opposite direction), an overexpressing strain was available, and bacterial NAD-AIDHs have rarely been characterized. Phenylacetaldehyde and 2-phenylethylamine are very good substrates for PAD and ECAO, respectively. The apparent *K_m* values for these substrates suggest them to be the natural substrates for PAD and ECAO. This finding, together with the observation that the gene of ECAO is adjacent to that of PAD in *E. coli*, suggest that the aromatic amine oxidation in *E. coli* proceeds via ECAO and PAD. The amino acid sequence of PAD, which suggests the enzyme to be cytoplasmatic, shows the highest levels of similarity to NAD-AIDHs from *E. coli* (50-95%).

Upon purification, PAD is quite labile, like certain other NAD-AIDHs. Highest enzyme stability is found for samples that are stored at very low temperatures (-80 °C) and pH 7.0. This pH value has also been found to be the most favourable one for storage of other NAD-AIDHs [150]. PAD has a molecular mass of 155-190 kDa, consists of 54 kDa subunits, and is proposed to be a homotetramer. For the few prokaryotic NAD-AIDHs which have been characterized, only tetrameric structures have been observed so far (with molecular masses of 160-220 kDa and consisting of 40-55 kDa subunits) [145-149].

NAD(P)-dependent oxidoreductases exhibit a strong preference for either NAD or NADP as coenzyme. The PAD activity is 20-fold higher with NAD than with NADP suggesting NAD to be the natural coenzyme. All the glycine residues at the region postulated as the putative NAD-binding site in the majority of NAD-AIDHs is conserved in PAD (positions 250-255: GSTATG).

The pH optimum of the PAD activity is 9.0 whereas the temperature optimum is at 65-70 °C, which is quite high compared to other NAD-AIDHs. Steady-state activity measurements show that the enzyme, NAD and aldehyde form a ternary complex before the substrate is converted. PAD has a wide substrate specificity, with a preference for long-chain aliphatic aldehydes and aromatic aldehydes and showed a very low enantioselectivity towards the racemic aldehydes tested. For many NAD-AIDHs it is known that a cysteine and glutamate, at positions 302 and 268, respectively, are involved in catalysis [151-155]. Since the cysteine and glutamate are also conserved in PAD, at position 306 and 272, respectively - the same 34 amino acid residue distance - and the enzyme is also inhibited by bromoacetophenone, this suggests a similar catalytic cycle for PAD as for other NAD-AIDHs.
Like other NAD-AIDHs, PAD shows dehydrogenase as well as esterase activity. The esterase activity is 17-fold higher in the presence than in the absence of NADH. This characteristic stimulation of esterase activity by NAD and NADH has been found for NAD-AIDHs from various sources, although the stimulation varied only from 2 to 11 fold [146,162-168]. The aldehyde is competitive with p-nitrophenylacetate in the active site indicating no distinct active sites for the dehydrogenase and esterase reaction and further that the ester could be an intermediate in the dehydrogenase reaction. Reducing agents stimulate dehydrogenase as well as esterase activities. These results indicate that PAD is most active when the catalytic thiol is in the reduced form. Bromoacetophenone, phenylmethanesulfonylfluoride (PMSF) and Hg^{2+} are effective inhibitors for the dehydrogenase and esterase activity of PAD. PMSF can react with serine as well as cysteine residues. It appears that removal of a serine residue (mutant enzyme) in NAD-AIDHs affects the coenzyme binding and the active site cysteine residue even in the absence of coenzyme since the mutant enzyme has a lowered specific esterase activity [158]. Addition of a reducing agent after the addition of these inhibitors produced partial reversal of enzyme inhibition which also indicates that the enzyme requires the presence of -SH groups (cysteine) for activity.

PAD appears to be quite similar to many other, including eukaryotic, NAD-AIDHs with respect to its sequence, structure, active site and mechanism of action. Since PAD can also be easily expressed in E. coli, the enzyme can be used as a model for eukaryotic NAD-AIDHs.

5. Applications of aldehyde dehydrogenases

5.1 Potentiometric detection of formaldehyde in air by an AIDH field-effect transistor

Biosensors have found limited exploitation in the quantitative and time-resolved monitoring of atmospheric pollutants. Formaldehyde, for example, a suspected carcinogen [199], is an automotive exhaust gas [200] and an important chemical in the manufacturing of a variety of consumer products and in performance of medical services [201]. For determining trace amounts of this aldehyde simple and sensitive methods are needed in the field of environmental control. Since several analytical methods require expensive and bulky instrumentation with high power demand and well-trained operators, a widespread detection and quantitation of
formaldehyde are not routinely performed. This explains the interest in development of a portable lowcost biosensor for real-time monitoring of this gas-phase pollutant. Recently a system for sampling atmospheric formaldehyde by dissolution followed by the monitoring of the aldehyde using an ion-sensitive field-effect transistor (ISFET) in conjunction with an enzyme specific for this pollutant has been developed [202]. Since formaldehyde is characterized by a high solubility in water, it was stripped from the atmosphere by pumping it concurrently with water. The enzyme, formaldehyde dehydrogenase from Pseudomonas putida, was chosen on the basis of its kinetic parameters (with formaldehyde as substrate) and generates the production of two protons, which can be sensed by the ISFET. Since several problems occur by immobilization of the enzyme, the enzyme was chosen to be added directly to the sampled solution. NAD was also added to the solution since the enzyme uses NAD as coenzyme. The working conditions were chosen to obtain a linear response of the sensor up to 200 µM formaldehyde. On the basis of the results obtained by the sampling system, the detection limit of 10 µM in aqueous solution, achieved by the ISFET biosensor, corresponds to an atmospheric concentration of the formaldehyde in the ppb range.

5.2 An aldehyde biosensor based on the determination of NADH enzymatically generated by AIDH

Several procedures as well as modified electrodes [203-206] have been reported for the electrocatalytic oxidation of NADH. Recently [207,208], an aldehyde biosensor based on the determination of NADH generated by the enzymatic activity of immobilized (on a nylon mesh membrane) AIDH, from baker’s yeast, has been described. The enzymatically generated NADH is, in turn, electrocatalytically oxidized at a glassy carbon electrode modified with an electopolymerized film of 3,4-dihydroxybenzaldehyde (3,4-DHB). This film exhibits very high and persistent electrocatalytic activity for the oxidation of NADH. The biosensor exhibits high sensitivity and a limit of detection in the micro molar range (5.0 µM), as well as a sufficiently rapid response (60 s to reach 90 % of its steady state value). Aromatic aldehydes were found to give superior results.
5.3 Class 1 and Class 3 AIDH levels in human tumor cell lines currently used by the National Cancer Institute of the U.S.A. to screen for potentially useful antitumor agents

To discover new anticancer agents, the National Cancer Institute of the U.S.A. uses a semiautomatic procedure [209] to annually evaluate thousands of compounds for their ability to inhibit the growth of each of a panel of 60 human tumor cell lines [210]. Class 1 and 3 AIDHs are established molecular determinants of cellular sensitivity to a subgroup of nitrogen mustards that are of substantial value in the treatment of several cancers and that, collectively, are known as oxazaphosphorines [211,212]. Class 1 and 3 AIDHs catalyze the detoxification of these agents (Fig. 11). As compared to nitrogen mustards that are not detoxified by these enzymes and, in the case of certain cancers, other antitumor agents that are also not detoxified by these enzymes, oxazaphosphorines usually exhibit a more favourable therapeutic index. This is largely because the AIDHs are present in the cytosol of certain, otherwise vulnerable, critical normal cells and because these enzymes are ordinarily absent, or present at very low levels, in certain types of tumors [211-215]. Quantification of AIDH expression in each of the National Cancer Institute’s panel of 60 human tumor cell lines and submission of this information to computer analysis appears to lead to the identification of cytotoxic agents bioinactivated (as well as bioactivated) by AIDH-1 and/or AIDH-3. These agents could be equally, or even more, therapeutically useful than are the oxazaphosphorines [216].

5.4 Yeast AIDH sensitivity to inhibition by chlorpropamide analogues as an indicator of human AIDH sensitivity to these agents

Human AIDH is a polymorphic enzyme that is relatively substrate-nonspecific. Several isoenzymes are found in human tissues. These catalyze the biotransformation (bioactivation and/or bioinactivation) of a broad spectrum of endogenous (biogenic) and exogenous (xenobiotic) aldehydes that are physiologically and/or pharmacologically important [20,211,212,217,218]. Inhibitors of these enzymes would be of experimental, and, in some cases, even of clinical value. Like disulfiram and cyanamide, the oral hypoglycemic agent chlorpropamide is thought to be a pro-inhibitor of the AIDHs which catalyze the oxidation of ethanol-derived acetaldehyde [219,220]. Based on this, a number of N1-substituted chlorprop-
Fig. 11. Metabolism of oxazaphosphorines including cyclophosphamide, mafosfamide and 4-hydroperoxycyclophosphamide. These prodrugs give rise to 4-hydroxycyclophosphamide which exists in equilibrium with aldophosphamide. These latter two compounds are without cytotoxic activity. However, aldophosphamide gives rise to acrolein and phosphoramidate mustards which are cytotoxic. Alternatively, aldophosphamide can be further oxidized to carboxyphosphamide by AlDHs. Carboxyphosphamide is without cytotoxic activity nor does it give rise to a cytotoxic metabolite. In this way the latter oxidation can be viewed as an enzyme-catalyzed bioinactivation (detoxification) of the oxazaphosphorines.

chlorpropamide analogues have been designed and synthesized as potential alcohol deterrents [221-224]. These compounds have been used to inhibit yeast-AlDH-catalyzed oxidation of acetaldehyde as the initial indicator of potentially useful alcohol-deterrent activity [221-225].
acids of the catalytic site are conserved [226], yeast and human AIDHs differ substantially with respect to their primary structure and catalytic properties [227,228]. Thus, the sensitivity of yeast-AIDH to a candidate inhibitor may not be identical to that of human AIDH, and the use of yeast-AIDH to screen for potential alcohol deterrents is not necessarily predictive. The yeast-AIDH model does, however, offer some advantages compared to human AIDH because it is commercially available, relatively inexpensive, and, in most cases, relatively more sensitive to candidate inhibitors [229]. Perhaps the most economical and rewarding approach would be to use yeast-AIDH in primary screening, thereby identifying virtually all candidates that have any possibility of being of value, and then submitting the agents thus identified to secondary screening in which inhibition of the human AIDH of interest would be evaluated.

5.5 Food component modification

AIDHs are also of interest to investigators in food science and technology. The enzymes are involved in reactions that reduce the levels of undesirable aldehydes and associated odors and in the formation of esters, major flavor compounds in some fruits [230]. Aldehydes can be produced from the action of lipoxygenase on polyunsaturated fatty acids as well as during metabolisms in plants and animals. In part, these aldehydes are unwanted as they may cause off-flavors in soybeans, for example [231]. AO has been proposed to oxidize the aldehydes to acids, with loss of odor. The same reaction can also be accomplished by a NAD-AIDH. However, whether application of these aldehyde oxidoreductases can work in practice is questionable (depends on production quantities and production costs). Conversion of aldehydes to acids or alcohols and subsequent ester formation is not only important in flavor development in several fruits, but also during the fermentation of wine.

6. Characterization techniques for redox enzymes

To obtain more insight into the structure and mechanism of action of the bacterial AIDHs (AIDH, FEDH, DL-AIDH and PAD), many different biochemical and biophysical techniques were applied. These techniques include cloning and expression of a gene, cultivation of bacteria (on a small, medium or large (fermentor) scale), isolation (cell disruption) and purification of
enzymes by using different kinds of chromatography (anionic- and cationic exchange chromatography, affinity chromatography, hydrophobic interaction chromatography and gel filtration chromatography), enzyme activity measurements, protein determinations, electrophoresis (native-PAGE, SDS-PAGE, activity staining, IEF), UV/Vis spectroscopy, HPLC and reversed-phase HPLC in combination with a photo-diode-array detector, gas chromatography, chemical analyses (determination of molybdenum, FAD, iron and acid-labile sulphur), EPR spectroscopy, redox potentiometry (in combination with UV/Vis or EPR spectroscopy), enzyme kinetics and determination of the N-terminal amino acid sequences which were subsequently compared with sequences present in protein Databanks. Although all these techniques were important for an extended enzymological characterization of the AIDHs, UV/Vis spectroscopy, EPR spectroscopy and redox potentiometry will be discussed in more detail.

6.1 Ultraviolet/Visible (UV/Vis) spectroscopy

With UV/Vis spectroscopy it is possible to monitor the transitions between ground- and excited states of molecules which can exist in a variety of excited states. Electronic transitions for organic molecules involve absorption of ultraviolet or visible radiation by electrons in $n$ (nonbonding), $\sigma$ or $\pi$ orbitals and result in their promotion to some higher energy antibonding orbital (excited state) [232]. The visible and ultraviolet spectra of proteins are associated only with transitions between electronic energy levels of certain types or groups of atoms within the protein and do not characterize the protein as a whole. The UV/Vis spectra of the bacterial AIDHs were recorded with an UV/Vis spectrophotometer which spans the wavelength region 190 to 800 nm by employing a deuterium lamp for the ultraviolet region and a tungsten lamp for the visible region.

UV/Vis spectra of enzymes can be used to determine the identity and specific properties of the cofactor(s). The first studies on AIDH, FEDH and DL-AIDH, for example, already suggested the possible presence of FAD and iron-sulphur clusters as UV/Vis spectra of the purified enzymes showed maxima at 280, 338, and 450 nm and a shoulder at 550 nm [Chapter 2 and 3]. Difference spectra of the purified enzymes minus the substrate- or dithionite reduced enzymes strongly supported this suggestion. The ratios $A_{350}/A_{550}$, determined from the UV/Vis spectra of the purified AIDHs, even indicated a comparable value to that reported for
molybdoprotein hydroxylases with a full complement of cofactors [233].

The fact that FAD and the iron-sulphur clusters, and to a lesser extend the Moco, show absorbance in the region from 310 to 600 nm can be very helpful to obtain more information on the bacterial AIDHs. In this way UV/Vis spectra can help to localize the cofactors in the enzyme and to establish the removal of a cofactor. For example, after isolation of the γ-subunit of AIDH, UV/Vis spectroscopy indicated the presence of a plant-type [2Fe-2S] cluster in this subunit [Chapter 2]. Furthermore, an UV/Vis spectrum of deflavo-AIDH confirmed the removal of FAD but also that of one [2Fe-2S] cluster [Chapter 2]. Restoration of the UV/Vis absorbance change under several different conditions in presence of FAD was not possible indicating that reconstitution was also not possible. UV/Vis spectroscopy can also be used to determine the redox state of an enzyme or a cofactor. In this way UV/Vis spectroscopy was a suitable method to help to determine the redox potentials of the FAD and the two different [2Fe-2S] clusters of AIDH [Chapter 2]. In case of a colourless enzyme such as PAD, which shows only a protein maximum at 280 nm [Chapter 5], UV/Vis spectroscopy is less helpful to characterize an enzyme.

6.2 Electron paramagnetic resonance spectroscopy (EPR)

EPR has been one of the core techniques in biochemistry and biophysics for nearly four decades [234]. The principle of EPR, also termed as electron spin resonance spectroscopy (ESR), is similar to that of nuclear magnetic resonance spectroscopy (NMR), but involving electron spins instead of nuclear spins. It describes the resonant absorption of microwave radiation by a paramagnetic substance in a static magnetic field [235]. Free radicals and transition-metal ions generally contain one or more unpaired electrons and can be studied by EPR [234]. The spectrometer consists of a resonant cavity which contains the sample. In an EPR experiment, the microwave frequency (ν) is fixed and the magnetic field (B₀) is varied. The most common frequency is X band (ν ~ 9-10 GHz, B₀ ~ 330 mT), but L-band (ν ~ 1-2 GHz), S-band (ν ~ 2-4 GHz), C-band (ν ~ 6-9 GHz), P-band (ν ~ 15 GHz), K-band (ν ~ 20 GHz), Q-band (ν ~ 35 GHz), W-band (ν ~ 95 GHz), D-band (ν ~ 140 GHz) and bands at even higher frequency (250-450 GHz) also exist. For technical reasons the derivative spectrum is displayed. The energy (ΔE) at which resonant absorption by an unpaired electron occurs is given by equation (1).
\[ \Delta E = h \nu = g \mu_B B_0 \]  

(1)

The proportionality constants \( h \) and \( \mu_b \) are Planck’s constant and the Bohr magneton for the electron, respectively [236]. Thus the resonance magnetic field is inversely related to the \( g \) factor which is easily calculated by rearranging the above equation:

\[ g = \frac{(71.4484 \nu)}{B_0} \]  

(2)

In proteins the paramagnetic species are transition metal ions or organic radicals. For enzymes containing more than one paramagnetic centre, magnetic coupling between two or more paramagnetic centres may occur. The strength of this magnetic coupling is a measure of the distance between the paramagnets [237-239]. EPR experiments of (metallo)proteins are usually performed at low temperatures in frozen solutions to slow down relaxation. As a consequence, temperature effects and anisotropy effects are important parameters to understand the EPR spectra [238].

EPR has also been applied in the study of the molybdoprotein AIDHs. Reduction of these enzymes, for example, leads to the formation of [2Fe-2S]\(^+\) which contains an unpaired electron so that it can be easily detected by means of EPR [Chapter 2 and 3]. Due to different relaxation properties and \( g \)-values it was possible to identify two nonidentical [2Fe-2S] clusters with EPR [Chapter 2]. In this way EPR has been used to identify some of the cofactors in AIDH, FEDH and DL-AIDH. EPR is also a quantitative technique. The intensity of the signals is directly related to the concentration allowing determination of the absolute and relative concentrations of the cofactors per enzyme molecule. The finding that the EPR signal of one the [2Fe-2S] clusters was affected by that of the other indicates magnetic interaction between the clusters suggesting the clusters to be near to each other [Chapter 2]. To elucidate the structure of a new paramagnetic centre with an unknown EPR signal, it can be very helpful to perform isotope substitution experiments. This method was used to elucidate the nature of the organic radical that was observed with EPR for the bacterial molybdoprotein AIDHs [Chapter 4]. After substitution of \(^{14}\)N with \(^{15}\)N and/or replacing \( \text{H}_2\text{O} \) for \( \text{D}_2\text{O} \), and \( ^{95}\text{Mo} \) or \( ^{98}\text{Mo} \) in AIDH, the EPR signal could be assigned to a trihydropterin radical. EPR can also be helpful to determine the redox potentials of paramagnetic centres. In this way the redox potentials of the cofactors
present in ALDH have also been determined (see section 6.3, [Chapter 2]).

6.3 Redox potentiometry

To understand the energetics of biological electron-transfer processes redox potentiometry can be very helpful [240,241]. With redox potentiometry the midpoint potentials of the oxidation-reduction components, present in biological electron-transfer systems, can be determined. The midpoint potential is a thermodynamic parameter and its value determines the position of the equilibrium of a redox system. This can be accomplished by measuring the potential, $E_n$, and the corresponding state of oxidation or reduction of a redox couple. The redox state is measured by some form of spectrometry, e.g., EPR or UV/Vis, the $E_n$ is measured by electrodes [240]. These electrodes include a measuring and a reference electrode. The first one is a strip of platinum fused into a glass rod but in direct contact with the aqueous solution containing the redox system under assay. The reference electrode, usually a standard saturated KCl calomel electrode, is made up of a metal in contact with an insoluble salt in turn in contact with a solution containing the chloride. In this case contact with the solution containing the redox system under assay is made via a salt bridge [240].

$E_n$ is expressed in the well-known Nernst equation which has been derived from the equation for the standard free energy, $\Delta G^\circ$, of a reaction [240,242]:

$$E_n = E_0 + \left(\frac{RT}{nF}\right) \ln (\text{Ox}/\text{Red})$$  \hspace{1cm} (3)

In this equation, $E_0$ is the standard redox potential (in V), R, gas constant (8.3144 J/mol.K), T, temperature (in K), n, number of electrons transferred in the reaction, F, the Faraday constant (96485 C/mol), Ox, oxidized fraction of the redox couple, and Red, the reduced fraction of the redox couple. In general for work done at $\sim 29$ °C the $RT/F$ term together with a change to log$_{10}$ simplifies the expression to:

$$E_n = E_0 + (0.06/n) \log (\text{Ox}/\text{Red})$$  \hspace{1cm} (4)

The symbol for the standard redox potential, $E_0$, under conditions other than pH zero or unit
activities is $E_m$ or $E_{\text{max}}$ where $m$ stands for midpoint and $x$ is the pH. Since in many redox equilibria protons cannot be ignored, the $E_m$ is often pH dependent. For a redox reaction in which the pH is in between widely separate values for $pK_{\text{ox}}$ and $pK_{\text{red}}$ and in which the number of protons and electrons is the same [240]:

$$E_{\text{max}} = E_{m0} - 0.06 \text{ pH}$$ (5)

Since a redox centre of a protein is often shielded by the protein itself, it generally cannot make proper contact with the electrode surface. To achieve proper contact, redox mediators are required to act as go-betweens between the measuring electrode and the redox centre [240]. These mediators are small organic (e.g., quinones) or inorganic redox agents which have to meet certain requirements to be effective. Except that mediators (concentration range $10^{-6}$-$10^{-3}$ M) must react effectively and reversibly with the electrode and the biological redox component, it is important that the rates of the electrode-mediator-biological component reaction must be rapid enough to achieve an equilibrium [242]. The effectiveness of relatively weakly interacting mediators can be improved by increasing their concentration. Mediators must also be stable and not decompose on oxidation or reduction. Furthermore, mediators must not chemically modify the biological component and should not interfere with accompanying measurements of the state of reduction of the redox couple under assay [240].

To obtain information on the redox properties of the prosthetic groups of AIDH, redox potentiometry was performed. AIDH contains one molybdenum centre, one FAD, and two nonidentical [2Fe-2S] clusters. Midpoint potentials of these latter cofactors were determined using room temperature UV/Vis spectroscopy and low temperature EPR to follow the change in oxidation state of the redox centres [Chapter 2]. UV/Vis spectroscopy appeared to be a suitable method to determine the redox potentials of the FAD and [2Fe-2S] clusters of AIDH since the redox properties could be determined by selection of specific wavelengths of the complete spectrum. Whereas at 450 nm all these latter cofactors show absorbance, at 550 nm the absorbance is only due to the two [2Fe-2S] clusters. With the following equation, derived from equation (4), the absorbance at 450 nm at different potentials was simulated:
\[ A_{450} = A_{\text{red}} + \Delta A_{\text{FAD/FADH}^0} (10^8 (((E_n + E_{m\text{FAD/FADH}^0}) n)/58.54))/(1+(10^8 (((E_n + E_{m\text{FAD/FADH}^0}) n)/58.54))) + \]
\[ \Delta A_{\text{FADH/FADH}^2} (10^8 (((E_n + E_{m\text{FADH/FADH}^2}) n)/58.54))/(1+(10^8 (((E_n + E_{m\text{FADH/FADH}^2}) n)/58.54))) + \]
\[ \Delta A_{\text{[2Fe-2S]}^{-1}} (10^8 (((E_n + E_{m[2Fe-2S]^{-1}}) n)/58.54))/(1+(10^8 (((E_n + E_{m[2Fe-2S]^{-1}}) n)/58.54))) + \]
\[ \Delta A_{\text{[2Fe-2S]}^{-2}} (10^8 (((E_n + E_{m[2Fe-2S]^{-2}}) n)/58.54))/(1+(10^8 (((E_n + E_{m[2Fe-2S]^{-2}}) n)/58.54))) \] (6)

in which \( \Delta A_{\text{FAD/FADH}^0} \), \( \Delta A_{\text{FADH/FADH}^2} \), \( \Delta A_{\text{[2Fe-2S]}^{-1}} \) and \( \Delta A_{\text{[2Fe-2S]}^{-2}} \), \( E_{m\text{FAD/FADH}^0} \), \( E_{m\text{FADH/FADH}^2} \), \( E_{m[2Fe-2S]^{-1}} \), and \( E_{m[2Fe-2S]^{-2}} \) represent the relative optical absorbances (absorbance oxidized state minus absorbance reduced state) and midpoint potentials, respectively, of the redox couples FAD/FAD\(^+\), FADH\(^+\)/FADH\(^+\), [2Fe-2S]\(^{-1}\) and [2Fe-2S]\(^{-2}\), respectively. \( A_{\text{red}} \) represents the absorbance at 450 nm of fully reduced AIDH. Since the redox titrations were performed at room temperature and \( E_n \) and \( E_m \) are now in mV, the RT/F term becomes 58.54. In case of the simulation of the absorbance at 550 nm, equation (6) was simplified to:

\[ A_{550} = A_{\text{red}} + \]
\[ \Delta A_{\text{[2Fe-2S]}^{-1}} (10^8 (((E_n + E_{m[2Fe-2S]^{-1}}) n)/58.54))/(1+(10^8 (((E_n + E_{m[2Fe-2S]^{-1}}) n)/58.54))) + \]
\[ \Delta A_{\text{[2Fe-2S]}^{-2}} (10^8 (((E_n + E_{m[2Fe-2S]^{-2}}) n)/58.54))/(1+(10^8 (((E_n + E_{m[2Fe-2S]^{-2}}) n)/58.54))) \] (7)

Now, \( A_{\text{red}} \) represents the absorbance at 550 nm of fully reduced AIDH. Besides the absorbance at 450 and 550 nm due to the FAD and [2Fe-2S] clusters, UV/Vis absorbance at 620 nm indicates the formation of a blue neutral flavin semiquinone (FADH\(^+\)). The redox potentials of the [2Fe-2S] clusters and FADH\(^+\) could also be determined with EPR [Chapter 2]. By comparing the potentials obtained with the UV/Vis spectrophotometric method with those of EPR, complications due to, e.g., freezing the enzyme can be indicated. In case of AIDH similar redox potentials were obtained by using the two spectroscopic methods indicating no complications due
to freezing. Whereas it is not possible to determine the midpoint potential of the Mo(VI)/Mo(IV) couple by the optical method, as molybdenum shows no absorbance, it is possible with EPR via detecting Mo(V) [Chapter 2].

7. The outline of the thesis

Oxidation of alcohols and amines by quinoprotein and nicotinoprotein bacterial oxidoreductases is one of the main topics in our research group. Since the first oxidation step in both conversions yields aldehydes and the fate of the aldehydes is relevant in the outcome of the enzymatic kinetic resolution of racemic alcohols, it was decided in first instance to study the role of the already known molybdoprotein AIDH from C. testosteroni [243] in the kinetic resolution of alcohols by this organism. Since we found that this AIDH shows very low enantioselectivity for a variety of racemic aldehydes [Chapter 2], it was attempted to reveal the reason for this by performing structural and mechanistic studies on this enzyme as well as that of a quite different bacterium, A. methanolica [Chapter 2]. Since indications existed [244] that the latter organism contained a second dye-linked AIDH whose identity was unknown, this enzyme was isolated and characterized ([Chapter 3], in a joint effort with Dr. S.W. Kim). Since EPR of the as isolated AIDHs revealed for the first time a free radical form of the pterin moiety of the Moco, we attempted to elucidate its structure and to answer the question whether the pterin moiety has a structural or catalytic role [Chapter 4]. Studies conducted in our research group and that of Dr. Postma, University of Amsterdam, revealed that a gene for an AIDH might be present in E. coli next to the gene of quinoprotein amine oxidase [198]. Since an overexpressing strain was available, the enzyme was purified and characterized [Chapter 5]. During the final stage of this project, similar results were reported as ours.
REFERENCES


Chemicals to Humans in Some Industrial Chemicals and Dyestuffs, Vol. 29, Lyon, France.


96, 7152.


pp. 203-221, New York.


Chapter 2

Characterization of molybdoprotein aldehyde dehydrogenases from *Comamonas testosteroni* and *Amycolatopsis methanolica*

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**Abbreviations.** AIDH, aldehyde dehydrogenase from *Comamonas testosteroni*; DL-AIDH, (dye-linked) aldehyde dehydrogenase from *Amycolatopsis methanolica*; E, enantiomeric ratio; FEDH, formate ester dehydrogenase from *Amycolatopsis methanolica*; HMP, hypoxanthine monophosphate; MCD, molybdopterin cytosine dinucleotide; MGD, molybdopterin guanine dinucleotide; Moco, pterin molybdenum cofactor; MPT, molybdopterin; PVDF, polyvinylidene fluoride.

**Enzymes.** Aldehyde dehydrogenase (EC 1.2.99.3, haem c-containing); xanthine oxidase (EC 1.1.3.22); xanthine dehydrogenase (EC 1.1.1.204); aldehyde oxidase (EC 1.2.3.1).

The sequences of the stretches of amino acids determined for the subunits of AIDH and FEDH have been deposited at the EBI Data Library under accession numbers: p80704, p80705, p80706, and p80707.
The purification procedure of molybdoprotein aldehyde dehydrogenase from *Comamonas testosterone* (AIDH) has been improved and its properties have been compared to those of one of the molybdoprotein aldehyde dehydrogenases from *Amycolatopsis methanolica* (FEDH). Both enzymes consisted of three-subunits, in unit stoichiometry, with molecular masses of 86, 30, 16 kDa and 96, 38, 22 kDa, respectively. AIDH and FEDH contained FAD, iron, acid-labile sulphide, molybdenum and CMP in molar ratios of 1.0:4.3:3.9:0.9:0.8 and 1.0:3.5:3.5:0.8:0.9, respectively. The pterin molybdenum cofactor has been identified as a molybdopterin cytosine dinucleotide. The N-terminal sequences of the subunits of AIDH and FEDH showed greatest similarity with other molybdopterin cytosine dinucleotide-containing enzymes (32-55 %). AIDH contained two nonidentical [2Fe-2S] clusters which showed magnetic interaction with each other. Redox potentials for [2Fe-2S]-1 and [2Fe-2S]-2 were found to be -138 and -263 mV at pH 7.00, respectively, and -257 and -333 mV at pH 9.00, respectively, indicating protonation of a site close to these clusters upon reduction. An EPR signal of a blue flavosemiquinone, accounting maximally for 20 ± 5 % of the enzyme concentration, was present at pH 7.00 but not at pH 9.00. The redox potential of the FAD/FADH₂ couple was found to be -68 mV at pH 7.00 and -123 mV at pH 9.00. No significant amount of Mo(V) EPR signal was detected during redox titrations. The purified γ-subunit of AIDH contained only the plant-type [2Fe-2S] cluster as judged from the properties of the optical spectrum of the oxidized cluster. The second [2Fe-2S] cluster was lost during isolation. The reduced γ-subunit was unstable as concluded from bleaching of the optical spectrum and its EPR silence. Deflavo-AIDH still contained the plant-type [2Fe-2S] cluster but had lost the same [2Fe-2S] cluster as the purified γ-subunit. An optical difference spectrum (deflavo-AIDH minus γ-subunit) revealed an absorbance maximum at 370 nm, suggesting that in the deflavo-enzyme the pterin-moiety is in the fully oxidized state. As substrates AIDH and FEDH preferred aliphatic and aromatic aldehydes with a short aliphatic chain. Both enzymes showed a very low enantioselectivity towards racemic aldehydes. Benzaldehyde and 3-phenylpropanal behaved like competitive inhibitors of AIDH but were good substrates for FEDH, indicating structural differences between the active sites of AIDH and FEDH. Benzaldehydes substituted with an electron withdrawing group were, however, good substrates for AIDH. AIDH and FEDH exhibit structural, cofactor, spectroscopic and catalytic properties that
are in many respects similar to other molybdenum-containing hydroxylases of both bacterial and eukaryotic origin, although differences in the redox potentials of the prosthetic groups exist. The basis for the variation in the properties of the class of molybdoproteins is discussed.

*Keywords.* aldehyde dehydrogenase; molybdopterin cytosine dinucleotide; iron-sulphur clusters; EPR; redox potentiometry.
INTRODUCTION

Molybdenum containing enzymes play varied and important roles in prokaryotic and eukaryotic organisms. The molybdenum forms part of an FeMo-cofactor, present in nitrogenase (MoFe-protein), or of a pterin molybdenum cofactor (Moco) [1-5]. Moco containing enzymes catalyze hydroxylation and oxo-transfer reactions, these events taking place at the molybdenum centre. Additional redox centres, like iron-sulphur clusters, flavins or haem centres [2,6,7], may also be present and are assumed to be involved in intramolecular electron transfer. Reactions catalyzed by these molybdoenzymes are two-electron redox reactions in which the hydroxyl group incorporated into the product is derived from water [4]. The common component of all forms of the Moco is molybdopterin (MPT). It was demonstrated that all eukaryotic molybdoenzymes contain an unmodified form of MPT [8]. The first variant form of the Moco was reported for carbonmonoxide dehydrogenase from Pseudomonas carboxydoflava which was determined to be a molybdopterin cytosine dinucleotide (MCD) [9-12]. Later it appeared that other prokaryotic molybdoenzymes contain an alternative version of the Moco with either an AMP [13], GMP [13-16] or HMP [13] nucleotide attached to the MPT via a pyrophosphate linkage. However, an apparent exception is xanthine dehydrogenase from prokaryotic organisms [13,17] which like the eukaryotic enzyme contains MPT without a nucleotide.

The dimeric xanthine oxidase from bovine milk [18], a widely studied representative member of the family of molybdoenzymes, contains the unmodified MPT, two different [2Fe-2S] clusters and one non-covalently bound FAD per subunit. On the basis of a variety of spectroscopic studies, mechanisms for the hydroxylation reaction have been proposed but have suffered from lack of knowledge on the 3-D structure. Recently, the structures of the aldehyde oxidoreductase from Pyrococcus furiosus [19], containing a bis-pterin tungsten cofactor, the DMSO reductase from Rhodobacter sphaeroides [20], containing two molybdopterin guanine dinucleotide (MGD) cofactors, the formate dehydrogenase H from E. coli [21], containing two MGD cofactors, and the aldehyde oxidoreductase from Desulfovibrio gigas [22], containing a MCD, have been determined by X-ray crystallography. The latter enzyme is structurally related to xanthine oxidase, is a homodimer and catalyzes the oxidation of aldehydes to carboxylic acids. In addition to MCD, the enzyme contains also two different types of [2Fe-2S] clusters but lacks the FAD. For this enzyme the MCD is found to be deeply buried in the protein and accessible
through a 15 Å deep tunnel along which aldehydes are suggested to pass [22,23].

Molybdenum aldehyde oxidoreductases have also been found in aerobic bacteria, in Gram-negative as well as in Gram-positive representatives [7,24-26]. Although the natural electron acceptors of these enzymes are unknown, from the fact that artificial dyes but not O₂ act as electron acceptor, they are called dehydrogenases. Further differentiation in names has been based on the observation that the enzymes convert aldehydes into acids (AIDH [24] and (dye-linked) aldehyde dehydrogenase from Amycolatopsis methanolica (DL-AIDH) [26], being dissimilar in several properties) and formate esters into pyrocatechol esters (FEDH, formate ester dehydrogenase; this name was introduced [25] when it was found for the first time that such a type of aldehyde dehydrogenase exhibited this activity, the property thought to be unique; the latter appears to be incorrect now since all three aldehyde dehydrogenases show it [26, vide infra]; however, the name is still used here for practical reasons). Preliminary characterizations revealed that the enzymes contain one Moco, four iron, four sulphur and one FAD [24,25], i.e., they belong to the class of molybdenum hydroxylases. To enable comparison of these aldehyde dehydrogenases with the better known molybdenum proteins, such as xanthine oxidase [18] and aldehyde oxidoreductase from D. gigas [22], a more detailed characterization of AIDH and FEDH was carried out including potentiometric titrations of the various prosthetic groups of AIDH at low temperature and room temperature. To obtain insight into how the structural differences observed amongst the aldehyde dehydrogenases is reflected in the mechanistic features, the substrate specificity and enantiomericity of AIDH were determined and compared with those of FEDH.

MATERIALS & METHODS

Microorganisms and culture conditions. Comamonas testosteroni strain ATCC 15667 was cultivated aerobically at 30°C on a mineral medium, supplemented with 0.3 % (v/v) butanol [27]. The mineral medium contained (per litre) [27]: K₂HPO₄, 11.8 g; KH₂PO₄, 4.5 g; (NH₄)₂SO₄, 3 g; FeCl₃·6H₂O, 0.04 g; tricine, 0.05 g; CaCl₂·2H₂O, 0.015 g; MgCl₂·6H₂O, 0.5 g; trace-element solution, 0.1 ml [28]. The trace-element solution contained (per 100 ml): CuSO₄·5H₂O, 7.8 mg; H₃BO₃, 10 mg; MnSO₄, 10 mg; ZnSO₄·7H₂O, 70 mg; Na₂MoO₄·2H₂O, 17 mg. Amycolatopsis methanolica NCIB 11946 was cultivated as described by Van Opheum et al. [25]. Cells were
harvested at the end of the exponential growth phase, washed with 20 mM Mops buffer, pH 7.5, and disrupted twice using a French pressure cell at 10 MPa. The viscosity of the suspension was lowered by adding DNase (Fluka) and MgCl₂ (1 mM). The suspension was centrifuged at 250,000 x g for 90 min at 4 °C, the supernatant yielding the cell-free extract.

**Enzyme assays.** Activities for AIDH were determined aerobically at room temperature by measuring the rate of Wurster’s blue reduction at 600 nm (ε = 9000 M⁻¹cm⁻¹) [29] in a 1 cm optical path spectrophotometer cell. The optimized assay mixture (1 ml) consisted of 50 mM potassium phosphate buffer, pH 8.2, 100 μM Wurster’s blue and 10 μl of enzyme solution. The reaction was started by the addition of 10 μl acetaldehyde (100 μM). Under these experimental conditions 1 enzymatic unit corresponds to 2 μmol of Wurster’s blue reduced per minute. In case of cell-free extract, the assay mixture also contained 1 mM KCN in order to prevent the reoxidation of reduced Wurster’s blue via components of the respiratory chain. The enzyme assay for FEDH was as described by Van Ophem et al. [25], except that ethyl formate (10 mM) was replaced by acetaldehyde (0.1 mM) as substrate. Protein determinations were performed according to Bradford [30] and Pierce [31] with desalted bovine serum albumin as standard. Absorption coefficients of the purified proteins were determined according to van Iersel et al. [32] using the equation: 

\[
A_{280}^{0.1%} = 34.14 \frac{A_{280}}{A_{205}} - 0.02.
\]

Substrate specificities of AIDH and FEDH were examined with their standard enzyme assays in which acetaldehyde was replaced by the substrates to be tested. The concentrations of the substrates varied from 0.01 to 100 mM.

To test putative inhibitors for their effect on the enzymatic activity, they were mixed together with the AIDH in 50 mM potassium phosphate buffer, pH 8.2, containing 100 μM Wurster’s blue, followed by the addition of propanol (0.1, 0.3, 0.5, 1.0 mM). The inhibitors tested were benzaldehyde and 3-phenylpropanal in concentrations of 0, 0.25, 0.50, 1.00, 1.50, 2.00 and 3.00 μM.

**Enantioselectivity determinations.** The enantioselectivity of AIDH and FEDH towards racemic aldehydes has been determined for racemic 2-ethylhexanal, 3,5,5-trimethylhexanal, 3-phenylbutanal and 2-phenylpropanal and the enantiomeric ratios (E) have been calculated according to the equation reported by Chen et al. [33]. For that purpose, during the conversion of the substrate the ratio between the R,S-enantiomers of the remaining substrate has been analyzed by GC. The R,S-enantiomers of 2-ethylhexanal and 3,5,5-trimethylhexanal could be
separated on a Chiraldex G-TA capillary column (20 m x 0.25 mm I.D. filmthickness 0.125 μm; Astec, Whippany, NJ, USA) and of 2-phenylpropanal and 3-phenylbutanal on a CP-cyclodextrin-2,3,6-M-19 capillary column (25 m x 0.25 mm I.D. filmthickness 0.25 μm; Chrompack, Middelburg, The Netherlands). Chromatography was performed with a Hewlett-Packard Model 5890 Series II gas chromatograph with a split injector, a flame ionization detector and an integrator (Hewlett-Packard 335 Chemstation). The columns were operated at 65 °C. Nitrogen was used as the carrier gas and the inlet pressure was 56 kPa for the column. A split flow of 100:1 was used. The injector and detector temperatures were kept constant at 200 and 250 °C, respectively.

The conversion of the racemic aldehydes started with a mixture consisting of 15.7 ml 50 mM potassium phosphate buffer, pH 8.2, 160 μl Wurster’s blue (100 μM) and 160 μl of a racemic aldehyde (1 mM). A 4 ml fraction of the mixture was sampled at the start (0 % conversion). To the 12 ml mixture, 2.5 μl enzyme (30 nM) was added. After all Wurster’s blue was reduced (colourless solution) again a 4 ml fraction was collected. To the remaining 8 ml mixture again 160 μl Wurster’s blue was added. After decolouration again 4 ml was collected. Finally 160 μl Wurster’s blue and 2 μl enzyme solutions were added to the remaining 4 ml assay mixture. After decolouration all four 4 ml fractions were analyzed by GC. Samples were obtained by extracting the R,S-aldehydes from the 4 ml assay mixtures with 5 ml of a heptanone/dry diethyl ether (10:1:25 (v/v)) mixture. Subsequently, the diethyl ether extract was dried with MgSO₄ and the solvent was evaporated with a stream of nitrogen gas until a residue of approximately 50 μl remained. The injection volume of the sample was 0.2 μl. The conversion of the aldehydes was determined by comparing the integrated peaks of the R,S-aldehydes with the integrated peak of heptanone.

Enzyme purification. All purification steps for ALDH were performed at 5 °C. After centrifuging the crude extract, the supernatant was loaded on a DEAE Bio-Gel agarose column (5 x 20 cm, Bio-Rad) equilibrated with 20 mM Mops buffer, containing 10 mM NaCl, pH 7.5. After charging, the column was washed with the same buffer. Proteins were eluted with 3 bedvolumes of a linear gradient of NaCl (10 mM - 400 mM) in 20 mM Mops buffer, pH 7.5, at a flow rate of 1 ml/min. Active fractions were pooled and applied to a DEAE-Sepharose column (2.5 x 24 cm, Pharmacia) also equilibrated with 20 mM Mops buffer, containing 10 mM NaCl, pH 7.5. The column was washed with the same buffer. The adsorbed enzyme was eluted with 3
bedvolumes of a linear NaCl gradient (10 mM - 400 mM) in 20 mM Mops buffer, pH 7.5, at a flow rate of 1 ml/min. Fractions exhibiting enzymatic activity were combined and applied to a hydroxylapatite column (2.5 x 10 cm, Bio-Rad) equilibrated with 25 mM potassium phosphate buffer, pH 7.5. A flow rate of 0.5 ml/min was applied. The enzyme passed through under these conditions, so the column was washed with 25 mM potassium phosphate buffer till activity was absent from the eluate. After pooling the active fractions, ammonium sulfate (0.5 M) was added to the solution before it was loaded on a phenyl-Sepharose CL-4B column (2.5 x 9 cm, Pharmacia) equilibrated with 20 mM Mops buffer, pH 7.5, containing 0.5 M ammonium sulfate. The protein was eluted with 15 bedvolumes of decreasing ammonium sulfate gradients from 0.5 to 0.1 M and 0.1 to 0 M in 5 mM Mops buffer, pH 7.5, at a flow rate of 1 ml/min. The active fractions were pooled, concentrated to a final enzyme concentration of 30 mg/ml and stored at -80 °C. FEDH has been purified as described by Van Ophem et al. [25].

**Gel electrophoresis.** The homogeneity of the native AIDH and FEDH was determined by PAGE on commercial gradient gels (8-25 %, Pharmacia) using the Phast system equipment (Pharmacia). The gel was calibrated with native high molecular mass markers (Pharmacia). The subunit compositions and masses of pure AIDH and FEDH were determined by SDS-PAGE, using low molecular mass protein standards from Pharmacia. Denaturation of the proteins occurred by bringing the solution at 5 % (w/v) SDS and 10 % (v/v) β-mercaptoethanol and heating the mixture for 20 min at 100 °C.

**Subunit stoichiometry.** For AIDH, 3 μl (6.7 mg/ml) of enzyme solution was mixed with 5 μl of 10 % (w/v) SDS and 20 % (v/v) β-mercaptoethanol after which the mixture was heated for 20 min at 100 °C. After cooling, the solution was applied to a gel filtration column (Superdex-200, 1 x 30 cm, Pharmacia) equilibrated with 50 mM sodium phosphate buffer, pH 7.2, containing 0.1 % (w/v) SDS and 0.1 M NaCl. Chromatography occurred at a flow rate of 0.5 ml/min and the eluate was monitored with a photodiode array detector at 205 nm. Since the absorbance value at 205 nm is directly related to the protein concentration [32], the molar ratios of the subunits of AIDH were calculated by dividing the surface area of their peaks in the chromatogram by their respective molecular masses. For FEDH, 2 μl (6.9 mg/ml) of enzyme solution was mixed with 5 μl denaturation mixture as described for AIDH, following the same procedure.

**Metal and cofactor determination.** The FAD content was determined after denaturing
the enzyme with trichloroacetic acid (5%), removing aggregated protein by centrifugation [24], and measuring the absorbance of the supernatant at 450 nm (using a molar absorption coefficient of 11300 M⁻¹ cm⁻¹ [34]). The content of iron was determined colorimetrically by the method of Pierik et al. [35]. Ferrene was used as an iron chelator [36]. Acid-labile sulphide was extracted by zinc acetate treatment and determined by methylene blue as described by Beinert [37]. The spectrophotometric method described by Cardenas and Mortenson [38] was used for the quantitative determination of molybdenum. For the identification of the Moco, the methods described by Hettrich et al. [12] and Johnson et al. [14] were used. The denaturated protein was removed by centrifugation and the supernatant was applied to a reversed-phase HPLC column (Lichrospher 100 RP-18, 5 μm, Merck) using 20 mM KH₂PO₄, pH 3.5, as eluant at a flow rate of 0.5 ml/min. The identity of the nucleotide, assumed to be part of the Moco, was determined by chromatographic comparison, as indicated by Frunzke and Meyer [39].

Localization of the cofactors. AIDH was applied to a gel filtration column (Superdex-200, 1 x 30 cm, Pharmacia) equilibrated with 50 mM sodium phosphate buffer, pH 7.5, containing 0.1 M NaCl and 3 M guanidine. The enzyme solution (15 μl, 47.0 mg/ml) was directly applied to the column. Chromatography occurred at a flow rate of 0.5 ml/min and the eluate was monitored with a photodiode array detector at 280, 450 and 550 nm.

Preparation of deflavo-AIDH. AIDH (20 μl, 33.2 mg/ml) was mixed aerobically with 580 μl 0.1 M Tris/HCl buffer, pH 7.5, containing 0.2 mM EDTA. Then 400 μl 5 M CaCl₂ (Suprapur, Merck) was added to give a final concentration of 2 M [40]. The enzyme solution was kept on ice for 180 min and subsequently passed through a gel filtration column (Sephadex G-25 M, PD-10, Pharmacia) equilibrated with 0.1 M Tris/HCl, pH 7.5, containing 0.4 mM EDTA, at 5 °C. The collected coloured fractions were incubated with 10 mM cysteine overnight in ice and passed through a second gel filtration column (Sephadex G-25 M, PD-10, Pharmacia) equilibrated with 50 mM potassium phosphate buffer, pH 7.5, containing 0.2 mM EDTA, at 5 °C. The deflavo-enzyme was concentrated with a Microcon-10 concentrator (Amicon) at 5 °C.

Absorption spectra. UV/Vis-spectra were measured in cells of 1 cm path length at room temperature with a Hewlett-Packard HP 8452 A Diode Array spectrophotometer.

EPR spectroscopy. EPR spectra at X-band frequencies were recorded with a Varian E-9 spectrometer. The magnetic field and microwave frequency were determined with an AEG Kernresonanz-Magnetfeldmesser and a HP 5245L electronic counter, respectively. This
spectrometer is equipped with a home-built He-flow system.

**Potentiometric titrations.** Potentiometric titrations were carried out at room temperature in a borosilicate glass cell similar to that described by Dutton [41] using modifications of techniques detailed earlier [42]. The cell was fitted with a platinum electrode and a calomel reference electrode (Radiometer, REF 401) calibrated against a standard solution of quinhydrone (Fluka) with a pH meter (Metrohm 632). Redox equilibration between the protein and the indicating electrode was achieved by the use of a mixture of dye mediators. The following mediator dyes were used: phenazine methosulfate (80 mV, Sigma), phenazine ethosulfate (55 mV, Sigma), indigo tetrasulfonic acid (-46 mV, Aldrich), indigocarmin (-125 mV, Aldrich), 2-hydroxy-1,4-naphthoquinone (-145 mV, Sigma), anthraquinone-2,6-disulfonic acid (-185 mV, Fluka), anthraquinone-2-sulfonic acid (-225 mV, Fluka), phenoasfranine (-255 mV, Sigma), benzylviologen (-311 mV, Sigma), bromocresol purple (-410 mV, J.T. Baker) and methylviologen (-440 mV, Sigma). AIDH was first passed through a gel filtration column (Sephadex G-25 M, PD-10, Pharmacia) equilibrated with pH 7.00 buffer (26 mM KH₂PO₄ + 41 mM Na₂HPO₄) or pH 9.00 buffer (50 mM H₂BO₃ + 50 mM KCl + 22 mM NaOH). The reduction-oxidation titrations were carried out with 5.5 ml of 12.5 µM AIDH in pH 7.00 or 9.00 buffer, containing the mediator dyes (10 µM each). The solution was stirred throughout the experiment by a magnetic stirrer. The redox potential of the system was adjusted by addition of small volumes of 0.1 M dithionite (Merck) or 0.1 M ferricyanide (Sigma) via a Hamilton gastight syringe. The solution of enzyme and mediators was made anaerobic by thoroughly flushing with nitrogen gas and the components allowed to reach equilibrium before the first UV/Vis spectrum was taken. Then a small volume of dithionite was added to the solution. After equilibrium was attained (constant readings of absorbance and potential), a spectrum was recorded and the next portion of dithionite was added. The process was repeated until both AIDH and the redox indicator dyes were completely reduced. Now small amounts of ferricyanide were added to the solution and in between spectra were recorded until AIDH and the redox indicator dyes were completely reoxidized. This reduction-reoxidation titration was repeated with the same solution except that it lacked the enzyme. Spectra were recorded at the same potentials as during the titration in presence of AIDH. Finally, the latter spectra were subtracted from the spectra obtained in presence of AIDH and the mediator dyes.

**EPR titration.** This titration was carried out in a standard potentiometric vessel described
by Dutton [41] in which samples were taken anaerobically with a gastight Hamilton syringe fitted with a 25 cm stainless steel needle, transferred to a nitrogen-flushed EPR tube, and frozen in liquid nitrogen. The reduction titration was carried out in 3.4 ml of 45 μM AlDH in pH 7.00 buffer, containing the same mediator dyes (30 μM each) as described in “Potentiometric Titrations”. Dithionite (0.1 and 1.0 M) was used to adjust the redox potential. A sample was taken before adding the mediator dyes to the solution. Then samples were taken at potentials of 114, 42, 0, -50, -95, -150, -200, -250, -300, -340 and -390 mV. Finally, EPR spectra of these samples were recorded at 22 K.

**N-terminal amino acid sequences.** The enzyme (1 mg) was denatured in the presence of 10 % (w/v) SDS and 20 % (v/v) β-mercaptoethanol for 20 min at 100 °C. The subunits were separated on a gel filtration column (Superdex-200, 1 x 30 cm, Pharmacia) (see, e.g., Fig. 1) equilibrated with 20 mM Mops buffer, containing 0.1 % SDS and 0.1 M NaCl. Chromatography occurred at a flow rate of 0.5 ml/min. Fractions of 250 μl were collected. The fractions containing the subunits were identified by SDS-PAGE using a 10 % polyacrylamide gel (Bio-Rad). Protein staining of the gel was performed with Coomassie brilliant blue R-250. The subunits were precipitated with 80 % ethanol at -80 °C. After centrifugation the pellets were dried for 20 h. Removal of all salts and free amino acids was done by the method of Sheer [43]. N-terminal amino acid sequences were determined with automatic Edman degradation (Applied Biosystems, Sequencer Model 477A and HPLC Model 120A). The sequences were compared with those published in the literature by eye or with those in the protein sequence databanks using the program BLAST [44].

**RESULTS**

**Purification of aldehyde dehydrogenase.** The purification of AlDH was modified compared to the previous method [24] by introducing an extra anion-exchange chromatography purification step (DEAE Bio-gel agarose). Further, gel filtration was omitted, instead hydrophobic interaction chromatography (phenyl-Sepharose) was applied. Table 1 summarizes the results of the new purification procedure. The enzyme was purified 86-fold (63-fold for the old procedure [24]) and appeared to be homogeneous as judged by native PAGE which showed a single band (not shown).
Table 1. Purification of AIDH from *Comamonas testosteroni*.

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Total protein (mg)</th>
<th>Specific activity (U/mg)</th>
<th>Yield (%)</th>
<th>Purification (fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell-free extract</td>
<td>16500</td>
<td>0.1</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>DEAE Bio-Gel agarose</td>
<td>1500</td>
<td>0.9</td>
<td>82</td>
<td>9</td>
</tr>
<tr>
<td>DEAE-Sepharose</td>
<td>350</td>
<td>3.2</td>
<td>68</td>
<td>32</td>
</tr>
<tr>
<td>Hydroxylapatite</td>
<td>115</td>
<td>5.0</td>
<td>35</td>
<td>50</td>
</tr>
<tr>
<td>Phenyl-Sepharose</td>
<td>50</td>
<td>8.6</td>
<td>26</td>
<td>86</td>
</tr>
</tbody>
</table>

Molecular masses and subunit composition. Determination of the native molecular masses by native PAGE of purified AIDH and FEDH yielded 170 kDa for both enzymes. SDS-PAGE revealed that both enzymes are composed of three different subunits with molecular masses of 86, 30, 16 kDa (cf. Fig. 5) and 96, 38, 22 kDa (not shown), respectively. Gel filtration chromatography of denatured AIDH or FEDH in the presence of SDS enabled the separation of the three subunits (Fig. 1) and the calculation of their relative concentrations. Furthermore, the molecular masses of the subunits, calculated from the retention times of the peaks of the separated subunits, agreed with those revealed by SDS-PAGE. Molar ratios of 1:0:0.9:1.0 and 1.0:1.0:0.8, respectively, were found, indicating that both enzymes have an α, β, γ composition. Assuming a 1:1:1 subunit stoichiometry, molecular masses for AIDH and FEDH of 132 kDa and 156 kDa, respectively, were calculated (Table 2). The difference between the calculated and measured native molecular mass (by native PAGE) may be due to an unusual behaviour of the proteins in the methods applied (cf. ref. [26]) so that no decision can be made with respect to the genuine molecular masses.

Cofactor analysis. The results on cofactor content, assuming a molecular mass of 132 kDa for AIDH and 156 kDa for FEDH, are given in Table 2. These data are consistent with a stoichiometry of one FAD, one Moco - present as MCD - four iron atoms and four acid-labile sulphides per enzyme molecule.

Absorption spectra. The UV/VIS spectrum of AIDH as isolated (Fig. 2) shows maxima at 280, 338 and 450 nm and a shoulder at 550 nm. The ratio $A_{338}/A_{550}$ was 3.0, a value comparable to that reported for molybdenoprotein hydroxylases with a full complement of cofactors [45]. Addition of the substrate acetaldehyde (2 mM) effected a decrease in absorbance in the
region from 310 to 600 nm. The difference spectrum, with a maximum at 450 nm and a shoulder at 350 nm, clearly shows (partial) reduction of FAD (at 450 nm) and of an iron-sulphur cluster (at 550 nm). The spectrum (between 400-600 nm) obtained after addition of an excess dithionite is typical of reduced iron-sulphur clusters (Fig. 2).

Fig. 1. Gel filtration profile of SDS-denatured AIDH and FEDH. The enzymes were denatured and chromatographed on a gel filtration column in the presence of SDS as described in "Materials & Methods". Quantitation was carried out by integrating the peaks. The solid line represents the elution profile of AIDH, the dotted line that of FEDH.

The specific absorption coefficient, $A_{280}^{0.1\%}$, of AIDH was calculated as $1.06 \pm 0.01$, instead of 0.96 calculated previously [24]. The difference between these values is probably due to the fact that more pure AIDH and a more sensitive photodiode array detector were applied here for the determination of $A_{280}^{0.1\%}$. For FEDH the specific absorption coefficient equals 1.03 [25].

**EPR spectroscopy.** Figure 3 (bottom trace) shows the EPR spectrum of AIDH reduced with propanal (900 μM). A rhombic signal was observed with g-values $g_{x,y,z} = 1.904, 1.941, 2.023$ similar to those of the iron-sulphur cluster designated as [2Fe-2S]-1 in xanthine oxidase [18].
Quantitation of the amount represented by this signal yielded 10% reduced cluster per enzyme molecule (see also Fig. 2). Reduction with excess dithionite resulted in the complete reduction of \([2\text{Fe-2S}]^{-1}\) and of a second cluster with \(g_{e_x} = 1.89\text{-}1.90, 1.98, 2.092\), designated as \([2\text{Fe-2S}]^{-2}\) for xanthine oxidase [18]. The two types of \([2\text{Fe-2S}]\) clusters have different relaxation properties (Fig. 3), i.e., \([2\text{Fe-2S}]^{-2}\) is broadened beyond detection at 56 K, \([2\text{Fe-2S}]^{-1}\) at about 80 K (not shown). The EPR signal of the \([2\text{Fe-2S}]^{-1}\) cluster, in particular in the \(g_z\) peak, is broader at 35 K than at 56 K owing to magnetic interaction of the cluster with the \([2\text{Fe-2S}]^{-2}\) cluster, similar to the situation in xanthine oxidase [46-49] (see also Fig. 8). Quantitation of the EPR signals (Table 2) shows that each \([2\text{Fe-2S}]\) cluster is present in amounts stoichiometric with the enzyme. For FEDH very similar signals were observed in the X-band EPR spectrum (not shown).

Table 2. Comparison of structural and spectroscopic properties of several MCD-containing oxidoreductases.
Abbreviations enzymes: DL-AIDH, (dye-linked) aldehyde dehydrogenase from *Amycolatopsis methanolica* [26]; ALOR, aldehyde oxidoreductase from *Desulfovibrio gigas* [6]; QOR, quinoline oxidoreductase from *Pseudomonas putida* 86 [45,73]; AALDH, acetaldehyde dehydrogenase complex from *Acetobacter europaeus* [7].

<table>
<thead>
<tr>
<th>Property</th>
<th>AIDH</th>
<th>FEDH</th>
<th>DL-AIDH</th>
<th>ALOR</th>
<th>QOR</th>
<th>AALDH</th>
</tr>
</thead>
<tbody>
<tr>
<td>(m) (kDa)</td>
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<td>156</td>
<td>156</td>
<td>200</td>
<td>300</td>
<td>150</td>
</tr>
<tr>
<td>subunits</td>
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<td>96,38,22</td>
<td>87,35,17</td>
<td>100</td>
<td>85,30,20</td>
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<tr>
<td>(\alpha:\beta:\gamma)</td>
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<td>1:1:0.8:0.8</td>
<td>1:0:8:1.7</td>
<td>.(^b)</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>Moco</td>
<td>0.8 MCD</td>
<td>0.9 MCD</td>
<td>MCD</td>
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<tr>
<td>Mo.</td>
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<tr>
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<tr>
<td>(S^2)</td>
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<td>3.7</td>
<td>8</td>
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<td>No</td>
<td>No</td>
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</tr>
<tr>
<td>[2Fe-2S]-1</td>
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<td>1.0(^a)</td>
<td>1.0(^a)</td>
<td>2</td>
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<tr>
<td>[2Fe-2S]-2</td>
<td>0.8(^a)</td>
<td>1.0(^a)</td>
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<tr>
<td>Mo(V)</td>
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<td>FAD(^c)</td>
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</tr>
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</table>

\(^a\) Determined by double integration of the EPR spectrum of the reduced enzyme

\(^b\) Single subunit

n.d. not determined.
Fig. 2. Absorption spectra of AlDH. AlDH as isolated was dissolved in 20 mM Mops buffer, pH 7.5. Inset, solid line, enzyme as isolated; dotted line, enzyme reduced with 2 mM acetaldehyde; dashed line, enzyme reduced with excess dithionite; dotted/dashed line, difference spectrum of enzyme as isolated and substrate-reduced enzyme.

Localization of the cofactors. By denaturing AlDH under mild conditions, it was possible to localize some of the cofactors in the enzyme. Gel filtration in the presence of guanidine resulted in an elution pattern (Fig. 4A) with four peaks. The optical spectrum of the third peak (t = 30.5 min) (Fig. 4B) resembles the optical spectrum of plant-type [2Fe-2S] ferredoxins [50-52]. This [2Fe-2S] cluster is located in the γ-subunit, as judged from SDS-PAGE (Fig. 5). The iron content and acid-labile sulphide content were calculated as 1.9 mol iron and 2.0 mol acid-labile sulphide, respectively, per mol of γ-subunit. Reduction of the γ-subunit with dithionite resulted in bleaching of the absorption spectrum; neither the oxidized nor the reduced γ-subunit showed EPR signals from the [2Fe-2S] clusters. Other reducing agents like sodium ascorbate or sodium borohydride were ineffective in reducing the [2Fe-2S] cluster in the γ-subunit. The peak eluting at 40.0 min has been identified as FAD. Furthermore, at 43.0 min a compound with an unknown optical spectrum was observed (Fig. 4). The corresponding fraction
did not contain molybdenum or acid-labile sulphide. It did, however, contain 2.0 mol iron per mol of AldH applied to the column. The optical spectrum presumably originates from an iron-complex of which the iron is suggested to belong to the second [2Fe-2S] cluster present in AldH.

![EPR Spectra](image)

**Fig. 3. X-band EPR spectra of AldH.** To the final preparation of AldH in 20 mM Mops buffer, pH 7.5, propionaldehyde (900 μM, bottom trace) or excess dithionite (top and middle traces) was added. Experimental conditions: microwave frequency, 9.234 GHz; microwave power, 2 mW; modulation amplitude, 0.4 mT; temperature, 56 K (middle and bottom traces), 35 K (top trace). NB: The gain is different for the different traces. The intense signal at g = 2 (329.5 mT), bottom trace, is from a pterin radical. Its properties will be described elsewhere.

**Deflavo-AldH.** Deflavo-AldH was prepared by treating AldH with calcium chloride ([40], Fig. 6). Native PAGE of deflavo-AldH showed a single band with a similar molecular mass as AldH (not shown). The iron as well as acid-labile sulphide content were determined as
Fig. 4. HPLC gel filtration profile of AIDH denatured in the presence of 3 M guanidine. A, HPLC elution profile of AIDH. The black line represents the elution profile monitored at 280 nm, the dark grey line line that at 450 nm and the grey line that at 550 nm. B, Absorption spectra of the substances eluting at 30.5 min (solid line), 40.0 min (dashed line) and 43.0 min (dotted line). NB: The gain is different for the different spectra.
2 mol per mol of deflavo-AIDH, indicating the presence of only one [2Fe-2S] cluster and the removal of the other cluster and FAD. The optical spectrum showed similarity with the spectrum of the isolated γ-subunit. By subtracting the spectrum of the latter one from the first one a difference spectrum was obtained (Fig. 6) which may represent the spectrum of the pterin cofactor present in deflavo-AIDH. The spectrum which shows a maximum at 370 nm suggests the presence of the pterin cofactor in the fully oxidized state (cf. refs. [14,53,54]). Addition of acetaldehyde to the deflavo-AIDH had no effect on the absorption spectrum whereas adding dithionite resulted in bleaching of the spectrum. The deflavo-AIDH was EPR-silent both in the oxidized and reduced state. Deflavo-AIDH was not active in the standard enzymatic assay, nor could activity be detected using the electron acceptors cytochrome c, 2,6-dichlorophenol-indophenol, ferricyanide, NAD(P) or phenazine methosulfate. Reconstitution under various conditions (FAD concentration, incubation time, temperature) of the deflavo-enzyme to an active or FAD-containing enzyme failed.

Fig. 5. SDS-PAGE of AIDH and the isolated γ-subunit. Lane 1, molecular mass standards (in kDa): phosphorylase b, 94; bovine serum albumin, 67; ovalbumin, 43; carbonic anhydrase, 30; soybean trypsin inhibitor, 20.1; lactalbumin, 14.4. Lane 2, AIDH. Lane 3, γ-subunit of AIDH.

Redox potentials. Potentiometric titrations of AIDH have been performed at room temperature at pH 7.00 and 9.00. Reduction and reoxidation of FAD and the [2Fe-2S] clusters were monitored by UV/Vis spectroscopy at 450, 550 and 620 nm and also by EPR. Figure 7 indicates that approximately half of the absorbance at 450 nm is due to the [2Fe-2S] clusters, the other half to FAD. At 550 nm all of the absorbance in the oxidized enzyme is due to the two [2Fe-2S] clusters. The increase at 620 nm may indicate the formation of a blue neutral flavin semiquinone. It was observed at pH 7.00 and not at pH 9.00. The formation of flavin radical was
Fig. 6. Absorption spectra of deflavo-AIDH (solid line), the isolated γ-subunit (dark grey line), and the Moco (grey line). The spectrum of the Moco was obtained from the difference spectrum (deflavo-AIDH) - (isolated γ-subunit) normalized at 450 nm.

apparently not reversible (Fig. 7) because it was not observed in the oxidative titration. The flavin semiquinone was also observed in the EPR-monitored titration (Fig. 8) at potentials between -50 mV and -150 mV, a potential range consistent with the presence of the 620 nm absorbance in the titration monitored optically. The amount of flavin-radical, determined by quantitation of the EPR signal, was maximally 20 ± 5 % of the enzyme concentration, consistent with the simulation of the optical titration (see Fig. 7). Figure 8 indicates that the midpoint potential of [2Fe-2S]-1 is higher than that of [2Fe-2S]-2 and further that the values for FAD and [2Fe-2S]-1 determined optically and by EPR are quite similar (Fig. 7, Table 3). Note that the g<sub>r</sub>-line of [2Fe-2S]-1 is broadened upon reduction of [2Fe-2S]-2 (Fig. 8, lower traces). The potentials of the redox centres are pH dependent to varying degrees. The FAD redox potential decreases by 55 mV in the pH range 7.00 to 9.00. The potential of [2Fe-2S]-2 decreases by 70 mV by changing the pH from 7.00 to 9.00 (Table 3) suggesting that the pK<sub>red</sub> value lies between these pH values. The
potential of [2Fe-2S]-1 decreases by 119 mV, i.e., $pK_{\text{red}} > 9$. For both clusters $pK_{\text{ox}} < 7$. So both [2Fe-2S] clusters behave as proton acceptors whose $pK_{\text{a}}$ value changes with the reduction state. Only a very small amount of Mo(V) was apparently formed (see, e.g., the trace at $E = -250$ mV, Fig. 8).

Fig. 7. Potentiometric titration of the various redox centres in AIDH at pH 7.00. Reductive (-) and oxidative (○) titrations of AIDH at pH 7.00 monitored by UV/Vis spectroscopy at 450 (higher curve), 550 (middle curve) and 620 nm (lower curve). The curves represent the best fits by using $n = 1.0$ for each redox center and the following relative optical absorbances and midpoint potentials in the Nernst equation: $\Delta A = 0.044, E_m = -79$ mV (FAD/FADH$^\text{+}$), $\Delta A = 0.044, E_m = -58$ mV (FADH/FADH$_2$), $\Delta A = 0.044, E_m = -138$ mV ([2Fe-2S]-1), $\Delta A = 0.047, E_m = -263$ mV ([2Fe-2S]-2) all at 450 nm, $\Delta A = 0.058, E_m = -138$ mV ([2Fe-2S]-1), $\Delta A = 0.023, E_m = -263$ mV ([2Fe-2S]-2) both at 550 nm. The dashed curve represents the best fit based upon reduction of [2Fe-2S]-1 (●) and [2Fe-2S]-2 (▲) monitored by EPR (Fig. 8). See also Table 3.

Catalytic properties. Purified AIDH and FEDH catalyzed the oxidation of a broad range of aldehydes (Table 4) including aliphatic aldehydes, branched-chain aliphatic aldehydes, aromatic aldehydes and formate esters. For both enzymes the activity decreased with increasing
chain length of the aldehyde. Short chain aldehydes with a phenyl group were good substrates. FEDH showed higher activity than AIDH for branched-chain aliphatic aldehydes whereas AIDH showed higher activity for formate esters. Both enzymes showed very low enantioselectivity towards racemic aldehydes. In case of AIDH, the substrates 2-ethylhexanal, 3,5,5-trimethylhexanal and 3-phenylbutanal showed E-values of 1.7, 2.3 and 2.0, respectively. In case of FEDH, an E-value of 1.0 was found for 2-phenylpropanal, i.e., it was not selective at all.

Table 3. Midpoint potentials of the prosthetic groups of AIDH determined optically at pH 7.00 and 9.00. The values for the midpoint potentials at the different pH values were obtained by non-linear fitting of the Nernst equations describing the redox behaviour of prosthetic groups.

<table>
<thead>
<tr>
<th>pH</th>
<th>FAD/FADH₂</th>
<th>[2Fe-2S]-1</th>
<th>[2Fe-2S]-2</th>
<th>Mo(IV)/Mo(VI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.00</td>
<td>-68⁴</td>
<td>-138, -125⁵</td>
<td>-263⁶</td>
<td>-250⁴</td>
</tr>
<tr>
<td>9.00</td>
<td>-123</td>
<td>-257</td>
<td>-333</td>
<td></td>
</tr>
</tbody>
</table>

⁴ On basis of EPR a maximum of 20 ± 5 % flavosemiquinone was found yielding midpoint potentials of -79 and -58 mV for the FAD/FADH² and FADH/FADH₂ couples, respectively.

⁵ Determined by EPR.

⁶ The value from EPR is between -250 and -300 mV.

⁷ On basis of the very low amount of Mo(V) (< 0.5 %) at -250 mV (Fig. 8).

Benzaldehyde and 3-phenylpropanal were oxidized by FEDH but behaved as inhibitors for AIDH. The lines obtained in a Dixon plot (Fig. 9A), with propanal as substrate, showed that benzaldehyde is a competitive inhibitor for AIDH with a Kᵢ of 0.1 μM. Benzaldehydes substituted with an electron withdrawing group were, however, good substrates for AIDH (Table 4) whereas these same substrates, at least when compared to benzaldehyde, were more slowly converted by FEDH. The extent of inhibition of AIDH activity by 3-phenylpropanal was not dependent on the concentration of the substrate propanal (Fig. 9B) indicating that the aromatic aldehyde binds so strongly to AIDH that it could not be replaced by the concentrations of propanal applied in the short time as it is present in the assay. This suggestion is in agreement with the fact that 3-phenyl propionic acid did not show inhibition in the AIDH assay, implying that no product inhibition occurred. The Kᵢ of 3-phenylpropanal was determined as 0.5 μM from the equation: activity = [enzyme] x (Kᵢ / [inhibitor] + Kᵢ) x constant. Optical spectra (not shown) recorded during inhibition of AIDH by benzaldehyde or 3-phenylpropanal revealed that AIDH became reduced, suggesting at least one turnover. Preincubation of AIDH in presence of 1 μM
Fig. 8. EPR redox-titration of AIDH at pH 7.00. Traces (from top to bottom) represent EPR spectra of AIDH samples measured at potentials of -50 (13 % reduced [2Fe-2S]-1, 0 % reduced [2Fe-2S]-2), -95 (30 % reduced [2Fe-2S]-1, 0 % reduced [2Fe-2S]-2), -150 (68 % reduced [2Fe-2S]-1, 0 % reduced [2Fe-2S]-2), -200 (100 % reduced [2Fe-2S]-1, 0 % reduced [2Fe-2S]-2), -250 (100 % reduced [2Fe-2S]-1, 0 % reduced [2Fe-2S]-2), -300 (100 % reduced [2Fe-2S]-1, 100 % reduced [2Fe-2S]-2) and -340 mV (100 % reduced [2Fe-2S]-1, 100 % reduced [2Fe-2S]-2), respectively. The signal around g = 2 (329.5 mT) represents the FADH⁺ only in the top three traces. In the other traces the g = 2 signal is due to the redox dyes. Experimental conditions: microwave frequency, 9.235 GHz; microwave power, 2 mW; modulation amplitude, 0.5 mT; temperature, 22 K. NB: Quantitation of the FADH⁺ was performed at 95 K and at non-saturating (20 μW) microwave power (see also text).
benzaldehyde or 3-phenylpropanal showed that activity was recovered after about 10 min indicating slow conversion of these aldehydes. The rate of conversion of these aldehydes could be measured by using very high concentrations of AIDH in the assay and equals 8.6 mU/mg, i.e., 0.1 % (Table 4). We suggest therefore that a combination of strong binding, slow dissociation of the product and/or slow conversion of the latter aldehydes causes the very low activities.

Table 4. Substrate specificity of AIDH and FEDH. Only maximal enzyme activities are reported here whereas substrate concentrations varied from 0.01 to 100 mM.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>AIDH Activity (%)</th>
<th>FEDH Activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Aliphatic aldehydes</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Formaldehyde</td>
<td>8&lt;sup&gt;c&lt;/sup&gt;</td>
<td>15&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>Acetaldehyde&lt;sup&gt;*&lt;/sup&gt;</td>
<td>100&lt;sup&gt;b&lt;/sup&gt;</td>
<td>100&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>2-Chloroacetaldehyde</td>
<td>120&lt;sup&gt;b&lt;/sup&gt;</td>
<td>85&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Propanal</td>
<td>90&lt;sup&gt;c&lt;/sup&gt;</td>
<td>76&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Butanal</td>
<td>43&lt;sup&gt;c&lt;/sup&gt;</td>
<td>73&lt;sup&gt;c&lt;/sup&gt;</td>
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<tr>
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<td>29&lt;sup&gt;c&lt;/sup&gt;</td>
<td>51&lt;sup&gt;c&lt;/sup&gt;</td>
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<tr>
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<td>10&lt;sup&gt;c&lt;/sup&gt;</td>
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<td><strong>Aromatic aldehydes</strong></td>
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<td>Benzaldehyde</td>
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<td>0</td>
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<td>0</td>
<td>38&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>4-Chlorobenzaldehyde</td>
<td>15&lt;sup&gt;a&lt;/sup&gt;</td>
<td>55&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td>4-Nitrobenzaldehyde</td>
<td>60&lt;sup&gt;b&lt;/sup&gt;</td>
<td>23&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
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<td>290&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>110&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
<td>3-Phenylpropanal</td>
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</tr>
<tr>
<td>3-Phenylbutanal</td>
<td>50&lt;sup&gt;b&lt;/sup&gt;</td>
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<td><strong>Branched-chain aldehydes</strong></td>
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<tr>
<td>Trimethylacetaldehyde</td>
<td>13&lt;sup&gt;a&lt;/sup&gt;</td>
<td>49&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td>2-Methylbutanal</td>
<td>14&lt;sup&gt;a&lt;/sup&gt;</td>
<td>65&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>1-methylpentanal</td>
<td>25&lt;sup&gt;b&lt;/sup&gt;</td>
<td>55&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
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<td>21&lt;sup&gt;a&lt;/sup&gt;</td>
<td>38&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>2,3-Dimethylpentanal</td>
<td>24&lt;sup&gt;a&lt;/sup&gt;</td>
<td>55&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>2-Ethylhexanal</td>
<td>13&lt;sup&gt;b&lt;/sup&gt;</td>
<td>28&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>3,5,5-Trimethylhexanal</td>
<td>43&lt;sup&gt;a&lt;/sup&gt;</td>
<td>11&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Vannilin</td>
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</tr>
<tr>
<td>Cinnamaldehyde</td>
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<td>0</td>
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<tr>
<td><strong>Formate esters</strong></td>
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<td></td>
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<tr>
<td>Methyl formate</td>
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<td>27&lt;sup&gt;e&lt;/sup&gt;</td>
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<tr>
<td>Ethyl formate</td>
<td>93&lt;sup&gt;d&lt;/sup&gt;</td>
<td>36&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>*</sup>For AIDH 100 % = 8.6 U/mg as for FEDH 100 % = 20.1 U/mg [25].
<sup>a</sup>0.01 mM, <sup>b</sup>0.1 mM, <sup>c</sup>1 mM, <sup>d</sup>10 mM, <sup>e</sup>100 mM.
Fig. 9. Effect of benzaldehyde or 3-phenylpropanal on AIDH activity assayed with propionaldehyde as substrate. Propionaldehyde concentrations used were 0.1 (○), 0.3 (□), 0.5 (△) and 1.0 (○) mM. A. Dixon plot for benzaldehyde as inhibitor. Concentration range of benzaldehyde, 0 to 3 μM (0, 0.25, 0.50, 1.00, 1.50, 2.00 and 3.00 μM). B. AIDH activity at different concentrations of 3-phenylpropanal varying from 0 to 10 μM (0, 0.25, 0.50, 1.00, 1.50, 2.00, 3.00 and 10.0 μM).
Table 5. Comparison of N-terminal amino acid sequences of the subunits of AIDH, FEDH and DL-AIDH with those of other bacterial Moco containing oxidoreductases and stretches detected in eukaryotic xanthine dehydrogenase (α, β, γ-domain). 1. AIDH; 2. FEDH; 3. DL-AIDH [26]; 4. Aldehyde oxidoreductase from Desulfovibrio gigas [58]; 5. Quinoline oxidoreductase from Pseudomonas putida 85 [75]; 6. Quinoline-4-carboxylic acid oxidoreductase from Agrobacterium spec.1B [76]; 7. Nicotine dehydrogenase from Arthrobacter nicotinovorans; 8. Carbonmonoxide dehydrogenase from Pseudomonas thermocarboxydovorans [59]; 9. Acetaldehyde dehydrogenase complex from Acetobacter europaeus [7]; 10. Xanthine dehydrogenase from humans [60].

<table>
<thead>
<tr>
<th>α-subunit</th>
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<tbody>
<tr>
<td>3. 2-V-GTRVRHIEDQQILTQGGTVEDL-25</td>
</tr>
<tr>
<td>7. 15-M-GERLRTEADARLTTGRKLNDI-39</td>
</tr>
<tr>
<td>8. 15-M-GERLRTEADARLTTGRKLNDI-39</td>
</tr>
<tr>
<td>9. 8-RLGK-DGRREEQASLSRR-GFLVTSL-30</td>
</tr>
<tr>
<td>10. 574-V-GRLPHLAAADMQSAGEAVYCDIDDI-597</td>
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<table>
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<th>β-subunit</th>
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<tbody>
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<td>1. 1-MYAFSYSTFR-TLDEVSAAS-19</td>
</tr>
<tr>
<td>2. 1-MIAPAFFDYVAP-STVDEAVQAL-22</td>
</tr>
<tr>
<td>3. 1-MIAPAFFDYVAP-STVDEAVQAL-22</td>
</tr>
<tr>
<td>5. 2-KFPA-FSYRAPAS-LQEVIQ-L-21</td>
</tr>
<tr>
<td>6. 2-KAPA-F?YA?PA-TLDETF-19</td>
</tr>
<tr>
<td>8. 3-PFAPAHYAPR-TLDPDAIR-19</td>
</tr>
<tr>
<td>10. 231-RERVTWIQ-ASTLKLDDLLKA-250</td>
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<table>
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<th>γ-subunit</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. 1-MNVOFTVNGRASS-1-D-VPPNT-LLV-23</td>
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<tr>
<td>2. 1-MRITVNVDGTSYTEDFPR-LLV-23</td>
</tr>
<tr>
<td>3. 1-MKVSIEINGTTSV-S-EV?DRT-LL-21</td>
</tr>
<tr>
<td>4. 3-QKV-ITVNGIEQNL-F-VDAEA-LLS-24</td>
</tr>
<tr>
<td>8. 4-HIVSTVNGRKE-E-AVEART-LLV-26</td>
</tr>
<tr>
<td>9. 1-MTT-FRLNGR-EVTVVDVPGFDTPLLLW-23</td>
</tr>
<tr>
<td>10. 5-KLV-FFVNGRKE-VKNAFDPETTLLA-28</td>
</tr>
</tbody>
</table>

**N-terminal amino acid sequences.** The sequences of the amino-termini of the β- and γ-subunit of AIDH and FEDH were compared to published sequences of other bacterial molybdenoenzymes and stretches found in eukaryotic molybdenoenzymes. The amino-termini of the α-subunit of AIDH and FEDH were blocked in contrast to the amino-terminus of the α-subunit of DL-AIDH [26]. Homology could be detected especially between the sequences of AIDH, FEDH, DL-AIDH and other MCD-containing enzymes (even without containing FAD [7]) (32-55 %). Similarity has also been observed between the amino-terminal sequences of the γ-subunit of AIDH and FEDH and a stretch in eukaryotic xanthine dehydrogenase (15-42 %) (Table 5,
DISCUSSION

Structural properties

The improved purification method for AIDH yielded enzyme of a higher purity as before [24], as evidenced by the increase of the specific activity from 6.3 to 8.6 U/mg and the decrease of the $A_{280}/A_{450}$ ratio from 4.9 to 4.6. PAGE under native conditions further confirmed the homogeneity of the AIDH preparation. In addition to a more pure AIDH, the enzyme appeared to be more stable, as the spectral changes observed before on storage [24] did not occur.

The determination of metal and cofactor contents yielded a Mo:Fe:S:FAD ratio of 1:4:4:1 for AIDH as well as FEDH, a typical cofactor composition for molybdo-iron/sulphur-flavoproteins (Table 2). Furthermore, the Moco was identified as a MCD. SDS-PAGE revealed that purified AIDH and FEDH both are composed of three different subunits, in contradiction with earlier results [24,25] in which both enzymes were proposed to be homodimers. This is in all likelihood due to different denaturation methods. In our earlier work the enzymes were boiled for 5 min, now for 20 min. Several other bacterial molybdenum containing oxidoreductases carrying a MCD have also been reported to be three-subunit complexes with subunit sizes similar to those of AIDH and FEDH (Table 2). Eukaryotic molybdenum containing hydroxylases, like xanthine dehydrogenase from Drosophila melanogaster and rat liver, show native molecular masses approximately equal to that of two times the ($\alpha+\beta+\gamma$) structure of the prokaryotic molybdoenzymes and have been reported to consist of three different domains per monomer corresponding to the three subunits of the prokaryotic hydroxylases [55-58].

EPR studies and the analytical iron and acid-labile sulphide content of AIDH and FEDH support the conclusion that these enzymes contain two distinct [2Fe-2S] clusters showing magnetic interaction, as evidenced by the line-broadening in the EPR spectrum of [2Fe-2S]-1 recorded at 35 K (see also Fig. 3) or upon reduction of [2Fe-2S]-2 (Fig. 8). The extent of line broadening is similar as found in xanthine oxidase and other (bacterial) molybdoproteins [46-49] and suggests that the spatial distribution of these cofactors is conserved between these enzymes.

Primary sequence data of molybdoenzymes like carbonmonoxide dehydrogenase [59] and
xanthine dehydrogenase [55,60] indicate that the γ-subunit or γ-domain, respectively, binds both [2Fe-2S] clusters. The N-terminal sequences of the γ-subunits of AIDH and FEDH show similarity to those of other molybdoenzymes (Table 5) suggesting that the two clusters of AIDH and FEDH are also located in the γ-subunit. However, the isolated γ-subunit of AIDH contained only one cluster, as determined by the iron and acid-labile sulphide content, with an optical spectrum resembling that of the plant ferredoxin [2Fe-2S] cluster (Fig. 4). Apparently the other [2Fe-2S] cluster is easily lost. Unfortunately, we could not relate the EPR spectrum to the optical spectrum of this cluster because reduction of the cluster by dithionite led to bleaching of the optical spectrum and an EPR silent preparation. The apparent instability of the reduced cluster in the isolated γ-subunit may be due to a diminished stabilization after dissociation of the enzyme into subunits.

The extraction procedure to remove FAD from AIDH yielded an enzyme form with an optical spectrum similar to that of the isolated γ-subunit of AIDH (Fig. 6), indicating the loss of the same [2Fe-2S] cluster that was absent in the purified γ-subunit. From the difference spectrum, deflavoo-AIDH minus the isolated γ-subunit, theoretically the spectrum of the Moco should be obtained. The absorbance maximum (370 nm) suggests indeed that the Moco is still present in deflavoo-AIDH and is in the fully oxidized state [14,53,54]. Determination of the redox state of the Moco of sulfite oxidase indicated, however, a dihydropterin reduction level [61,62]. The same conclusion holds for the aldehyde oxidoreductase from D. gigas, as suggested from the non-planarity of the Moco [19,20,22,63,64]. Assuming that the native AIDH contains the Moco in the same redox state, the calcium treatment, leading to loss of FAD and an [2Fe-2S] cluster, may result in oxidation of the pterin either by direct exposure to oxygen or by the [2Fe-2S] cluster during unfolding.

The deflavoo-forms of eukaryotic xanthine oxidase and xanthine dehydrogenase do not catalyze the reactions performed by the holo-enzymes [40]. Since they do so when reconstituted with FAD [66], apparently the procedure applied for obtaining the deflavoo-form does not affect other structural alterations other than FAD removal crucial for activity. The deflavoo-form of xanthine oxidase shows xanthine dehydrogenase activity with artificial electron acceptors [40], suggesting that the presence of FAD is not essential for substrate oxidation but plays a role in electron transfer from reduced molybdenum to O₂. Since the deflavoo-form of AIDH did not show aldehyde dehydrogenase activity with a variety of artificial electron acceptors and reconstitution
with FAD was not achieved (which could be due to a variety of reasons), no clue was obtained with respect to the role of FAD in this enzyme [65,67]. However, since FAD is lacking in aldehyde oxidoreductase from *D. gigas*, it is clear that FAD does not play an essential role in the aldehyde oxidation step by molybdocproteins. The absence of the [2Fe-2S] cluster and the higher oxidation state of the pterin moiety of Moco in deflavo-AIDH, as compared to the situation in holo-AIDH, indicates that this molybdocprotein is more vulnerable to structural damage caused by the deflavinylation procedure than eukaryotic xanthine oxidase and xanthine dehydrogenase.

**Redox properties**

UV/Vis spectroscopy appeared to be a suitable method to determine the redox potentials of the FAD and [2Fe-2S] clusters of AIDH since the redox properties could be determined by selection of specific wavelengths of the complete spectrum (Fig. 7). The potentials of the [2Fe-2S] clusters of AIDH determined with EPR were comparable to the values obtained with the UV/Vis spectrophotometric method. This indicated that no complications are introduced due to freezing the AIDH. In many molybdocproteins, like in xanthine oxidase and xanthine dehydrogenase [68,69], the [2Fe-2S]-2 has a more positive redox potential than [2Fe-2S]-1, as judged from low temperature EPR spectra [70,71]. However, the reverse is true for AIDH, as shown by EPR redox potentiometry (Fig. 8, Table 3). Further, addition of substrate resulted in 10% reduction of [2Fe-2S]-1 and no reduction of [2Fe-2S]-2. Thus, whereas at pH 7.00 the potential of [2Fe-2S]-2 of AIDH is similar to that in xanthine oxidase and xanthine dehydrogenase, the potential of [2Fe-2S]-1 of AIDH is much higher [68,69].

At pH 7.00, during reduction of AIDH, the increase of absorbance at 620 nm (Fig. 7) indicates the formation of a blue neutral flavin semiquinone, which could also be observed with EPR. As was calculated, a maximum of 20 ± 5% of the semiquinone could be generated in AIDH whereas 10 and 100% have been reported in xanthine oxidase and xanthine dehydrogenase, respectively [68,69]. The potentials of the FAD/FADH⁺ and FADH⁺/FADH₂ couples in AIDH were determined to be -79 and -58 mV, respectively. These values are much higher than those found for xanthine oxidase (-280 and -230 mV, respectively) and xanthine dehydrogenase (-270 and -410 mV, respectively) from cows' milk at pH 7.5 [68,69]. So compared to xanthine oxidase and xanthine dehydrogenase, all the potentials of the redox couples
in AIDH (including those for the Mo(VI)/Mo(V) and Mo(V)/Mo(IV) couples) are higher, except the potential of the [2Fe-2S]-2 cluster.

The potentials of both [2Fe-2S] clusters in AIDH are pH dependent (see Table 3) which is surprising since at least one of the two clusters may be deeply buried in the protein and will probably be inaccessible to water (cf. aldehyde oxidoreductase from *D. gigas* [22]). This pH dependency is most simply explained by the presence of an acid/base group in electrostatic contact and/or close to the [2Fe-2S] cluster (cf. ref. [68]). The nature of this acid/base group and its possible function in catalysis remain to be determined.

**Active site and substrate (enantio)specificity**

Although AIDH and FEDH show several similar structural- and spectroscopic properties, their substrate specificity is different, indicating somewhat different active sites. Benzaldehyde and 3-phenylpropanal, for example, appeared to be good substrates for FEDH whereas they behaved like inhibitors in the AIDH enzyme assay. Furthermore, low activity with 3-phenylpropanal as substrate for AIDH contrasts with the high activity obtained with 2-phenylpropanal and 3-phenylbutanal. This means that the position of the phenyl-group and the chain length of the aliphatic moiety, containing the aldehyde group, are very important for activity. Probably the phenyl-group of 3-phenylpropanal interacts outside of the active site with an (aromatic) amino acid of the protein, explaining the inhibition behaviour of this aldehyde. In case of 2-phenylpropanal the phenyl-group probably cannot reach this protein site as for 3-phenylbutanal the C-(δ)-methyl-group disturbs the interaction between the phenyl-group and the protein site.

The fact that benzaldehyde was a poor substrate and behaved as a competitive inhibitor for AIDH might be explained by taking the resonance structures of this aldehyde into account. These resonance structures indicate a positively charged phenyl-group which may interact with a negatively charged amino acid in the active site of AIDH. This interpretation is consistent with the finding that introduction of an electron withdrawing group to the phenyl-ring of benzaldehyde (4-nitrobenzaldehyde, 4-chlorobenzaldehyde) increased AIDH activity (Table 4) whereas benzaldehydes with an electron donating group (2,4,6-trimethylbenzaldehyde, 4-isopropylbenzaldehyde) showed no detectable AIDH activity (Table 4). The fact that
benzaldehyde was a good substrate for FEDH but that lower FEDH activity was obtained by introducing an electron withdrawing group to the phenyl-ring of benzaldehyde indicates structural differences between the active sites of AIDH and FEDH.

Accepting the present view on the mechanism of molybdenum hydroxylases, the aldehyde dehydrogenases discussed here oxidize aldehydes and formate esters by catalyzing hydride transfer from the Cl-atom of the substrate to the sulphido or oxo group linked to the molybdenum centre and attack of a hydroxyl group (derived from H₂O) on the Cl-atom in concert [23]. In view of the substrate specificities of the two dehydrogenases studied here and of DL-AIDH [26], a wide spectrum of aldehydes, including special aldehydes like formaldehyde and benzaldehyde and aldehyde analogs like formate esters, can be oxidized according to this mechanism. However, aldehyde oxidoreductase from D. gigas, for unknown reasons, cannot convert formate esters (unpublished results). The very low enantioselectivity of AIDH and FEDH implies that the steric conformation of the residues attached to the asymmetric C-atom is irrelevant with respect to discrimination between the enantiomers in binding (Kᵣ values) as well as in turnover rates (Vₘₐₓ values). This is surprising since this atom concerns the 2- as well as the 3-position in the aliphatic aldehyde chain and aliphatic as well as phenyl substituents. The lack of discrimination could be explained by assuming that the site where the catalytic event takes place is at the outside of the enzyme molecule so that scarcely any interaction would occur between the enzyme molecule and the chain attached to the carbonyl group of the substrate. However, this is very unlikely for the following reasons. The structure of aldehyde oxidoreductase from D. gigas [22] indicates that the aldehyde has to pass a deep tunnel before conversion of it takes place. Assuming that the catalytic site in the aldehyde dehydrogenases is buried in a similar way, the chain should make contact with the tunnel walls. The latter is supported by the fact that AIDH and FEDH show restricted substrate specificities (Table 4). The following observations indicate indeed that the nature of the chain is relevant for binding and/or turnover: benzaldehyde binds very well but is a poor substrate for AIDH (although it is a very good substrate for FEDH); similarly, although 3-phenylpropanal binds so strongly to AIDH that it cannot be replaced by propanal, it is a very poor substrate for the enzyme (although it is reasonably well for FEDH); benzaldehyde becomes a good substrate for AIDH when this compound is substituted with an electron withdrawing group at the p-position (although lower FEDH activity was obtained). All these examples show that interaction occurs between the chain
and the enzyme, suggesting that the steric conformation of the substituents to the chain, should affect the strength of this interaction. Since this does apparently not occur, further investigations are required to explain why AIDH and FEDH show such a poor enantioselectivity for the racemates investigated.

Mechanistic implications

In conclusion, we have presented experimental data which indicate that the two aldehyde dehydrogenases studied share many characteristics with other molybdoproteins from prokaryotic and eukaryotic origin. Properties of AIDH that are different from other molybdoproteins are for example the higher value of $E_m$ of [2Fe-2S]-1 compared to that of [2Fe-2S]-2, the high value of $E_m$ of the FAD and the very low amount of Mo(V) formed in equilibrium potentiometric titrations or upon addition of substrate. What the possible consequences of these differences are for (e.g., the rate of) catalysis by AIDH is described below.

The molybdoproteins in all likelihood possess the same basic mechanism of action [23]. In most enzymes, the $E_m$ of the Mo(VI)/Mo(IV) redox couple is lower than that of the other prosthetic groups ensuring that the intramolecular single-electron transfers are thermodynamically downhill. The rate of equilibration among all redox centres has been established to be (much) faster than the rate of turnover, the latter being in the millisecond time range [72]. As a matter of fact the rates of internal electron transfer are in the nanosecond to microsecond time domain [73] as calculated from the edge-to-edge distances between the molybdenum centre and the nearest [2Fe-2S] cluster (~13 Å or ~5 Å from the pterin moiety to the [2Fe-2S] cluster) and between the two [2Fe-2S] clusters (~10 Å) as determined from the crystal structure of D. gigas aldehyde oxidoreductase [22]. The distance between FAD and the [2Fe-2S] clusters has been estimated as 16 Å [47], also yielding high rates of internal electronic equilibration. The distances between the various prosthetic groups are apparently well conserved in all molybdoproteins given the similarities in the strength of the magnetic interaction between the redox centres [26,70, vide supra] and are probably not or no longer subject to evolutionary change. A variation in the values of $E_m$ of the molybdenum centre, the [2Fe-2S] clusters or FAD by 0.1-0.2 V uphill would slow down internal electron transfer a thousand fold, but the absolute rate may still be significantly higher than the overall turnover rate and so the values of $E_m$ of the
various prosthetic groups are not subject to strong evolutionary pressure. Thus, the observations that the values of $E_{m}$ of [2Fe-2S]-1 in AIDH is higher than that of [2Fe-2S]-2, that the stabilities of FADH$'$ and of the Mo(V) are quite low compared to other molybdoproteins and as a matter of fact vary widely within the group of molybdoproteins, are understandable in terms of the conserved spatial arrangement of the prosthetic groups allowing rapid internal electronic equilibration. Apparently, once the first molybdoprotein had evolved many variations on the overall structural theme were allowed - including the absence of FAD, the presence of haems $c$ and $b$ instead of FAD [7], union of the three subunits to a single large subunit, monomeric or dimeric enzymes, apparent unfavourable stabilities of FADH$'$ and Mo(V), thermodynamically 'unfavourable' $E_{m}$'s, a short Mo=S or Mo=O bond - as long as the rate of internal electron transfer was high relative to the overall rate of turnover. In time differences in substrate binding domains (also of the electron acceptor which, however, is unknown for most of these enzymes) evolved widening the spectrum of catalysis performed by this class of enzymes, while leaving intact the spatial arrangement of its very core, the Moco and the two different [2Fe-2S] clusters. It is expected that elucidation of the 3-D structure of these enzymes (crystallization of AIDH is in progress) will provide a strong contribution in removal of the uncertainties still left.

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REFERENCES


Chapter 3

A second molybdoprotein aldehyde dehydrogenases
from *Amycolatopsis methanolica* NCIB 11946

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Abbreviations. (F)AIDH(s), (form)aldehyde dehydrogenase(s); DCPIP, 2,6-dichlorophenol-indophenol; DL-AIDH, (dye-linked) aldehyde dehydrogenase; FEDH, formate ester/aldehyde dehydrogenase; Moco, pterin molybdenum cofactor; Mops, 4-morpholinepropanesulfonic acid; PAGE, polyacrylamide gel electrophoresis; PVDF, polyvinylidene fluoride; SDS, sodium dodecyl sulfate; SPPB, 10 mM sodium pyrophosphate buffer, pH 9.0, 25 mM ethanol.

Enzymes. Aldehyde dehydrogenase (EC 1.2.99.3, haem-containing); (dye-linked) aldehyde dehydrogenase (DL-AIDH; EC 1.2.99.-); formate ester/aldehyde dehydrogenase (FEDH; EC 1.2.99.-); xanthine dehydrogenase (EC 1.1.1.204); xanthine oxidase (EC 1.1.3.22).

The sequences of the stretches of amino acids determined for the subunits of DL-AIDH have been deposited at the EBI Data Library under accession numbers: p80413 and p80414.
Methanol-grown *Amycolatopsis methanolica* NCIB 11946 contains a molybdoprotein dehydrogenase, active with aldehydes and formate esters as substrates and with Wurster's blue as electron acceptor, the so-called formate ester dehydrogenase (FEDH) [van Ophem, P.W., Bystrykh, L.V. and Duine, J.A. (1992) *Eur. J. Biochem.* 206, 519-525]. It appears now that another molybdoprotein dehydrogenase is present in this organism. This enzyme, indicated here as dye-linked aldehyde dehydrogenase (DL-AIDH), has the same set of cofactors and converts the same type of substrates but with different specificity, and uses 2,6-dichlorophenol-indophenol as sole artificial electron acceptor for these conversions. The enzymes also differ in their quaternary structure, FEDH having an α, β, γ and DL-AIDH having an α, β, γ₁ composition. Furthermore, differences exist with respect to the sizes and N-terminal amino acid sequences of their subunits, indicating that the enzymes derive from different genes. However, neither their substrate specificity nor their induction pattern give a clear indication for distinct physiological roles. Just like other bacterial molybdoprotein dehydrogenases, DL-AIDH consists of three different subunits (87, 35, and 17 kDa) and contains FAD, molybdopterin- cytosine-dinucleotide cofactor, Fe, and acid-labile sulphide in a molar ratio of 1:1:4:4. Although eukaryotic xanthine oxidase and xanthine dehydrogenase differ from these prokaryotic dehydrogenases in size and number of their subunits, certain stretches of amino acid sequences show similarity and the magnetic coupling between Mo(V) and the [2Fe-2S]-1 cluster in DL-AIDH and bovine milk xanthine oxidase is of the same magnitude. In view of this similarity, the topology of the cofactors in the active site of this type of molybdoproteins might be conserved among enzymes from prokaryotic as well as eukaryotic organisms.

**Keywords.** Aldehyde dehydrogenase; *Amycolatopsis methanolica*; formate ester dehydrogenase; molybdoprotein.
INTRODUCTION

Oxidation of aldehydes *in vitro* can be catalyzed by a number of different types of (form)aldehyde oxidoreductases: a molybdoprotein oxidase (EC 1.2.3.1); NAD(P)-dependent dehydrogenases (EC 1.2.1.-); NAD-dependent dehydrogenases requiring a co-substrate which can be either CoA (e.g., EC 1.2.1.10), GSH (EC 1.2.1.1), or an unknown factor [1,2]; and dye-linked dehydrogenases present in cell-free extracts of bacteria and thus most probably coupled to respiratory chains of these organisms. Since it is generally thought that (form)aldehyde oxidation occurs via (NAD(P) + co-substrate)-dependent dehydrogenases, what could be the role of the other types, especially the dye-linked dehydrogenases?

Several different bacterial dye-linked (form)aldehyde dehydrogenases ((F)AIDHs) appear to exist: a haemoprotein (haem c-containing) dehydrogenase (EC 1.2.99.3) [3,4]; quinoprotein dehydrogenases supposed to contain either PQQ [5] or an unknown, covalently bound quinone [6]; nicotinoprotein (enzymes containing firmly bound NAD(P)) dehydrogenases [7,8] which are able to dismutate aldehydes to the corresponding alcohol and carboxylic acid (e.g., formaldehyde dismutase (EC 1.2.99.4)); molybdoprotein dehydrogenases which contain either the full set of cofactors (pterin molybdenum cofactor (Moco), FAD, iron-sulphur clusters) as in the enzyme from *Comamonas testosteroni* [9] and *Amycolatopsis methanolica* [10] or the cofactors without FAD, as in the enzyme from *Desulfovibrio gigas* [11] and *Clostridium formicoaceticum* [12]; and tungstoprotein dehydrogenases from *C. formicoaceticum* [13], *C. thermoaceticum* [14], *Pyrococcus furiosus* [15], and *D. gigas* [16].

It has already been reported [10] that *A. methanolic* NCIB 11946 contains a 2,6-dichlorophenol-indophenol (DCPIP)-dependent AIDH in addition to the aldehyde/formate ester-oxidizing, Wurster's blue-linked, molybdoprotein dehydrogenase, designated as "formate ester dehydrogenase (FEDH)". In view of the possibility of finding a new or another type of aldehyde oxidoreductase in this organism and our general interest in the significance of diversity of enzyme types catalyzing one and the same reaction, this "dye-linked AIDH (DL-AIDH)" was purified and characterized. Surprisingly, it appeared to be a molybdoprotein too, although its structural and catalytic properties differ from those of FEDH.
MATERIALS & METHODS

Cultivation of the organism. *A. methanolica* NCIB 11946 (LMD 80.32) was cultured on a mineral medium [17] supplemented with 1% (by vol.) methanol as a carbon and energy source in a 20-litre fermentor at 37 °C. The pH was maintained at 6.7 and good aeration was provided. Cells were harvested at the end of the exponential growth phase and the cell paste was stored at -20 °C.

Purification scheme. Frozen cell paste (183 g) was suspended in an equal volume of 10 mM sodium pyrophosphate buffer, pH 9.0, containing 25 mM ethanol (SPPB). After adding some DNase (to avoid a viscous mass), the cells were disrupted in a French pressure cell at 10 MPa. The suspension was centrifuged (1 h at 100,000 x g) at 4 °C, the supernatant yielding the cell-free extract.

The extract was directly applied to a DEAE Bio-Gel agarose column (5 x 19.5 cm, Bio-Rad) equilibrated with SPPB. The column was washed with the same buffer and the adsorbed proteins were eluted with a linear gradient from 0 to 1 M KCl in SPPB in 12 h at a flow rate of 1.2 ml/min. Active fractions were pooled and concentrated (Centriprep-100 concentrator, Amicon).

The concentrate was brought to 1 M KCl and applied to a phenyl Sepharose column (2.6 x 20 cm; Pharmacia) equilibrated with 10 mM potassium phosphate buffer, pH 7.9, containing 1 M KCl and 25 mM ethanol. Proteins were eluted in 3 h with a linear gradient from 1 to 0 M KCl in this buffer at a flow rate of 1 ml/min. Active fractions were pooled and concentrated. Subsequently buffer exchange occurred on a PD-10 gel filtration column (Pharmacia) equilibrated with SPPB.

Aliquots of the concentrate were applied to a Mono-Q HR 10/10 column (Pharmacia) equilibrated with SPPB. The column was washed with the same buffer and proteins were eluted with this buffer containing 0.6 M KCl at a flow rate of 1 ml/min. Active fractions were pooled and concentrated as above and buffer exchange occurred against 20 mM Mops buffer, pH 7.5, containing 25 mM ethanol.

Aliquots of the concentrate were applied to a Superdex-200 column (Pharmacia) equilibrated with 20 mM Mops buffer, pH 7.5, containing 25 mM ethanol and 0.15 M KCl.
Elution occurred with the same buffer at a flow rate of 0.4 ml/min. Active fractions were pooled and stored at -80 °C.

All manipulations were carried out at room temperature, except DEAE-chromatography (at 4 °C). Protein determinations during the purification were performed according to Bradford [18] using desalted bovine serum albumin as a standard.

**Enzyme assay.** DL-AldH activity was measured by following the rate of reduction of DCPIP (60 µM) at 600 nm (using a molar absorption coefficient of 22,000 M⁻¹cm⁻¹ [19]) in 0.12 M sodium pyrophosphate buffer, pH 9.2. After incubating the mixture at 37 °C for 1 min, the reaction was started by adding formaldehyde (10 mM).

**Molecular mass determination.** The molecular mass of the native enzyme was determined by gel filtration with the same column and conditions as used in the last purification step. It was also determined by PAGE on commercial gradient gels (Pharmacia, 8-25 %). The following markers were used: catalase (232 kDa), aldolase (158 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), chymotrypsinogen A (25 kDa), and ribonuclease (13.7 kDa). Denaturation of the proteins occurred by bringing the solutions at 10 % (by mass) SDS and 20 % (by vol.) 2-mercaptoethanol and heating the mixture at 100 °C for 10 min. The molecular mass of the subunits was determined by SDS-PAGE on the gradient gels according to the instructions given by the manufacturer (Phast Systems, using the low molecular weight calibration kit, Pharmacia).

**Subunit stoichiometry.** Enzyme solution (3 µl, 50 mg/ml) was mixed with an equal volume of a solution containing 5 % SDS and 10 % 2-mercaptoethanol. The mixture was heated for 10 min at 100 °C and applied to a gel filtration column (Superdex-200, 30 x 1 cm) equilibrated with 50 mM sodium phosphate buffer, pH 7.4, containing 0.1 % SDS and 0.1 M NaCl. Chromatography occurred at a flow rate of 0.5 ml/min and the eluate was monitored with a photodiode array detector at 205 nm (the absorbance at this wavelength is directly related to the protein concentration [20]). The peak areas were integrated and divided by the molecular mass of the subunits, providing the concentrations of the subunits.

**Cofactor identity and content.** The FAD content was measured (see ref. [9] for details) in the supernatant at 450 nm (using a molar absorption coefficient of 11,300 M⁻¹cm⁻¹[19]) after denaturing the enzyme with trichloroacetic acid and removing aggregated protein by centrifugation. It was also determined by applying an aliquot to an HPLC reversed-phase column
(Novapak 5 μm, Waters) after denaturing the enzyme by boiling the solution in the dark and removing aggregated protein by centrifugation. The amount of FAD was calculated from a curve relating peak area to known amounts of FAD. The other cofactors were determined by a colorimetric method: molybdenum [21], iron [22], using ferrene as a chelator, and acid-labile sulphide [23]. The Moco was determined by two different methods [24,25]. The identity of the nucleotide moiety in it was assessed by chromatographic comparison as developed by Frunzke and Meyer [25]. The denatured protein was removed by centrifugation and the supernatant was applied to a reversed-phase HPLC column (Lichrospher RP-18, 5 μm, Merck) using 20 mM Na H₂PO₄, pH 3.5, as the eluant.

To calculate the cofactor/enzyme ratios, a specific absorption coefficient was used for the enzyme of 0.98 at 280 nm, the latter determined with a method previously described [20].

**N-terminal amino acid sequences.** The purity of the subunit fractions, prepared as indicated under “Subunit stoichiometry”, was checked by SDS-PAGE. Proteins were adsorbed to PVDF membranes according to Sheer [26]. N-terminal amino acid sequences were determined with automatic Edman degradation (Applied Biosystems, Sequencer model 477A and HPLC model 120A). The sequences were compared with those published in the literature by eye or with those in the protein sequence databanks, using the program BLAST [27].

**EPR spectroscopy.** EPR spectroscopy was performed with a Varian E-9 spectrometer operating at X-band frequency and equipped with a home-built He-flow system.

## RESULTS

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Total protein (mg)</th>
<th>Total activity (U)</th>
<th>Specific activity (U/mg)</th>
<th>Yield (%)</th>
<th>Purification (-fold)</th>
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</thead>
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<td>Cell-free extract</td>
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<tr>
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<td>0.70</td>
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<td>Phenyl-Sepharose</td>
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<td>125</td>
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<tr>
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<td>12</td>
<td>83</td>
<td>6.93</td>
<td>73</td>
<td>346</td>
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<tr>
<td>Superdex-200</td>
<td>6</td>
<td>45</td>
<td>7.56</td>
<td>40</td>
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</table>
Purification and some biochemical properties. Table 1 shows the scheme and yield of purification. The final preparation was homogeneous, as judged from gel filtration (identical absorption spectra taken at different positions in the eluting peak) and native-PAGE (one band with protein staining). Purification required the presence of ethanol in the buffers otherwise, a dramatic drop in activity occurred. The pH optimum of the assay appeared to be 9.2. Only DCPIP was active as electron acceptor, not phenazine methosulphate, methylene blue, ferricyanide, Wurster’s blue, tetranirotblue tetrazolium chloride, horse heart cytochrome c, 1,2-naphtoquinone, 1,4-naphtoquinone, NAD, NADP or O₂.

Table 2. Substrate specificity of DL-AldH. Substrates were assayed as indicated in section “Materials & Methods”. Only maximal enzyme activities are reported here whereas substrate concentrations varied from 0.01 to 100 mM.

<table>
<thead>
<tr>
<th>Substrates</th>
<th>Substrate concentration (mM)</th>
<th>Activity (%)</th>
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</thead>
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<tr>
<td><strong>Aliphatic aldehydes</strong></td>
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<tr>
<td>Formaldehyde*</td>
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<tr>
<td>Propionaldehyde</td>
<td>1</td>
<td>447</td>
</tr>
<tr>
<td>Butyraldehyde</td>
<td>0.1</td>
<td>437</td>
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<tr>
<td>Hexaldehyde</td>
<td>1</td>
<td>246</td>
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<td>Heptaldehyde</td>
<td>1</td>
<td>137</td>
</tr>
<tr>
<td>Octanal</td>
<td>10</td>
<td>125</td>
</tr>
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<tr>
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<td>Isobutyraldehyde</td>
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<td>24</td>
</tr>
<tr>
<td>Ethyl formate</td>
<td>100</td>
<td>116</td>
</tr>
</tbody>
</table>

*For DL-AldH 100 % = 7.6 U/mg.

The substrate specificity appeared to be very broad: aliphatic aldehydes, even those containing a phenyl moiety, as well as formate esters, are substrates (Table 2), some of them exhibiting substrate inhibition (Table 3). Quinoline, xanthine or primary alcohols are not substrates. Methanol is not an inhibitor but Hg²⁺, Fe³⁺, NH₄⁺, and CN⁻ are (Table 4).
Table 3. Apparent kinetic parameters of DL-AIDH. Substrates were assayed in the concentration range 0-25 mM with the standard concentration of DCPIP (60 μM). Kinetic parameter values were calculated according to the Simfit program 1.01 designed on our laboratory by Dr. J.B.A. van Tol.

<table>
<thead>
<tr>
<th>Substrates</th>
<th>$K'_m$ (mM)</th>
<th>$V'_{max}$ (mM s$^{-1}$)</th>
<th>$K'_i$ (mM)</th>
<th>$V'_{max}/K'_m$ (s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Formaldehyde</td>
<td>63</td>
<td>7</td>
<td></td>
<td>0.11</td>
</tr>
<tr>
<td>Acetaldehyde</td>
<td>0.02</td>
<td>11</td>
<td>92</td>
<td>565</td>
</tr>
<tr>
<td>Propionaldehyde</td>
<td>0.13</td>
<td>15</td>
<td>261</td>
<td>114</td>
</tr>
<tr>
<td>Butyraldehyde</td>
<td>0.04</td>
<td>11</td>
<td>4</td>
<td>265</td>
</tr>
<tr>
<td>Benzaldehyde</td>
<td>0.02</td>
<td>2</td>
<td>20</td>
<td>105</td>
</tr>
</tbody>
</table>

Table 4. Inhibitors of DL-AIDH. Incubation with inhibitor was carried out for 1 min before the reaction was started by adding formaldehyde (10 mM). Abbreviations used: PMSF, phenylmethysulfonylfluoride; DTNB, 5,5'-dithio-bis(2-nitrobenzoic acid); NEM, N-ethylmaleimide.

<table>
<thead>
<tr>
<th>Inhibitors</th>
<th>Concentration (mM)</th>
<th>Relative activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None*</td>
<td>-</td>
<td>100</td>
</tr>
<tr>
<td>PMSF</td>
<td>1</td>
<td>87</td>
</tr>
<tr>
<td>DTNB</td>
<td>1</td>
<td>78</td>
</tr>
<tr>
<td>NEM</td>
<td>1</td>
<td>84</td>
</tr>
<tr>
<td>EDTA</td>
<td>1</td>
<td>96</td>
</tr>
<tr>
<td>Ni</td>
<td>1</td>
<td>91</td>
</tr>
<tr>
<td>Fe$^{3+}$</td>
<td>1</td>
<td>95</td>
</tr>
<tr>
<td>Fe$^{2+}$</td>
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<td>3</td>
</tr>
<tr>
<td>Hg$^{2+}$</td>
<td>0.1</td>
<td>0</td>
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<tr>
<td>KCN</td>
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<td>0</td>
</tr>
<tr>
<td>H$_2$O$_2$</td>
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<td>113</td>
</tr>
<tr>
<td>NH$_4^+$</td>
<td>330</td>
<td>15</td>
</tr>
<tr>
<td>Primary alcohols</td>
<td></td>
<td></td>
</tr>
<tr>
<td>methanol</td>
<td>100</td>
<td>87</td>
</tr>
<tr>
<td>ethanol</td>
<td>100</td>
<td>108</td>
</tr>
<tr>
<td>propanol</td>
<td>100</td>
<td>87</td>
</tr>
<tr>
<td>butanol</td>
<td>100</td>
<td>85</td>
</tr>
</tbody>
</table>

*For DL-AIDH 100 % = 7.6 U/mg.

Spectroscopic and structural properties. The final preparation showed an absorption spectrum (Fig. 1) reminiscent of that of molybdoproteins [28] with an $A_{280}/A_{450}$ and $A_{450}/A_{550}$ ratio of 4.3 and 3.0, respectively. Formaldehyde addition resulted in disappearance of the FAD contribution to the spectrum (Fig. 1).
Figure 1. Absorption spectra of DL-AlDH. The absorption spectrum of the final preparation in 20 mM Mops buffer, pH 7.5, was measured at room temperature. The inset shows the spectrum in the absence (solid line) and in the presence (dotted line) of formaldehyde (1 mM).

Figure 2 shows the EPR spectrum of DL-AlDH reduced by formaldehyde or dithionite. The top trace is characteristic of a rhombic Mo(V) signal with 3 g-values below that of the free-electron value (Table 5). Both the $g_z$ and the $g_z$ resonances are split, most likely due to hyperfine interaction with a proton in close proximity, as has been observed for xanthine oxidase [29,30]. The lines in the high-field part of the spectrum are the $g_y$ resonances of the molybdenum centre originating from the $^{95,97}$Mo isotopes with $I = 5/2$ ($A_y = 2.8$ mT).

When the spectra were measured at lower temperature (Fig. 2, middle and bottom traces), an additional rhombic signal was observed. The g-values (Table 5) and relaxation behaviour of this signal are very similar to that of the iron-sulphur cluster designated as [2Fe-2S]-1 for xanthine oxidase [29,30]. Quantitation (Table 5) of this signal induced by dithionite reduction of the enzyme revealed equimolar amounts of enzyme and cluster. The signal of the cluster designated as [2Fe-2S]-2 in xanthine oxidase was not observed, although several attempts were
Fig. 2. X-band EPR spectra of DL-AIDH. To the final preparation in 20 mM Mops buffer, pH 7.5, formaldehyde (1 mM, top and middle traces) or excess dithionite (bottom trace) were added. Experimental conditions: microwave frequency, 9.230 GHz; microwave power, 0.2 mW; modulation amplitude, 0.2 mT; temperature, 98 K (top trace), 23 K (middle and bottom traces). N.B.: The gain is different for the different traces.

made to detect it at 7 K with varying microwave power, using an enzyme preparation treated with excess dithionite at room temperature for 15 min. Since the chemical determination of iron and acid-labile sulphur (see below) suggests that such a cluster could be present, its escape from detection could be due to a low redox potential or to a lack of redox equilibration with the other
EPR-detectable centres in the enzyme. The latter possibility might result from the presence of two $\gamma$-subunits per enzyme molecule (see below), one of which does not participate in electronic communication.

Table 5. EPR parameters and spin quantitation of the Mo(V) and [2Fe-2S]-1 centres in DL-AIDH.
Measurements were performed as indicated in the legend of Fig. 2.

<table>
<thead>
<tr>
<th></th>
<th>$g_x$</th>
<th>$g_y$</th>
<th>$g_z$</th>
<th>$g_{yw}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mo(V)</td>
<td>1.962</td>
<td>1.968</td>
<td>1.994</td>
<td>1.974</td>
</tr>
<tr>
<td>[2Fe-2S]-1</td>
<td>1.904</td>
<td>1.940</td>
<td>2.023</td>
<td>1.956</td>
</tr>
</tbody>
</table>

Spin quantitation

<table>
<thead>
<tr>
<th></th>
<th>Formaldehyde</th>
<th>Formaldehyde</th>
<th>Dithionite</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temperature</td>
<td>98 K</td>
<td>23 K</td>
<td>23 K</td>
</tr>
<tr>
<td>Mo(V)</td>
<td>0.33$^a$</td>
<td>0.43</td>
<td>-</td>
</tr>
<tr>
<td>[2Fe-2S]-1</td>
<td>n.d.$^b$</td>
<td>0.42</td>
<td>1.04</td>
</tr>
</tbody>
</table>

$^a$ Mol $S = (1/2)mol$ enzyme.

$^b$n.d.: not detectable at this temperature.

From Fig. 2 it is clearly visible that the signals in the middle trace differ from those in the top and bottom traces, especially those belonging to Mo(V). The additional signals are probably due to magnetic coupling between Mo(V) and the [2Fe-2S]-1 cluster, as has been observed for other molybdoproteins [31,32]. The interaction is fairly isotropic (0.8-1.1 mT) and of similar strength as in xanthine oxidase [31]. The larger line widths of the [2Fe-2S]-1 resonances (0.4, 0.1 and 0.4 mT for $g_x$, $g_y$, and $g_z$, respectively) in the middle trace compared to the bottom trace are also in line with this magnetic interaction. Furthermore, the increase in the Mo(V) spin concentration in going from 98 to 23 K (Table 5) is also indicative of this, since it suggests that part of the Mo(V) spins are coupled to the [2Fe-2S]-1 spins, leading to a similar relaxation behaviour.

Native enzyme showed a molecular mass of 160 kDa with gel filtration and of 87 kDa with PAGE. The enzyme has three different subunits of 87, 35, and 17 kDa, as judged from SDS-PAGE. When heating was omitted during the denaturation step, one single band of 67 kDa was
observed with SDS-PAGE. Gel filtration chromatography of denatured enzyme in the presence of SDS and monitoring the eluate at 205 nm enabled the separation of the three subunits (Fig. 3) and the calculation of their concentration. From this it appeared that the α, β, and γ subunits occur in a molar ratio of 1.0:0.8:1.7. Since amino acid sequencing did not reveal any heterogeneity in the N-terminal sequences of the subunits (see below), this suggests that the enzyme has an α, β, γ_2 structure. Since such a composition for this type of molybdoproteins has not been reported before, FEDH was used as a reference molybdoprotein since the sum of the molecular masses of its subunits is in line with that of the native form (to be published elsewhere). From the ratios calculated from the profile of FEDH in Fig. 3 (1.0:1.0:0.8), it appeared indeed that this enzyme has an α, β, γ composition.

![Graph showing separation of subunits](image)

**Fig. 3. Separation and quantitation of the DL-AIDH and FEDH subunits.** The enzymes were denatured and chromatographed on a gel filtration column in the presence of SDS. Quantitation was carried out by integrating the peaks. The solid line represents the elution profile of DL-AIDH, the dotted line that of FEDH.
To calculate the stoichiometric ratios of cofactors to enzyme, a molecular mass of 160 kDa was assumed to apply to the enzyme. Ratios of 1.0 and 1.2 were obtained for FAD with the colorimetric and the HPLC method, respectively. Molybdenum, iron, and acid-labile sulphur appeared to be present in ratios of 0.9, 4.1, and 3.7, respectively. HPLC of denatured enzyme revealed a peak coinciding with that of the Moco detached from xanthine oxidase. Subsequent treatment with acid or nucleotide pyrophosphatase changed this to a peak coinciding with that of CMP, indicating the presence of the molybdopterin cytosine dinucleotide cofactor in this enzyme, just as in many other bacterial molybdoproteins.

The separated subunits could be sequenced, indicating that they are not terminally blocked. The N-terminal stretches of amino acids determined (not shown) showed similarity with those of published sequences of other molybdoproteins and that of the γ-subunit of a hydrogen dehydrogenase. Since the latter enzyme contains FMN and iron-sulphur clusters, the sequence similarity may indicate that both subunits contain a similar cofactor, most probably an iron-sulphur cluster.

DISCUSSION

In the past few years, several bacterial molybdoprotein dehydrogenases have been discovered. The enzyme described here belongs to this group as it has the typical set of cofactors and sizes of the protein subunits. On the other hand, the α, β, γ composition is unique and intriguing, especially because FEDH in this organism has an α, β, γ composition. According to the substrate specificity observed, it is a member of the subgroup of molybdoprotein AIDHs. However, in addition to its specific catalytic features, DL-AIDH has some other peculiarities as methanol is no inhibitor and ethanol has a stabilizing effect on it. The latter has also been observed for the hitherto uncharacterized, dye-linked AIDH from *Hyphomicrobium* X [33]. Methanol is an inhibitor for FEDH, whereas ethanol is not a stabilizer for it. Thus, not only the overall structure but also that of the active site of DL-AIDH will be different from that of FEDH. FEDH, as well as DL-AIDH, is distinct from the molybdoprotein aldehyde oxidoreductase from *D. gigas* [11] since the latter does not oxidize formate esters (unpublished results), has only two subunits (of 100 kDa) different in size from those of the dehydrogenases, and lacks FAD but contains much more iron and acid-labile sulphur.
No consistent picture was obtained with respect to the molecular mass of native enzyme, as determined with gel filtration (160 kDa) and gradient PAGE (87 kDa) (a similar situation exists with respect to the molybdoprotein quinone-4-carboxylic acid oxidoreductase from *Agrobacterium sp.* 1B [34]). Based on the ratio in which the subunits are found to occur in the enzyme and assuming that their molecular masses, as determined with SDS-PAGE (87, 35, 17 kDa), are correct, a molecular mass of 156 kDa is calculated for native enzyme, in line with the value found for it by gel filtration. The value of 87 kDa suggests that under the conditions of gradient PAGE, dissociation of the enzyme occurs.

In view of their differences in substrate and electron acceptor specificities, in stabilization and inhibition, in N-terminal amino acid sequences of their subunits, and in quaternary structure, it is clear that FEDH and DL-AIDH from *A. methanolica* are different enzymes encoded by different genes. However, neither the induction pattern (both enzymes are induced at growth on methanol) nor the specificities provide a clue for distinct physiological roles.

The presence of two dye-linked (F)AIDHs in one and the same organism has also been found in other cases: *Methyllobacterium extorquens* [36]; *Paracoccus denitrificans* [37], one of these having the properties of a molybdoprotein (unpublished results); and *Hyphomicrobiium zavarzinii* [6]. In the latter case, induction of one FAIDH occurred at growth on methanol, the other at growth on methylamine [6,38]. In the case of *P. denitrificans*, dye-linked FAIDHs seem unable to cope with formaldehyde since a mutant strain lacking NAD/GSH-dependent FAIDH (EC 1.2.1.1) did not exhibit growth on methanol or methylamine [39]. However, this does not exclude an assisting role of the dye-linked AIDHs in formaldehyde or formate ester oxidation at growth on methanol and a crucial role in dissimilation of other aldehydes. This appears to be the case for *Saccharomyces cerevisae* where a FAIDH other than the NAD/GSH-dependent one can take over so that the mutant can survive application of low formaldehyde concentrations [40]. Therefore, in the absence of any further information, it is clear that no conclusion can be drawn with respect even to the physiological role of dye-linked (F)AIDHs in PQQ- and haem c-containing AIDH [5] with a quite different size and subunit composition compared to the originally discovered haem c-containing AIDHs [3,4] (nevertheless, both enzymes are indicated under the same number (EC 1.2.99.3) in the latest edition of *Enzyme Nomenclature*). However, the sequence of the gene for dye-linked AIDH from *Acetobacter polyoxygens* has no similarity with those for PQQ-containing enzymes [41] but it has so with molybdoproteins [42]. Therefore,
this organism contains either a quinoprotein as well as a molybdoprotein AlDH or only the molybdoprotein one, the latter implying that the statement that the AlDH found in this organism is a quinoprotein [36] is incorrect. The absorption spectrum shown for the purified enzyme [43] is indeed indicative for a molybdoprotein and not for a haem c-containing quinoprotein.

In view of the examples given above, it appears now that molybdoprotein AlDHs occur in the major groups of bacteria. However, as far as we are aware, these enzymes have not been discovered in eukaryotes. The latter contain aldehyde oxidase (EC 1.2.3.1), which has the same set of cofactors in the same molar ratio as DL-AlDH, but has not been found in bacteria [29,30]. Given these similarities and dissimilarities, it would be interesting to compare the structures of both types of molybdoprotein aldehyde oxidoreductase. Despite large differences in subunit size and composition and a nondetectable signal of a [2Fe-2S]-2 cluster in DL-AlDH, structural and topological equivalence may exist since similarity (not shown) is observed between the N-terminal sequence of the γ-subunit of DL-AlDH and a stretch in eukaryotic xanthine dehydrogenase and the magnetic coupling between Mo(V) and [2Fe-2S]-1 in DL-AlDH (Fig. 2) is similar to that in mammalian xanthine oxidase [29,30]. Since such a similarity has also been observed for the stretches in the cloned genes for the subunits of molybdoprotein carbonmonoxide dehydrogenase [44,45], the topology of the cofactor sites might be conserved among enzymes originating from eukaryotic as well as prokaryotic organisms.

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REFERENCES


Chapter 4

Molybdopterin radical in bacterial aldehyde dehydrogenases

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Abbreviations. AlDH, aldehyde dehydrogenase from *Comamonas testosteroni*; DL-AIDH, (dye-linked) aldehyde dehydrogenase from *Amycolatopsis methanolica*; FEDH, formate ester dehydrogenase from *Amycolatopsis methanolica*; MCD, molybdopterin cytosine dinucleotide; Moco, pterin molybdenum cofactor; MPT, molybdopterin.

A note on nomenclature. The nomenclature of molybdopterins is ambiguous with respect to the redox state of the pterin moiety which may lead to confusion concerning some of the conclusions drawn in this paper. Recent findings (referred to in the text and see the legend to Fig. 9) have shown that the pterin-ring system is a three-ringed and not a two-ringed system. As a consequence, the redox state of the molybdopterin in situ is the same as that of, e.g., tetrahydrobiopterin. The molybdopterin radical that we describe below is one electron more oxidized than the tetrahydro-redox state and is therefore called a Mo(VI)-trihydropterin radical.

Furthermore, we use the old numbering of the atoms of the pterin ring. Since the pterin-ring system is a three-ringed system, numbering should actually start at the pyran oxygen atom.
The EPR spectra of three different molybdoprotein aldehyde dehydrogenases, one purified from *Comamonas testosteroni* and two purified from *Amycolatopsis methanolica* showed in their oxidized state a novel type of signal. These three enzymes contain two different [2Fe-2S] clusters, one FAD and one molybdopterin cytosine dinucleotide as cofactors all of which are expected to be EPR silent in the oxidized state. The new EPR signal is isotropic with $g = 2.004$ both at X-band and Q-band frequencies, consists of six partially resolved lines and shows Curie temperature behaviour suggesting that the signal is due to an organic radical with $S = 1/2$. The EPR spectra of *Comamonas testosteroni* aldehyde dehydrogenase obtained after cultivation in media containing $^{15}$NH$_4$Cl and/or after substitution of H$_2$O for D$_2$O show the presence of both nitrogen and proton hyperfine interactions. Simulations of the spectra of the four possible isotope combinations yield a single set of hyperfine coupling constants. The electron spin shows hyperfine interaction with a single $I = 1$ (0.9 mT) ascribed to a N-nucleus, with a single $I = 1/2$ (1.5 mT) ascribed to one non-exchangeable H-nucleus and with two, exchangeable, identical $I = 1/2$ spins (0.6 mT), ascribed to two identical exchangeable protons. Taken together, the observations and simulations rule out amino-acid residues or FAD as the origin of the radical. The values of the various hyperfine coupling constants are consistent with the properties expected for a molybdenum(VI)-trihydropterin radical in which the N5-atom is engaged in two hydrogen bonding interactions with the protein. The majority of the electron (spin) density of the radical is located at and around the N5-atom and at the proton bound to the C6-atom of the pterin ring. The EPR spectrum of the molybdopterin radical broadens above 65 K and is no longer detectable above 168 K indicating that it is not magnetically isolated. The linebroadening is ascribed to cross-relaxation with a nearby, rapidly relaxing, oxidized [2Fe-2S]-cluster involving its magnetic $S = 1$ excited state in this process. The amount of radical was apparently not changed by addition of aldehydes or oxidants but it disappeared upon reduction by sodium dithionite. Therefore, whether the molybdenum(VI)-trihydropterin radical as detected here is a functional intermediate in catalysis remains to be investigated further.

*Keywords.* Aldehyde dehydrogenase; electron transfer; EPR; magnetic coupling; molybdopterin; radical.
INTRODUCTION

Enzymes containing the molybdenum- or tungsten-pterin cofactor are found in eubacteria, archaebacteria, in lower and higher eukaryotes and function as redox enzymes in many different metabolic pathways. They catalyze oxidative hydroxylation reactions of, e.g., aromatic heterocycles, aldehydes, formate, carbon monoxide or sulfite [1,2]. They also catalyze the reverse of this reaction, the so-called reductive dehydroxylation of, e.g., nitrate or DMSO. Both types of hydroxylation reactions are two-electron redox reactions. In the oxidative hydroxylation the hydroxyl group being incorporated into the product is derived from water and not from oxygen. Similarly, in the reductive dehydroxylation, the (hydro)oxo-group transferred ends up in water.

The common component of all forms of the pterin molybdenum cofactor (Moco) is molybdopterin (MPT). A first proposal for the structure of MPT was given by Rajagopalan and co-workers [3,4]. A variant form of the Moco was first detected in carbon monoxide dehydrogenase [5,6] and had been identified as molybdopterin cytosine dinucleotide (MCD) in which a nucleotide is attached to the MPT via a pyrophosphate linkage (cf. Fig. 9). Later, more variants in the type of nucleotide have been detected, i.e., adenosine, guanosine or hypoxanthine (see [7] for an overview). So far MPTs attached to a nucleotide have been found exclusively in prokaryotes. Enzymes containing the MPT without a nucleotide have been found both in prokaryotes and eukaryotes.

Recently, the knowledge on the structure and functioning of molybdo- or tungstopterin-containing enzymes has greatly increased owing to the determination of the 3D-structures of four of these enzymes, the aldehyde ferredoxin oxidoreductase from Pyrococcus furiosus [8], the aldehyde oxidoreductase from Desulfovibrio gigas [9], the DMSO reductase from Rhodobacter sphaeroides [10], and the formate dehydrogenase H from Escherichia coli [11]. Two important new findings with respect to the structure of the pterin moiety have emerged from these studies. First, the enzyme-bound pterin system is a non-planar three-ringed structure (cf. Fig. 9) and not a two-ringed system as proposed for the free pterin [4,12]. The redox state of the pterin-ring system is formally the same as that of the tetrahydrobiopterin. Pterin-model compounds in the tetrahydro-redox state are non-planar [13,14]. The observation in all four crystal structures that the three-ringed pterin is non-planar is consistent with the tetrahydro-redox state of the pterin and not with a dihydro-redox state (a possible exception may be the Q-MPT ring in DMSO reductase
[15]. In all four crystal structures, the pterin-ring system is observed to be engaged in many hydrogen bonds with residues from the protein. The second new finding—in three of these enzymes but not in the *D. gigas* enzyme—was the presence of a so-called bis-MPT structure in which the Mo- or W-atom is coordinated by four sulphur atoms derived from two pterin rings [8,16].

The aldehyde oxidoreductase from *D. gigas* is a homodimer and catalyzes the oxidation of aldehydes to carboxylic acids [17,18] This enzyme which shares many characteristics with xanthine oxidase, contains two different types of [2Fe-2S] clusters but lacks FAD. The molybdenum atom is coordinated to two diithiolene sulphur atoms derived from a single pterin (the MCD) and further to two oxygen ligands and one sulphido group [9]. Recently, a detailed proposal for its mechanism of action describing the chemistry at the molybdenum centre has been put forward on basis of the crystal structures of various forms of the enzyme [19].

We have previously described the purification and characterization of the NAD-independent aldehyde dehydrogenases from *Comamonas testosteroni* (AIDH) [20] and from *Amycolatopsis methanolica* (FEDH [21] and DL-AIDH [22]). These enzymes can be classified as molybdenum hydroxylases. They catalyze dye-linked oxidation of aldehydes to the corresponding carboxylic acids. These three enzymes have different substrate specificities and their natural electron acceptors are unknown. Each one consists of three different subunits, contains one MCD, one FAD and two EPR distinguishable [2Fe-2S] clusters [23]. The three enzymes show sequence similarity (32-55 %) with the aldehyde oxidoreductase from *D. gigas*. The major apparent difference is the absence of FAD in the *D. gigas* enzyme.

In spite of the enormous progress made regarding the structure of molybdo-(tungsto) proteins, the role(s) of the pterin moiety itself during catalysis remains to be elucidated. So far indications exist that its redox state is important for overall activity [24] and/or in electron transfer [25]. However, whether MPT acts similarly as tetrahydrobiopterin, i.e., as electron donor (cf. ref. [26]) in reactions catalyzed by, e.g., aromatic amino acid hydroxylases [27], is not known. Furthermore, why the molybdenum atom is coordinated to diithiolene sulphur atoms and not to potential ligands of the protein (cysteine) is unknown. Is this related to function or to the pathway of biosynthesis of the cofactor [28-30], e.g., cysteine might not able to replace the Mo-O bond as present in molybdate by a Mo-S bond. To answer part of these questions we study the properties of bacterial aldehyde dehydrogenases. In this paper we describe the properties of a
novel EPR signal present in three aldehyde dehydrogenases 'as isolated'. Since all known prosthetic groups in these three enzymes should be EPR silent -the 'as isolated' enzyme is oxidized - which is the case for other molybdenum hydroxylases except for the presence of some Mo(V), the detection of a novel EPR signal was unexpected. This paper deals with the structural characterization of the species yielding this new EPR signal. We have performed various isotope substitution experiments, EPR spectroscopy at X- and Q-band frequencies and EPR spectral simulations leading to the proposal that the EPR signal is due to a molybdenum(VI)-trihydropterin radical. A possible role of this radical in catalysis, in particular in electron transfer, will be discussed.

MATERIALS & METHODS

Growth of C. testosteroni (ATCC 15667) and isolation of the AIDH was as described in [20,23]. The $^{15}$N-containing AIDH was obtained by cultivation of C. testosteroni on mineral medium containing, per litre, 1 g of $^{15}$NH$_4$Cl and 4 g of K$_2$SO$_4$ replacing 3 g of (NH$_4$)$_2$SO$_4$. The $^{95}$Mo- or $^{98}$Mo-derivatives of AIDH were obtained by growing C. testosteroni in 2 µM (Na)$_2$$^{95}$MoO$_4$ or (Na)$_2$$^{98}$MoO$_4$, respectively, instead of 0.1 µM (Na)$_2$MoO$_4$. (Na)$_2$$^{95}$MoO$_4$ and (Na)$_2$$^{98}$MoO$_4$ were prepared from $^{95}$Mo or $^{98}$Mo. $^{95}$Mo or $^{98}$Mo (each 0.1 mmol) was dissolved in 1 ml of nitric acid. After centrifugation of the sample at 14,000 rpm for 10 min, the supernatant was removed. The disodium molybdate isotopes were obtained by dissolving the remaining pellet in 0.5 ml of 1 M NaOH. Quantitative determination of molybdenum was as in [31]. Samples of the C. testosteroni AIDH in D$_2$O were obtained by dilution in 20 mM Mops (pH 7.0) made up in 99.8 % D$_2$O followed by concentration until a final D$_2$O concentration > 99 % had been obtained. This procedure lasted about 40' after which the EPR sample was frozen. To study whether protons were exchanged at a longer time scale, the sample was thawed and incubated in ice for another 7 hours. This shape of the EPR signal did not change further (see also text). A. methanolicola (NCIB 11946) was cultivated as described in [21] to obtain FEDH and cultivated as described in [22] to obtain DL-AIDH.

UV/Vis-spectra were measured in cells of 1 cm path length at room temperature with a Hewlett-Packard HP 8452 A Diode Array spectrophotometer. EPR spectra at X-band frequencies were recorded with a Varian E-9 spectrometer. The magnetic field and microwave frequency
were determined with an AEG Kernresonanz-Magnetfeldmesser and a HP 5245L electronic counter, respectively. This spectrometer has been equipped with a home-built He-flow system. Q-band EPR spectra were recorded as described in [32].

EPR spectra were simulated with a program specifically written for the Macintosh PowerPC (68k processor) using CodeWarrior 5, Metroworks Pascal 1.0 software. The expression for the linewidth used to simulate the anisotropic linewidth of the EPR signal was:

\[
W = W_i (1 + BM_i + C(M_i)^2)
\]

In this expression \(M_i\) refers to the 'central nucleus' which in the simulations shown is either the \(^{14}\)N or the \(^{15}\)N nucleus (cf. refs. [33,34]). Since the one nucleus has \(I = 1\) and the other \(I = 0.5\), the values of the linewidth parameters (\(W_i\, B\) and \(C\)) used in the various simulations are somewhat different (see legends to Fig. 4 and Table 1).

**RESULTS**

Figure 1 shows the X-band EPR spectra in the \(g = 2\) region of the three different bacterial aldehyde dehydrogenases as isolated. As we have shown previously [20,23], these enzymes contain the same complement of prosthetic groups as, e.g., xanthine oxidase, namely FAD, two different [2Fe-2S] clusters and a Moco, the cytosine-dinucleotide derivatized MPT. Control samples monitored optically and by EPR in which ferricyanide and/or Wurster's blue were added as oxidants indicate that FAD and the two [2Fe-2S] clusters in all three enzymes were completely oxidized; addition of these oxidants did not change the intensity of the \(g = 2\) signal (data not shown). Neither FAD nor [2Fe-2S] clusters show EPR signals when oxidized, hence the presence of an EPR signal in these three aldehyde dehydrogenases was unexpected. The major EPR signal which has a peculiar lineshape is centred at \(g = 2.004\), which suggests that it is due to an organic radical. Minor, derivative-like signals (\(g_{xx}\)-resonance) around 333 mT are observed which originate from Mo(V), the intensity of which is dependent on the type (and somewhat on the batch) of enzyme. The corresponding \(g_x\)-resonance of this Mo(V) species is at approximately 328 mT. The maximal amount of Mo(V) in the enzymes as isolated (middle trace) corresponds to less than 0.5% of the enzyme concentration.

The EPR-signal intensity as a function of temperature graphically represented in the Curie plot of Figure 2, shows that the \(g = 2.004\) signal behaves like a \(S = 1/2\) system between temperatures of 5 and 100 K. Above about 100 K the signal intensity apparently decreases some-
Fig. 1. X-band EPR spectra of the three different bacterial aldehyde dehydrogenases as isolated. EPR conditions: frequency: 9.236 GHz; modulation amplitude: 0.2 mT; microwave power: 80 µW; temperature 38 K. N.B.: Only the $g = 2$ region is shown. No other EPR signals were present in the enzymes as isolated except for a small signal at $g = 4.3$ due to adventitious iron. The signal around 333 mT in, e.g., the middle trace is due to some Mo(V) present. See text for further details.

what, an effect in part caused by the increase in linewidth and change in lineshape (vide infra). At temperatures above 168 K the signal has broadened beyond detection.

Figure 3 shows a comparison between the X-band and the Q-band spectrum of the $g = 2.004$ signal. The observation that the Q-band spectrum is very similar to the X-band spectrum further supports the proposal that the EPR signal originates from a $S = 1/2$ system for the following reasons. The finding that the overall 'width' of the spectrum, at least when plotted on a magnetic field scale (cf. Fig. 3), is independent of frequency, leads to the conclusion that the
The various lines observed are due to (e.g., proton and nitrogen) hyperfine interactions - the strength of which is frequency independent. Although a spectrum, in terms of overall shape - like the spectrum shown in Figure 3 might be due to $S = 1$ system with fairly small zero-field splitting parameters (D and E), the observation that at Q-band frequency the shape of the spectrum -in particular the spacing between the lines- is basically the same as at X-band frequency, argues against a $S = 1$ system. The major difference between the X-band and the Q-band spectrum is the

\[ \text{Signal intensity (au)} \]

\[ 100/T \quad (100/K) \]

**Fig. 2. Curie plot for the $g = 2.004$ EPR signal from aldehyde dehydrogenase from *C. testosteroni*.** The signal intensity was determined by double integration of the EPR spectrum of the $g = 2.004$ signal at various temperatures. To compare the values of the double integral, appropriate corrections were made for differences in experimental conditions such as differences in gain, modulation amplitude and microwave power. Determinations below 15K are not included because the signal was already saturated at the lowest microwave power (~ 1 $\mu$W at X-band) experimentally accessible precluding an accurate calculation of the signal intensity.

N.B.: Essentially similar relations between temperature and signal intensity were found for the two aldehyde dehydrogenases from *A. methanolica* (data not included).

The peak-to-peak width of the resolved lines. The finding that the various lines in the Q-band spectrum are somewhat broader compared to the X-band spectrum, is simply explained by the fact that the relative contribution of g-strain to the overall linewidth is greater at Q-band frequency [35].
Determination of the concentration of the $g = 2.004$ signal, assuming it is a $S = 1/2$ system, yields 12% of the enzyme concentration for the enzyme from *C. testosteroni* and 15% and 2% for FEDH and DL-AldH, respectively, from *A. methanolica*.

Flavin or some amino-acid side chains may yield EPR signals at or around $g = 2$. The EPR spectra shown in Figures 1 and 3 are widely different from the EPR spectra of FAD-radicals (either the protonated blue or the red anionic radical) in terms of the total width of the spectrum, the peak to peak width or the number of resolved lines [36-38]. The X-band spectrum is in terms of overall appearance and lineshape most similar to that of the tyrosine-radical in, e.g., ribonucleotide reductase or Photosystem II [39,40]. However, the X-band and Q-band EPR
spectra of tyrosine-radicals are very different owing to g-anisotropy which is partly resolved at Q-band. The species in the aldehyde dehydrogenases responsible for the g = 2.004 signal shows so little g-anisotropy that it is not resolvable at 35 GHz.

In order to investigate the properties and identity of the radical further, various isotope substitution experiments have been performed. Exchanging water for D₂O leads to a simplification of the EPR spectrum, yielding a spectrum with four well-resolved lines (Fig. 4A, lower trace), indicating the presence of exchangeable protons. Prolonged incubation up to 7 hours did not change the shape of the spectrum further.

The EPR spectrum of the AIDH purified from C. testosteroni cultured in the presence of ¹⁵N ammonium chloride is distinctly different from the ¹⁴N spectrum (Fig 4B, upper trace). This indicates that the electron spin interacts with one or more nitrogen nuclei. As a consequence, this observation effectively rules out all amino-acid-based radicals without a N-nucleus, in particular tyrosine, as possible candidates yielding the EPR signal. Exchanging water for D₂O in this ¹⁵N sample again led to simplification of the EPR signal, in this case consisting of three discernible lines (Fig. 4B, lower trace).

The final isotope substitution experiment we have performed involved molybdenum. Natural molybdenum consists of a mixture of ⁹⁵,⁹⁷Mo with I = 5/2 and ⁹⁶,⁹⁸Mo (I = 0). To study possible magnetic interactions between the electron spin of the g = 2.004 signal and the Mo(VI) nucleus (not Mo(V)!), C. testosteroni was cultivated on the pure isotopes ⁹⁵Mo and ⁹⁸Mo and the AIDH was purified subsequently. EPR spectra of the two types of preparations are shown in Figure 5. In case of a sufficiently strong magnetic interaction, the ⁹⁵Mo-containing enzyme should display a (somewhat) broader g = 2.004 EPR signal. The overlay of the EPR spectra of the ⁹⁵Mo- and ⁹⁸Mo-containing enzymes does not show a significant discernible difference in overall linewidth; the small differences that are, however, observed can be attributed to small differences in Mo(V) content between the two preparations.

Figure 6 shows that the lineshape and overall linewidth of the radical signal are dependent on temperature. Between 4.2 K and 50 K the lineshape is fairly constant but above 50 K the signal starts to broaden. The resolution decreases and the signal coalesces into a single featureless signal (Fig. 6). A plot of the linewidth of the radical versus the temperature shows a break at about 65K (Fig. 7A). Above 168 K the signal has broadened beyond detection. With increasing temperatures, both the linewidth and the relative rate of relaxation of the g = 2.004 signal -which
Fig. 4. X-band EPR spectra of the aldehyde dehydrogenase from *C. testosteroni* grown at $^{14}$N (A) or $^{15}$N (B) ammonium chloride and the effect of H$_2$O/D$_2$O exchange. The lower trace of each pair of spectra is a computer simulation (see Tables 1 and 2). EPR conditions for all experimental spectra as in Figure 1.
is proportional to the square root of the microwave power needed for half-saturation, \((P_{1/2})^{1/2}\) [41]- increase (Fig. 7B). The plot of the relative rate of relaxation versus temperature, significantly, shows a break around 65K, just like the plot of the linewidth of the \(g = 2.004\) signal versus the temperature. Below 65 K the slope is proportional to \(T^2\) consistent with the behaviour of an isolated radical, above 65 K the slope is proportional to \(T^6\), suggesting magnetic interaction with a rapidly relaxing paramagnet [39].

Figure 8 shows EPR spectra of dithionite reduced AIDH from *C. testosteroni*. EPR signals of two different iron-sulphur clusters are seen, labeled [2Fe-2S]-1 \((g_{x,y,z} = 1.904, 1.941, 2.023)\) and [2Fe-2S]-2 \((g_{x,y,z} = 1.89-1.90, 1.98, 2.092)\) [20,23]. As we have shown before, these
iron-sulphur clusters have different relaxation properties. The upper panel indicates the loss of signal intensity of [2Fe-2S]-2 in the temperature range between 14 and 52 K, most conspicuous in the decrease of the intensity of the $g_x$-line at $g = 2.09$ and the $g_y$-line at $g = 1.98$. Above 52 K the lines of [2Fe-2S]-2 are too broad to be detected. The linebroadening of [2Fe-2S]-2 is due to the increase in the rate of relaxation with temperature. Similarly, the lines of [2Fe-2S]-1 start to broaden due to the increased relaxation rate at temperatures above 55 K (Fig. 8, lower panel); at
90 K individual g-values are no longer resolved and above 100 K the resonances are too broad to be detected. The plot of the increase in linewidth of the g_z-line of [2Fe-2S]-1 versus temperature (Fig. 7A), shows a clear brake around 65 K.

DISCUSSION

Elucidation of the structure of the species yielding the \( g = 2.004 \) EPR signal

We have observed a new \( g = 2.004 \) EPR signal in three different bacterial aldehyde dehydrogenases. The EPR signal is concluded to be due to a \( S = 1/2 \) spin system since the signal intensity closely follows Curie's law (Fig. 2). The observation that at two different frequencies, X-band and Q-band, the EPR signal is very similar in terms of its g-value, overall lineshape and overall linewidth (measured in magnetic field units) is consistent with this proposal. Furthermore the fact that the number and spacing of the various resolved lines are the same in the X-band and Q-band spectra (Fig. 3) supports the view that the signal is due to a \( S = 1/2 \) system interacting with other nuclei. The nature of these interacting nuclei has been investigated by cultivation of \( C. \) testosteroni in a medium containing \(^{15}\)NH\(_4\)Cl as nitrogen source and also by H\(_2\)O/D\(_2\)O exchange experiments with the purified enzyme. The results obtained in Figure 4 unequivocally show that the electron spin interacts with one or more nitrogen and hydrogen nuclei. Combined the experimental observations listed above rule out FAD, iron-sulphur clusters, amino-acid residues as the origin of the radical. The only other cofactor associated with these three aldehyde dehydrogenases is the cytosine dinucleotide derivative of the MPT the structure of which is shown in Figure 9. We'll argue in the following that the \( g = 2.004 \) EPR signal is due to a molybdenum(VI)-trihydropterin radical.

Figure 4 shows simulations of the EPR spectra of the radical as present in AIDH prepared under 4 different conditions. Although the g-anisotropy of the signal is very small (cf. the X-band and Q-band spectra), the linewidth anisotropy is extremely large. This is clearly seen in the \(^{14}\)N/D\(_2\)O spectrum in Figure 4 by comparing the difference in linewidth between the two outer and the two inner lines. Such a large linewidth anisotropy is, however, present in all 4 spectra of Figure 4 and gives rise to large values of the linewidth parameters B and C used in the simulations (see Table 1, cf. ref. [42]). Owing to the large linewidth anisotropy, simulation of the signal was not a simple and straightforward matter, even though the signal has an isotropic
A

Linewidth (mT)

Temperature (K)

[2Fe-2S]-1

g = 2.004

B

P_{1/2}(mW)^{1/2}

Temperature (K)

\sim T^6

\sim T^2
Fig. 7. (A) Temperature dependence of the linewidth of the $g = 2.004$ signal (filled squares) and the $g_{\text{r}}$-line of [2Fe-2S]-1 (filled circles); (B) temperature dependence of the power saturation behaviour of the $g = 2.004$ signal. A) The linewidth is the width (mT) between the point of maximum amplitude of the $g_{\text{r}}$-line of [2Fe-2S]-1 and its half-maximal amplitude, measured at the low-field side of the spectrum (cf. Fig. 8). The linewidth of the $g = 2.004$ signal is the difference (mT) between the point of the maximum and the minimum amplitude of the signal. B) $(P_{\text{sat}})^{1/2}$, the square root of the microwave power (mW)$^{1/2}$ needed for half-saturation, is proportional to the rate of relaxation (cf. ref. [39]). The microwave power needed for half-saturation of the EPR signal was determined from power-plots (cf. ref. [41]) at different temperatures. N.B.: Determinations of the linewidth of the $g = 2.004$ signal between 15 and 4.2 K, yielding the same linewidth as at, e.g., 17 K are not included in A because the EPR signal was already saturated at the lowest microwave power experimentally accessible (~1 μW at X-band). The break in the curves in A and B occurs in all cases at 65 K. Lines with slopes proportional to $T^2$ and $T^4$ are also shown in B.

g-value. Preparation of the AIDH with 4 different isotopic substitutions proved to be vital to understand how, e.g., the number of lines and other details of the spectrum come about. The presence of three lines in the D$_2$O/14N spectrum and of four lines in the D$_2$O/15N spectrum are indicative of a strong coupling (1.5 mT) of the electron spin with a I = 1/2 nucleus in both spectra plus an additional strong coupling (1.2 mT or 0.9 mT) with a I = 1/2 or 1 nucleus, respectively, in the D$_2$O/13N spectrum or the D$_2$O/14N spectrum. As can be seen in Table 1, in all simulations the strong coupling of 1.5 mT (I = 1/2) had to be included. The difference between the spectra in H$_2$O and D$_2$O is caused by the presence of exchangeable protons interacting with the paramagnetic centre. Only when two (almost) identical I = 1/2 nuclei were included, could a satisfactory simulation of the spectra recorded in H$_2$O be obtained. This concerned, in particular, reproduction of the near absence of intensity (at least in the derivative spectrum) in the central part of the H$_2$O/14N spectrum. The values for the hyperfine coupling constants of the two nuclei are 0.6 mT (H$_2$O/14N spectrum) and 0.7 mT (H$_2$O/15N spectrum) and should be considered the same given the limits of accuracy of the simulation procedure (see Table 1).

The values for the various hyperfine coupling constants given in Table 1 are apparently different for the different isotope substitutions, but they form in fact a single set, i.e., the ratio of the values of the hyperfine coupling parameters for 14N- and 15N-containing samples obtained by simulation is, given the limits of accuracy of the simulation and the 'goodness of fit' close to the theoretical ratio of 1.40 and a similar reasoning applies to the 2D/2H ratio (0.15). We therefore conclude that the electron spin of the paramagnetic centre (in the H$_2$O/14N sample) interacts with a single strong nitrogen nucleus (I = 1, 0.9 mT), with a single strong non-exchangeable proton
Fig. 8. EPR signals of the two different [2Fe-2S] clusters in reduced aldehyde dehydrogenase from C. testosteroni measured at different temperatures. Upper panel shows the temperature range in which [2Fe-2S]-2 starts to broaden (14, 30, 38, 44 and 52 K), lower panel the temperature range for which broadening of [2Fe-2S]-1 occurs (52, 58, 60, 67, 75 and 82 K).

(I = 1/2, 1.5 mT) and with two magnetically indistinguishable H$_2$O/D$_2$O exchangeable protons (both I = 1/2, 0.6–0.7 mT).

Although observations on MPT radicals in free solution or associated with enzymes have not been reported to our knowledge, literature is available on the structure and properties of tetrahydrobiopterins or more generally on pterin-model systems and pterin-derivatives including EPR spectroscopic data on monohydro- and trihydro(bio)pterin radicals [43-49]. The EPR spectra of trihydro(bio)pterin radicals in solution are dominated by the strong coupling with the N5 nucleus, by the strong coupling with the exchangeable proton associated with this nucleus H(N5) and by the coupling with the non-exchangeable H(C6) (see Fig. 9). In general, the coupling with N8, H(N8) or H(C7) is a 3-4 fold lower than with N5, H(N5) or H(C6), respectively (see legend to Table 2). Usually, the values for the hyperfine coupling of N5 and
strength (the same holds for N8 and H(N8) in case they are resolved [48]). Spin density associated with the pyrimidine ring N- and H-atoms is too small to be resolved, just as in lumazin- or flavin-free radicals (cf. refs. [46,47]). Thus in trihydro(bio)pterin free radicals most of the unpaired spin density is located at and around the N5 and C6 nuclei. In contrast, in monohydro(bio)pterin radicals, the unpaired electron (spin) density is much more delocalized in the pyrazin-ring, as, e.g., reflected in the less than twofold difference in hyperfine coupling constants for N5, H(N5) compared to N8, H(N8), respectively [44].

Table 1. Hyperfine coupling constants used to simulate the four spectra of the Mo(VI)-trihydropterin radical shown in Figure 4.

<table>
<thead>
<tr>
<th>Nuclei</th>
<th>$^{14}$N</th>
<th>$^2$D</th>
<th>$^2$D</th>
<th>$^1$H</th>
<th>$^1$H</th>
<th>$^1$H</th>
<th>$^{15}$N</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$^{14}$N, H$_2$O</td>
<td>0.9$^a$</td>
<td>-</td>
<td>-</td>
<td>1.5</td>
<td>0.6</td>
<td>0.6</td>
<td>-</td>
</tr>
<tr>
<td>$^{14}$N, D$_2$O</td>
<td>0.9</td>
<td>0.1</td>
<td>0.1</td>
<td>1.5</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>$^{15}$N, H$_2$O</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1.5</td>
<td>0.7</td>
<td>0.7</td>
<td>1.2</td>
</tr>
<tr>
<td>$^{15}$N, D$_2$O</td>
<td>-</td>
<td>0.1</td>
<td>0.1</td>
<td>1.5</td>
<td>-</td>
<td>-</td>
<td>1.2</td>
</tr>
</tbody>
</table>

$^a$ values for the various hyperfine coupling constants expressed in mT are accurate to about ±0.08 mT. Larger deviations from the values listed, lead to unacceptable discrepancies (e.g., in the number of (partially) resolved lines) between experimental and simulated spectra. Range of the linewidth parameters (dependent on the simulation, see Materials & Methods): 0.6 mT < W, < 0.9 mT; -0.1 < B < -0.3; 0.5 < C < 1.0. An isotropic value of g = 2.004 was used in all simulations.

In general, EPR spectra of radicals associated with proteins, show somewhat greater hyperfine coupling constants for C$_v$- or N$_v$-protons (1.5 x) compared to the radicals in solution, because of the contribution of the anisotropic part of the hyperfine tensors [37]. This latter interaction is averaged out in the EPR spectra of free radicals recorded in solution due to their rapid tumbling. Given the EPR properties of free pterin radicals listed above, it is most reasonable to assign the I = 1, 0.9 mT hyperfine constant to coupling with N5 of the pterin ring and the I = 1/2, 1.5 mT to coupling with the non-exchangeable H(C6). To conform to the EPR characteristics of pterin radicals a single exchangeable proton with a coupling of 0.8-1.2 mT is expected. Instead, two identical, exchangeable protons each with a relatively small coupling (0.6-
Fig. 9. Proposal for the structure of the molybdenum(VI)-tetrahydropterin radical. Upper, structure of the MPT (Mo(VI)) cytosine dinucleotide (CDP) as present in aldehyde oxidoreductase from D. gigas [9]. The two [2Fe-2S]-clusters are also drawn. In the three aldehyde dehydrogenases from C. testosteroni and A. methanolicum the same MPT derivative is present [23]. Middle, structure of the molybdenum(VI)-trihydropterin radical. Dotted lines from the N5-atom indicate the two hydrogen bonds to the protein. The identity of potential H-donating and H-accepting...
amino-acid residues participating in hydrogen bonding at N5 is unknown. The Mo(VI)-trihydropterin radical is one electron more oxidized than the Mo(VI)-tetrahydropterin shown in the upper part of the figure. Lower, structure of trihydro- and monohydro-bioppterin radicals. N.B.: The redox state of the MPT cofactor—which is a nonplanar tricyclic pyranopterin—is the same as that of a tetrahydropterin.

0.7 mT) are required for a satisfactory simulation. To see whether these protons could be H(N5) and H(N8), the EPR spectra were simulated including these two protons, two I = 1 nuclei (representing N5 and N8) and the non-exchangeable H(C6). The trial simulations indicated that an additional I = 1 nucleus (N8) in the simulation with a coupling greater than about 0.1 mT did not produce satisfactory results (not shown). Consequently, coupling with H(N8) cannot be greater than 0.1 mT, a value significantly different from 0.6-0.7 mT. From this we conclude that the two exchangeable protons cannot be assigned to H(N5) and H(N8). We therefore propose that the two exchangeable protons are both bound to N5 (Fig. 9). Binding to the same atom, which implies a similar chemical environment, not only explains their similar hyperfine constants but also their relatively small value. The total coupling amounts to approximately 2 x 0.6-0.7 mT = 1.2-1.4 mT, a value expected for a single H(N5) in proteins on basis of the value observed in free pterins. In conclusion, we propose that the radical is a molybdenum(VI)-trihydropterin radical in which the N5-atom is engaged in two hydrogen bonding interactions with the protein. The majority of the electron (spin) density of the radical is located at and around the N5-atom and at the proton bound to C6-atom of the pterin ring.

The magnetic and structural properties found for H(N5) in the molybdenum(VI)-trihydropterin radical may be compared to those of the flavodoxin semiquinone. This latter flavosemiquinone is a blue semiquinone, i.e., protonated at the N5 position [50,51]. The crystal structure shows a single hydrogen bond (2.9 Å) between N5 and the main-chain carbonyl oxygen atom of Gly57 [52,53]. A comparison of the EPR spectra in H2O and D2O of the flavodoxin suggests a hyperfine coupling for the single proton at N5 of about 1.1-1.5 mT as compared to 0.8 mT for free flavin radicals [37]. The crystal structures of the four Mo- or W-pterin containing enzymes show that the pterin-ring system participates in numerous hydrogen bonding interactions with amino-acid side chains. Regarding protonation/hydrogen bonding at the N5 position, the crystal structures indicate that N5 may participate in two hydrogen-bonding interactions (P-pterin in DMSO reductase [10] and both pterins in formate dehydrogenase [11]), in one (Q-pterin in DMSO reductase [10]) or, apparently, in none [9]. Residues observed to be involved in hydrogen
bonding are Glu, Arg, His, Ser or water. Although EPR spectroscopy cannot determine the identity of the respective H-donating and H-accepting amino-acid residues participating in hydrogen bonding at N5, it predicts that in the aldehyde dehydrogenases from *C. testosteroni* or *A. methanolica* the N5-atom is engaged in two hydrogen bonds. Further confirmation of this and the residues involved has to await elucidation of their 3D-structure.

To obtain additional information on the nature of the radical Mo-substitution experiments have been performed. Mo(V) is known to affect the EPR spectrum of the [2Fe-2S]-1 via dipolar coupling, yielding splittings of about 1.0 mT in the resonances of this centre [22,54-56]. In our preparations, the molybdenum is, however, in the Mo(VI)-state and thus far less paramagnetic than Mo(V). Since the Bohr magneton of $^{95}\text{Mo}$(VI) ($I = 5/2$) is about three times smaller than that of a proton, the hyperfine coupling constant of Mo is about three times smaller. EPR spectral simulations indicate that including a hyperfine coupling constant of 0.1 mT ($I = 5/2$) or greater in addition to the constants listed in Table 2, would yield a measurable increase in the overall width of the spectrum; couplings of 0.03 mT ($I = 5/2$) would measurably change the lineshape of the central part of, e.g., the $^{14}\text{N}/\text{H}_2\text{O}$ spectrum of the radical. Within experimental resolution the EPR spectra of $^{95}\text{Mo}$ and $^{98}\text{Mo}$ are the same. This implies a very low electron (spin) density in the pyran ring, not unexpected since it is not conjugated to the pterin ring system. Apparently, the MPT cofactor behaves as a dinuclear redox centre in which electron (spin) density can be located at the molybdenum-atom and/or close to the N5 of the pterin.

<table>
<thead>
<tr>
<th>Nucleus</th>
<th>non-exchangeable</th>
<th>exchangeable</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>0.9</td>
<td>0.6</td>
</tr>
<tr>
<td>H</td>
<td>1.5</td>
<td>0.6</td>
</tr>
<tr>
<td>H</td>
<td>0.6</td>
<td>H(N5)</td>
</tr>
<tr>
<td>H</td>
<td>0.6</td>
<td>H(N5)</td>
</tr>
</tbody>
</table>

Values for trihydrobiopterin free radical [49]: N5: 0.7 mT; N8: 0.2 mT; H(N5): 0.8 mT; H(N8): 0.2 mT; H(C6): 0.9 mT; H(C7): 0.2 mT.

**Magnetic coupling between the Mo(VI)-trihydropterin radical and oxidized [2Fe-2S]-1**

The spectra in Figure 6 show that the EPR signal of the MPT radical starts to broaden at
temperatures above 55 K. Above 168 K the signal is undetectable. These phenomena are not expected for a magnetically isolated S = 1/2 radical which in general relaxes so slowly that it is detectable by EPR spectroscopy even at room temperature. Figure 7A shows that the temperature dependence of the linebroadening of the radical and its rate of relaxation (Fig. 7B) both show a break around 65 K. Figure 7A shows that the plot of the linebroadening of the EPR spectrum of reduced [2Fe-2S]-1 versus temperature also shows a break around 65 K. Linebroadening in this latter case is due to increase in the rate of spin-lattice relaxation (1/T1), i.e., the rate at which the absorbed microwave radiation is being transferred to the 'bath'. The linebroadening of the MPT radical and its apparent T⁰ dependency (Fig. 7B) above 65 K may be explained by the phenomenon of cross-relaxation, i.e., the radical relaxes via a nearby rapidly relaxing paramagnetic centre, i.e. [2Fe-2S]-1. It is important to realize that the linebroadening and increased rate of relaxation observed in the MPT radical occurs under conditions in which the [2Fe-2S]-1 is oxidized but that the increase in linewidth of [2Fe-2S]-1 and its relaxation behaviour have been determined for the reduced cluster (Fig. 8). So in order to understand the physical basis for the phenomena observed in the EPR spectra of the MPT radical, it must be shown that the oxidized [2Fe-2S]-1 is paramagnetic with a spectral envelope encompassing the g = 2 region and that it is rapidly relaxing with a similar temperature behaviour as the reduced iron-sulphur cluster. As to the latter, since EPR spectra of an oxidized [2Fe-2S] cluster have never been observed, we will assume that the pathways and/or the temperature dependence of the kinetics to transfer absorbed microwave radiation to the 'bath' are similar for the oxidized and reduced iron-sulphur cluster. The ground state of oxidized [2Fe-2S]-1 is S = 0, due to antiferromagnetic coupling between the two Fe(III) atoms [57]. Applying the simple Heisenberg exchange spin Hamiltonian for this system

\[ H = J S_1 S_2 \]

yields S = 1 as the first excited state separated at J from the ground state. Typical values for J in [2Fe-2S] proteins range between 300-400 cm⁻¹ although larger values have also been measured [58]. This means that in the temperature range of interest to us (65-168 K) the S = 1 level is populated for only 0.13-7.7 % (taking J = 300 cm⁻¹) whereas the higher spin multiplets (S = 2-5) are essentially non-populated. That the shape of the EPR signal of the whole population of the radical is affected (which itself represents maximally only 15 % of the enzyme or [2Fe-2S]-1 concentration), whereas only a small fraction of [2Fe-2S]-1 is in the S = 1 state, is possible when
the intrinsic relaxation rate of [2Fe-2S]-1 in the S = 1 state is much higher than the intrinsic relaxation rate of the MPT radical, a reasonable assumption. In this case, each radical will experience an iron-sulphur cluster with its magnetic moment resulting from an averaging over the different spin multiplets, a situation similar to that observed for the magnetic interaction between the tyrosyl radical and the diiron-oxo centre in ribonucleotide reductase [39] or for the interaction of dinuclear (antiferromagnetically coupled) Cu(II) centres with protons as revealed by 1H-NMR [59,60]. The average magnetic moment of [2Fe-2S]-1 and its rate of relaxation increase with increasing temperatures. Thus at relatively low temperatures (<65K) the population of the S = 1 level or the intrinsic rate of relaxation of [2Fe-2S]-1 or both factors together, are too small to induce observable linebroadening in the EPR spectrum of the MPT radical.

As noted above, the EPR spectrum of an [2Fe-2S] cluster in the S = 1 state has not been observed, but it is likely to encompass the g = 2 region. Both Fe(III) atoms are 12S_{1/2}-ions, with g-values very close to g = 2.0023 [41,57]. Fine structure terms due to the anisotropic part of the exchange interaction -although expected to be small- will undoubtedly contribute to the spectrum, but will not be able to shift the main intensity significantly away from the g = 2 region. The EPR spectrum of the S = 1 excited state of [2Fe-2S]-1 thus overlaps with the EPR spectrum of the MPT radical providing an efficient cross-relaxation pathway.

The observation of dipolar magnetic interaction between reduced [2Fe-2S]-1 and Mo(V) as described before in various molybdoenzymes including the AIDH from C. testosteroni [23] is consistent with the relatively short distance between these redox centres found in the crystal structure of the aldehyde oxidoreductase from D. gigas. The observation of magnetic interaction between oxidized [2Fe-2S]-1 and the new radical suggests that these centres are quite close and provides additional independent support for the identification of the radical as the MPT radical.

**Putative function of the molybdenum(VI)-trihydropterin radical**

To establish a role in catalysis of the molybdenum(VI)-trihydropterin radical transient kinetic experiments with any of the three aldehyde dehydrogenases should be performed. Sofar we have only performed equilibrium trial experiments (data not shown in this paper). Addition of aldehydes did not affect the amount of radical present. However, the redox potential of aldehydes may be too high to affect the amount of radical since they induced only partial
reduction of [2Fe-2S]-1 and FAD and no reduction of [2Fe-2S]-2 [22,23]. Addition of dithionite abolished the radical EPR signal. The activity of dithionite-reduced enzyme, which was subsequently reoxidized (no MPT radical), was the same as that of the untreated -as isolated-enzyme. This finding indicates either that enzyme molecules with a MPT radical are inactive and cannot be reactivated by this treatment or that the MPT radical is a true intermediate in catalysis. It is not clear in which structural and/or catalytic respect(s) the three bacterial aldehyde dehydrogenases described in this work are different from all other molybdo-(tungsto-)-pterin containing enzymes in which this MPT radical has not been observed so far. Note however, that the W-pterin containing aldehyde oxidoreductase from *P. furiosus* also seems to contain an odd number of electrons in the completely oxidized state [61]. The odd electron is located on the tungsten atom yielding a W(V)-tetrahydropterin. Clearly, this W(V)-tetrahydropterin contains two more reducing equivalents than the Mo(VI)-trihydropterin. The detection of these two different redox states containing an odd number of electrons may be indicative for a direct role in electron transfer of the pterin moiety, i.e., the pterin-ring system might act as a one-electron carrier -shuttling between its trihydro- and tetrahydro-redox states- mediating electron transfer between the molybdenum-ion and the nearest iron-sulphur cluster. The distance in the *D. gigas* aldehyde oxidoreductase between the Mo-atom and the nearest Fe-atom of the [2Fe-2S]-cluster is about 15Å, the distance between the Mo-atom and N5 about 5.5Å and from N5 to the Fe-atom about 10Å. Calculation of the rate of electron transfer as a function of the distance as described in [62] indicates that the two-step electron transfer pathway with the MPT radical as an intermediate is much faster than the single-step electron transfer. However, since also the lowest calculated rate (1000-5000/s) is still much higher than the rate of turnover (100/s), a two-step electron transfer pathway is not likely to be selected in evolution for its speed, although it may have been so for mechanistic or chemical reasons yet to be uncovered.

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REFERENCES


Chapter 5

Cloning of the gene encoding phenylacetaldehyde dehydrogenase from *Escherichia coli* and enzymological characterization of the overexpressed enzyme

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Abbreviations. AIDH, aldehyde dehydrogenase; E, enantiomeric ratio; EDTA, ethylenediaminetetraacetic acid; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IPTG, isopropyl β-D-thiogalactopyranoside; NAD(P), nicotinamide-adenine dinucleotide (phosphate) and its oxidized and reduced forms; NAD-AIDH(s), NAD(P)-dependent aldehyde dehydrogenase(s); PAD, phenylacetaldehyde dehydrogenase from *Escherichia coli*; PMSF, phenylmethanesulfonylfluoride; ECAO, aromatic amine oxidase from *Escherichia coli*; Tris, 2-amino-2-hydroxymethylpropane-1,3-diol.

Enzymes. Aldehyde dehydrogenase (EC 1.2.1.39); DNA ligase (EC 6.5.1.1); DNA polymerase I (Klenow fragment) (EC 2.7.7.7); DNA phosphatase (EC 3.1.3.1); DNA restriction endonucleases, *Bam*H1, *Cla*I, *Eco*RI, *Pst*I, *Sau*3AI (EC 3.1.24.4).
A gene encoding NAD-dependent phenylacetaldehyde dehydrogenase (PAD) was cloned from *Escherichia coli* W3350. The gene is situated adjacent to *maoA* encoding a quinoprotein aromatic amine oxidase (ECAO), but it is transcribed in the opposite direction. Partial DNA sequencing revealed that it is similar, if not identical, to the gene indicated as *feaB*, forming part of the *fea* cluster which is involved in the oxidation of 2-phenylethylamine by *E. coli* [Hanlon, S.P., Hill, T.K., Flavell, M.A., Stringfellow, J.M. & Cooper R.A. (1997) *Microbiology* 143, 513-518].

PAD was purified to homogeneity from *E. coli* TG2 containing the plasmid pBCP511. The enzyme has a molecular mass of 155-190 kDa and is proposed to be a homotetramer. Enzymatic activity as determined in the assay showed a pH optimum of 9.0 and a temperature optimum between 65 and 70 °C. In contrast to several other bacterial NAD(P)-dependent aldehyde dehydrogenases (NAD-AlDHs), PAD was not activated by potassium or phosphate ions. Steady-state activity measurements showed that the reaction of NAD and aldehyde with PAD proceeds in the normal way, i.e., according to a sequentially ordered process. PAD prefers long-chain aliphatic aldehydes or short-chain aliphatic aldehydes containing a phenyl group as a substrate. A very poor enantioselectivity was shown by the enzyme towards the racemic aldehydes tested. Apparent $K_m$ values for NAD and phenylacetaldehyde were found to be 0.18 mM and 10 μM, respectively, and activity with NAD was 20-fold higher than with NADP. These data, together with the localization of the *maoA* and *feaB* genes and the known substrate specificity of ECAO, indicate that PAD is a NAD-dependent aromatic aldehyde dehydrogenase involved in the metabolism of aromatic amines via ECAO. The occurrence of a similar, if not identical, enzyme in *E. coli* strain ATCC 11105 has been recently described [Ferrández, A., Prieto, M.A., García, J.L. & Díaz, E. (1997) *FEBS Letters* 406, 23-27].

Like other NAD-AlDHs, PAD showed aldehyde dehydrogenase as well as esterase activity. A 17-fold increase of esterase activity was observed in the presence of NADH. Phenylacetaldehyde and *p*-nitrophenylacetate, the substrate for the esterase activity assay, acted as competitive inhibitors for each other in the respective assays. This suggests that both activities derive from the same active site in which a cysteine residue forms a thiohemiketal adduct with the aldehyde or ester and the thiol ester subsequently formed is hydrolyzed in the final step of the catalytic cycle. In line with the proposed mechanism,
the inhibition of both activities by bromoacetophenone, phenylmethanesulfonylfluoride and Hg$^{2+}$ was elevated upon addition of a thiol reducing agent and a conserved cysteine is present in the enzyme. All these properties indicate that PAD is a normal NAD-AIDH.

**Keywords.** NAD(P)-dependent aldehyde dehydrogenase, phenylacetaldehyde dehydrogenase, *Escherichia coli.*
INTRODUCTION

NAD(P)-dependent aldehyde dehydrogenases (NAD-AIDHs) catalyze the oxidation of a variety of aliphatic and aromatic aldehyde substrates to their corresponding carboxylic acids, employing NAD(P) as coenzyme. These enzymes occur in prokaryotic as well as eukaryotic organisms and are presumed to function as an important tool for cellular defense against toxic aldehydes [1-3]. NAD-AIDHs have homotetrameric, sometimes homodimeric, quaternary structures with subunit molecular masses ranging from 50 to 65 kDa [4-8]. One of the notable properties of these enzymes is their instability during purification [9]. This usually has been overcome by adding a reducing agent [9] or and salt (0.1 M) [10]. In catalysis both a cysteine- and a glutamate residue are essential, as was concluded from a variety of inactivating chemical modifications and site directed mutagenesis studies [11-15]. A serine is believed to be involved in the enzyme-coenzyme interaction [16]. Several NAD-AIDHs are capable of catalyzing the hydrolysis of $p$-nitrophenylacetate, an extensively employed model substrate for esterases [17-20]. Detailed kinetic studies of the esterolytic activity of these enzymes contributed to the elucidation of the nature of a key acyl intermediate formed during the dehydrogenase reaction [18,19]. Mutational analyses of the NAD-AIDH, including the active site residues, glutamate and cysteine, all showed that there was a parallel loss of dehydrogenase and esterase activity indicating that the two reactions require the same active site components and occur at the same site [21].

During characterization of the maoA gene of *Escherichia coli* [22], encoding production of quinoprotein aromatic amine oxidase (ECAO), sequencing of an adjacent DNA revealed that this could encode a NAD-AIDH. To reveal the identity of the enzyme and to establish whether it has a role in oxidation of aldehydes generated by ECAO, a construct was made for overexpression of the putative aldehyde dehydrogenase and the enzyme was isolated and characterized. During these investigations, a report on the characterization of the gene, indicated as *feaB* by the authors, appeared [23] as well as on the characterization of the enzyme [24].

MATERIALS & METHODS

Growth media, bacterial strains and plasmids. The medium used was Luria-Bertani
medium (1 % tryptone, 0.5 % yeast extract, 0.5 % NaCl), prepared with demineralized water. Medium was solidified with 1.5 % Bactoagar.

E. coli TG2 supE hsdS5 thy Δ(lac-proAB) Δ(srb-reca)306:: Tn10 F' [traΔ36 proAB lacF' lacZΔM15] [25] was used as a host for cloning and expression. E. coli PPA207 has been described previously by Steinebach et al., 1995 [22].

Vectors used were pUC18 and M13mp19 [26] and pBlueScript II [Stratagene Inc.].

Other materials. Restriction endonucleases, T4 DNA ligase and E. coli DNA polymerase I (Klenow fragment) were from Pharmacia. The sequenase version II sequence kit was obtained from USB. Calf intestinal phosphatase and blocking reagent were purchased from Boehringer. [α-32P]dATP (3000 Ci mmol⁻¹; 11.1 Tbp mmol⁻¹) were from New England Nuclear. Genescreen plus was obtained from Du Pont. Geneclean was purchased from Bio101.

General methods. Methods for DNA manipulation were essentially as described by Maniatis et al. (1989) [27].

Cloning of the gene that encodes phenylacetaldehyde dehydrogenase (feaB). Chromosomal DNA from E. coli PPA207 was partially digested with Sau3AI and separated on a 0.8 % low melting agarose gel in 0.04 M Tris/acetate, pH 8.1 mM EDTA. Fragments ranging from 9 to 15 kpb were isolated from the gel by excising the proper agarose slice with subsequent melting at 60 °C and phenol extraction. The isolated fragments were ligated into BamH1 digested pJF119EH [28]. The resulting plasmids were used to transform E. coli TG2. Analysis of clones was done as described [22] using the radioactive labeled maoA-containing 7 kbp EcoR1/PstI insert of plasmid pBCP467 as a probe. One positive clone, pBCP509, was used for the isolation of DNA followed by detailed restriction enzyme analysis of the insert. A 5 kb ClaI fragment was cloned into ClaI digested pBlueScript SK resulting in plasmid pBCP511.

Expression of the feaB gene. In E. coli TG2 cells that harboured plasmid pBCP511, the feaB gene was expressed during growth in Luria-Bertani medium containing 100 μg/ml ampicillin. Routinely, E. coli TG2 cells were freshly transformed with plasmid pBCP511 to produce the PAD. 10-ml cultures of Luria-Bertani medium containing 100 μg/ml ampicillin were inoculated with a transformant and allowed to grow at 37 °C for 7 h. Cells were harvested by centrifugation, washed once to remove β-lactamase and used to inoculate 500 ml Luria-Bertani medium containing 100 μg/ml ampicillin. Cultures were grown overnight at 37 °C with vigorous shaking. Cells were harvested at the end of the exponential growth phase by centrifugation at
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24000 x g for 15 min, washed with 20 mM potassium phosphate buffer, pH 7.0, and disrupted three times using a French pressure cell at 4000 psi (max). The viscosity of the suspension was lowered by adding DNase (Fluka) in presence of 1 mM MgCl₂. The suspension was ultracentrifuged at 250000 x g for 90 min, supernatant yielding the cell-free extract.

**Enzyme assays.** The standard assay system used to measure PAD activity included an assay mixture with 50 mM sodium pyrophosphate buffer, pH 9.0, 2.5 mM NAD and 10 μl of enzyme solution in a total volume of 1.0 ml. Reactions were initiated by the addition of 10 μl phenylacetaldehyde (0.1 mM) after 1 min equilibration of the protein in the assay mixture. The assay was carried out at room temperature. NADH formation was monitored at 340 nm in a 1 cm optical path spectrophotometer cell. A molar absorption coefficient of 6220 M⁻¹·cm⁻¹ was used for NADH. Under these conditions, 1 unit corresponds to 1 μmol of NAD reduced per minute.

The substrate specificity of PAD was examined with the standard enzyme assay except that phenylacetaldehyde was replaced by the substrate to be tested. The concentrations of the substrates were varied from 0.01 to 100 mM.

In order to determine the electron acceptors used by PAD, NAD in the standard assay mixture was replaced by a variety of natural and artificial electron acceptors. The extinction coefficients used (mM⁻¹·cm⁻¹) were as follows: cytochrome *c*, ε₅₅₀nm = 21 [29]; 2,6-dichlorophenol-indophenol, ε₆₀₀nm = 21 [30]; ferricyanide, ε₄₂₀nm = 1.0 [31]; NADP, ε₃₄₀nm = 6.22 [32]; *p*-nitroso-*N*,*N*-dimethylaniline, ε₄₄₀nm = 35.4 [33]; phenazine methosulfate, ε₃₈₅nm = 22 [34]; nitroblue tetrazolium chloride, ε₅₆₆nm = 15.5 [32] and Wurster's blue, ε₆₀₀nm = 9 [35].

The pH optimum was determined using the standard assay replacing the sodium pyrophosphate buffer by 50 mM potassium phosphate buffer in the pH range 5-9 or 50 mM Tris-HCl buffer in the pH range 7-10.

The temperature optimum was determined using the standard assay at temperatures varying from 20 to 90 °C.

**Enantioslectivity measurements.** The enantioslectivity of PAD towards racemic aldehydes, like 2-ethylhexanal, 3,5,5-trimethylhexanal and 3-phenylbutanal, has been determined. To express the enantioslectivity, the enantiomeric ratios (E) for these chiral substrates have been calculated [36]. Therefore, during the conversion of the substrate the ratio between the R,S-enantiomers of the remaining substrate has been analyzed by gas chromatography. These enantiomers could be separated on a Chiraldex G-TA capillary column.
(20 m x 0.25 mm I.D. film thickness 0.125 μm; Astec, Whippany, NJ, USA). Chromatography was performed with a Hewlett-Packard Model 5890 Series II gas chromatograph with a split injector, a flame ionization detector and an integrator (Hewlett-Packard 335 Chemstation). The columns were operated at 85 °C. Nitrogen was used as the carrier gas and the inlet pressure was 56 kpa for the column. A split flow of 100:1 was used. The injector and detector temperatures were kept constant at 200 and 250 °C, respectively.

The starting assay mixture consisted of 14.9 ml 50 mM sodium pyrophosphate buffer, pH 9.0, 800 μl NAD (2.5 mM) and 160 μl substrate (1 mM). A 4 ml fraction of the assay mixture was collected (0 % conversion). To the remaining assay mixture 96 μl enzyme solution was added. The conversion was followed spectrophotometrically at 365 nm, starting with $A_{365} = 0.10$. Fractions of 4 ml were collected at $A_{365}$ values of 0.65 (± 50 % conversion), 0.98 (± 75 % conversion) and 1.4 (± 100 % conversion). The four collected fractions were analyzed separately by gas chromatography. Samples were obtained by extracting the R,S-aldehydes from the 4 ml assay mixtures with 5 ml of a 2-octanone/dry diethyl ether (10:3:25 (v/v/v)) mixture. Subsequently, the diethyl ether extract was dried with MgSO₄ and the solvent was evaporated with a stream of nitrogen gas until a residue of approximately 50 μl remained. The injection volume of the sample was 0.2 μl. The conversion of the aldehydes was determined by comparing the integrated peaks of the R,S-aldehydes with the integrated peak of 2-octanon.

**Esterase activity.** Esterase activity was measured in 50 mM sodium pyrophosphate buffer, pH 8.0, by following the hydrolysis of p-nitrophenyl acetate (35 μM) to p-nitrophenol at 400 nm with an extinction coefficient of 16.0 mM⁻¹ cm⁻¹ [37]. The p-nitrophenyl acetate was dissolved in acetone and diluted with water to a concentration of 3.5 mM. Adjustments were made to account for the possible spontaneous nonenzymic hydrolysis of the ester in the assay system. NAD(P)(H)-stimulated esterase activity was measured in the same assay system with 5-50 μM NAD(P)(H).

**Protein determination.** The enzyme concentration was determined using either the Bradford [38] or Pierce [39] method with bovine serum albumin as standard.

**Enzyme purification.** All purification steps for PAD were performed at 4 °C. The cell-free extract was fractionated with ammonium sulfate. PAD activity was in the 30-50 %-saturated fraction. After centrifugation the pellet was resuspended in a minimal volume of 20 mM potassium phosphate buffer, pH 7.0, and the sample was diluted (50 x) with the same buffer and
concentrated again (10 x) with a centriprep-100 concentrator (Amicon). The sample was applied onto a Macroprep-50 Q Sepharose column (1.5 x 18 cm, Bio-Rad) equilibrated with 20 mM potassium phosphate buffer containing 10 mM KCl, pH 7.0. After charging and washing the column with this buffer, proteins were eluted with 6 bedvolumes of a linear gradient of KCl (0.01-1 M) in 20 mM potassium phosphate buffer, pH 7.0, at a flow rate of 2 ml/min. Active fractions were pooled and applied to a hydroxylapatite column (2.5 x 10 cm, Bio-Rad) equilibrated with 10 mM potassium phosphate buffer, pH 7.0. After charging and washing the column with the same buffer, the proteins were eluted with 4 bedvolumes of a linear gradient of potassium phosphate (0.01-1 M), pH 7.0, at a flow rate of 1 ml/min. Fractions containing the enzyme activity were pooled, concentrated with a centriprep-100 concentrator (Amicon) and stored at -80 °C.

**Molecular mass determination.** The native molecular mass of the enzyme was determined by gel filtration on a Superdex-200 column (1 x 30 cm, Pharmacia) equilibrated with 10 mM potassium phosphate buffer containing 0.1 M KCl, pH 7.0. Elution occurred with the same buffer at a flow rate of 0.5 ml/min. Calibration standards used were as follows: blue dextran (2000 kDa), thyroglobulin (669 kDa), ferritin (440 kDa), catalase (232 kDa), aldolase (158 kDa), serum albumin (bovine, 67 kDa), vitamin B_{12} (1.36 kDa). The native molecular mass was also determined by PAGE on a commercial gradient gel (8-25 %, Pharmacia) using the Phast System equipment (Pharmacia). The gel was calibrated with a native high molecular mass marker (Pharmacia). The subunit composition and masses of pure PAD were determined by SDS-PAGE, using low molecular weight protein standards from Pharmacia. Denaturation of the protein occurred by incubating the enzyme solution in 5 % (w/v) SDS and 10 % (v/v) β-mercaptoethanol and heating the mixture for 20 min at 100 °C.

**Absorption spectra.** UV/Vis spectra were measured in cells of 1 cm path length at room temperature with a Hewlett-Packard HP 8452 A Diode Array spectrophotometer.

**N-terminal amino acid sequence.** Pure PAD was precipitated with 80 % ethanol at -80 °C. After centrifugation the pellet was dried for 20 h. Removal of all salts and free amino acids was done by the method of Sheer [40]. The N-terminal amino acid sequence was determined with automatic Edman degradation (Applied Biosystems, Sequencer Model 477A and HPLC Model 120A). The sequence was compared with those published in the literature by eye or with those in the protein sequence databanks, using the program BLAST [41].
RESULTS

A. pBCP509

B. pBCP511

Fig. 1. Schematic representation of plasmids pBCP509 and pBCP511. (A) a 5 kb EcoRI/PstI fragment in EcoRI/PstI-digested pJF119EH. (B) a 5 kb Clal fragment in Clal-digested pBlueScript SK. Thick lines indicate coding regions, P and O indicate the promoter and operator of the lacZ α-peptide coding region, respectively. The following abbreviations are used: C, Clal; E, EcoRI; P, PstI.

Cloning and expression of feaB. Sequence analysis of plasmid pBCP467 containing the maoA gene [22,23] and subsequent database searches revealed that downstream of the maoA gene a NAD-AlDH encoding gene is located that is transcribed in the opposite direction of the maoA gene. Since ECAO produces phenylacetaldehyde from its natural substrate 2-phenylethylamine it appeared logical to assume that the NAD-AlDH encoding gene that appeared to be clustered with the ECAO encoding maoA gene is in fact coding for PAD. This is evidenced
by the fact that recently Hanlon et al. [23] deposited a feaB sequence to which the determined partial DNA sequence and the N-terminal amino acid sequence of the here reported feaB are completely identical.

In order to clone the feaB gene the complete insert of plasmid pBCP467 was used as a probe to screen an E. coli PPA207 chromosomal library containing 9-15 kb partial Sau3AI inserts. A detailed restriction map of one of the positive clones obtained, pBCP509, is shown in Fig. 1A. It was estimated that the 5 kb ClalI digested pBlueScript SK yielding plasmid pBCP511 (Fig. 1B). The orientation of the gene is in the transcription direction of the vector encoded lac promoter. Transcription of the gene is under the direction of its own promoter as evidenced by the expression of the gene when cloned in the opposite direction in pBlueScript SK, however, a slight increase in expression of the gene was observed when isopropyl β-D-thiogalactopyranoside (IPTG) was included in the medium (results not shown).

Activity of the feaB gene is high in cells harbouring plasmid pBCP511 (4.1 U/mg of protein in cell free extract) while no detectable activity was observed in the control (E. coli TG2 cells harbouring pBlueScript SK). Native-PAGE confirmed this (results not shown) because protein staining revealed a heavy band at 190 kDa (which comigrated with the purified PAD) in the first and not in the latter case.

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Total protein (mg)</th>
<th>Specific activity (U/mg)</th>
<th>Yield (%)</th>
<th>Purification (-fold)</th>
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<tbody>
<tr>
<td>Cell-free extract</td>
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<td>4.1</td>
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</tr>
<tr>
<td>Ammonium sulfate</td>
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<td>49</td>
<td>1.2</td>
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<td>6.4</td>
<td>32.0</td>
<td>8.9</td>
<td>7.8</td>
</tr>
</tbody>
</table>

**Purification of PAD.** The overexpressed enzyme was purified 7.8-fold by ammonium sulfate precipitation, strong anion exchange chromatography on Macroprep-50 Q Sepharose and affinity chromatography on hydroxylapatite in a yield of 8.9%. Table 1 summarizes the results of the purification procedure. The preparation was homogeneous as revealed by the presence of
a single electrophoretic band on native-PAGE. This homogeneity was confirmed by gel filtration which yielded a single peak. The N-terminal amino acid sequence of the preparation established its purity and was identical to the amino acid sequence derived from the DNA sequence. The nucleotide sequence and derived amino acid sequence of the cloned gene displayed similarity with sequences of NAD-AIDHs from both bacterial and eukaryotic organisms (highest levels of similarity to other NAD-AIDHs from *E. coli* (55-95 %)).

**Molecular masses and subunit composition.** The native molecular mass of purified PAD was determined to 155 kDa by gel filtration. However, by native-PAGE this molecular mass was found to be 190 kDa. SDS-PAGE revealed that the enzyme is composed of identical subunits having a molecular mass of 50 kDa (not shown). The derived amino acid sequence of that subunit revealed a molecular mass of 54 kDa [23,42]. This indicates that the enzyme has an \( \alpha_3 \) or \( \alpha_4 \) composition.

**Absorption spectra.** The UV/Vis spectrum of the colourless PAD as isolated had a protein maximum at 280 nm. Addition of phenylacetaldehyde, acetaldehyde or sodium dithionite had no effect on the optical absorption spectrum (not shown).

**Stability and storage of the enzyme.** Upon purification, it was noted that the enzyme activity is quite labile. Highest enzyme activity was found in samples that were stored at very low temperatures (-80 °C) and pH 7.0. Storage at temperatures higher than -20 °C and outside the pH range of 6.5-7.5 caused inactivation of the enzyme within a few days. Incubation of PAD at room temperature affected the pattern on native-PAGE. After a few days the single band appeared to become sharper and the position of the band corresponded to a lower PAD molecular mass (160 kDa). Samples stored in presence of sodium, potassium or ammonium ions (0.5 M) resulted in a more stable enzyme. This effect has also been observed in the presence of 25 mM ethanol or 0.1%- Triton.

**Enzyme assay.** PAD catalyzed the conversion of aldehyde to the corresponding acid with concomitant reduction of NAD. The enzyme activity was 20-fold higher with NAD as electron acceptor than with NADP. No activity was detectable using the electron acceptors cytochrome c, 2,6-dichlorophenol-indophenol, ferricyanide, methylene blue, p-nitroso-\( N,N \)-dimethylaniline, phenazine methosulfate, tetrantrobile tetrazolium chloride and Wurster's blue. The pH optimum of PAD was 9.0 whereas the temperature optimum was in the range of 65 to 70 °C. The enzyme was not activated by potassium or phosphate ions in contrast to the activation observed for
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several other bacterial NAD-AlDHs [9].

**Table 2. Substrate specificity of PAD.** Only maximal enzyme activities are reported here whereas concentrations varied from 0.01 to 100 mM.

<table>
<thead>
<tr>
<th>Substrates</th>
<th>Concentration (mM)</th>
<th>PAD Activity (%)</th>
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<tbody>
<tr>
<td><strong>Aliphatic aldehydes</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Formaldehyde</td>
<td>10</td>
<td>1</td>
</tr>
<tr>
<td>Acetaldehyde</td>
<td>10</td>
<td>13</td>
</tr>
<tr>
<td>2-Chloroacetaldehyde</td>
<td>10</td>
<td>7</td>
</tr>
<tr>
<td>Propanal</td>
<td>10</td>
<td>9</td>
</tr>
<tr>
<td>Butanal</td>
<td>1</td>
<td>33</td>
</tr>
<tr>
<td>Hexanal</td>
<td>1</td>
<td>63</td>
</tr>
<tr>
<td>Octanal</td>
<td>1</td>
<td>80</td>
</tr>
<tr>
<td><strong>Aromatic aldehydes</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Benzaldehyde</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Phenylacetaldehyde*</td>
<td>0.1</td>
<td><strong>100</strong></td>
</tr>
<tr>
<td>2-Phenylpropanal</td>
<td>0.1</td>
<td>59</td>
</tr>
<tr>
<td>3-Phenylpropanal</td>
<td>0.1</td>
<td>35</td>
</tr>
<tr>
<td>3-Phenylbutanal</td>
<td>1</td>
<td>11</td>
</tr>
<tr>
<td><strong>Branched aldehydes</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Isobutanal</td>
<td>10</td>
<td>51</td>
</tr>
<tr>
<td>2-Methylbutanal</td>
<td>10</td>
<td>26</td>
</tr>
<tr>
<td>2,3-Dimethylpentanal</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>2-Ethylhexanal</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>3,5,5-Trimethylhexanal</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

*For PAD 100 % = 32.0 U/mg

**Substrate specificity.** Purified PAD catalyzed the oxidation of a broad range of aldehydes (Table 2) including aliphatic aldehydes, branched chain aliphatic aldehydes and aromatic aldehydes. For this enzyme the activity increased with increasing chain length of the aliphatic aldehyde. Short-chain aldehydes with a phenyl-group at the β-carbon atom were good substrates. Aldehydes lacking a hydrogen atom at the β-carbon atom like benzaldehyde and formaldehyde showed very low activity. Formate esters as well as benzoate esters showed no dehydrogenase activity.

From the calculated E-values, it appeared that PAD shows very low enantioselectivity towards the substrates 2-ethylhexanal, 3,5,5-trimethylhexanal and 3-phenylbutanal. The E-values were in the range of 1 to 2.

The apparent *Kₘ* values for NAD and phenylacetaldehyde, determined in the standard
assay and calculated in a Lineweaver-Burk plot, were 0.18 mM and 10 μM, respectively. Initial velocity studies were performed as a function of the NAD and acetaldehyde concentrations. Representative data are presented in Fig. 2. The fact that the lines drawn through the data converge at a common point is consistent with a ternary complex.

![Graph](image)

**Fig. 2. Initial velocity study for PAD activity: 1/(activity) versus 1/(acetaldehyde concentration) at different concentrations of NAD.** Acetaldehyde concentrations varied from 3 to 20 mM (3, 6, 10, 15 and 20 mM) whereas the following concentrations of NAD were used: 0.12 (○), 0.18 (□), 0.6 (△), 1.0 (×) and 2.5 (−) mM.

The enzyme was also assayed in the presence of 1 or 10 mM concentrations of dithiothreitol, glutathione, cysteine or β-mercaptoethanol. These reducing agents stimulated the dehydrogenase activity as is presented in Table 3.

**Inhibitors.** As shown in Table 4, bromoacetophenone, phenylmethanesulfonylfluoride (PMSF) and Hg²⁺ appeared to be effective inhibitors for the dehydrogenase activity. This inhibition was partially reversed upon addition of 10 mM dithiothreitol (Table 5). The following compounds did not inhibit the activity: 1 mM concentrations of Cu²⁺, Fe³⁺, Ni²⁺ and Zn²⁺ salts,
Phenylacetaldehyde dehydrogenase from *E. coli* 165

1 mM EDTA, 2 mM KCN, 2 mM NaN₃, 100 mM methanol and butanol.

**Table 3. Effect of reducing agents on the activity of PAD.** Pure enzyme was incubated for 1 min with 1 or 10 mM reducing agent in the common reaction mixture. The reaction was started by adding phenylacetaldehyde (0.1 mM).

<table>
<thead>
<tr>
<th>Reducing agent</th>
<th>1 mM PAD activity (%)</th>
<th>10 mM PAD activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Cysteine</td>
<td>100</td>
<td>196</td>
</tr>
<tr>
<td>Dithiothreitol</td>
<td>155</td>
<td>238</td>
</tr>
<tr>
<td>Glutathione</td>
<td>100</td>
<td>155</td>
</tr>
<tr>
<td>β-Mercaptoethanol</td>
<td>105</td>
<td>141</td>
</tr>
</tbody>
</table>

*For PAD 100 % = 32.0 U/mg*

**Table 4. Effect of potential inhibitors on the activity of PAD.** Pure enzyme was incubated for 1 min with the inhibitor in the common reaction mixture. The reaction was started by adding phenylacetaldehyde (0.1 mM).

<table>
<thead>
<tr>
<th>Inhibitors</th>
<th>Concentration (mM)</th>
<th>PAD activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethanol</td>
<td>100</td>
<td>68</td>
</tr>
<tr>
<td>Propanol</td>
<td>100</td>
<td>72</td>
</tr>
<tr>
<td>2-Propanol</td>
<td>100</td>
<td>82</td>
</tr>
<tr>
<td>Fe²⁺</td>
<td>0.1</td>
<td>80</td>
</tr>
<tr>
<td>Hg²⁺</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>PMSF</td>
<td>1</td>
<td>10</td>
</tr>
<tr>
<td>Isobutyramidine</td>
<td>10</td>
<td>78</td>
</tr>
<tr>
<td>Bromoacetophenone</td>
<td>0.1</td>
<td>4</td>
</tr>
</tbody>
</table>

*For PAD 100 % = 32.0 U/mg*

**Esterase activity.** PAD catalyzed the hydrolysis of *p*-nitrophenyl acetate. NAD (30 μM), the coenzyme for the dehydrogenase reaction, stimulated the hydrolysis 1.5-fold. NADH (50 μM), a product of the dehydrogenase reaction, activated ester hydrolysis even better, the activity being increased 17-fold. For NADP (30 μM) and NADPH (30 μM) the esterase activity increased 1.2 and 1.5-fold, respectively.

The effect of 0.2 mM phenylacetaldehyde on the esterase activity is shown in Fig. 3.
Phenylacetaldehyde appeared to be competitive with \( p \)-nitrophenyl acetate and the \( K_c \) calculated for phenylacetaldehyde from the change in the apparent \( K_m \) for \( p \)-nitrophenyl acetate is 16.6 \( \mu \)M.

![Graph](image)

**Fig. 3. Effect of phenylacetaldehyde on the enzymatic hydrolysis of \( p \)-nitrophenyl acetate.** The rate of hydrolysis of \( p \)-nitrophenyl acetate (concentrations of 6, 10, 15, 25 and 40 \( \mu \)M) was determined in the presence (○) and absence (●) of 0.2 mM phenylacetaldehyde.

The effect of bromoacetonophenone, PMSF or \( \text{Hg}^{2+} \) or/and dithiothreitol on the esterase activity is summarized in Table 5. As was observed for the dehydrogenase reaction, bromoacetonophenone, PMSF or \( \text{Hg}^{2+} \) also inhibited esterase activity and addition of dithiothreitol partially reversed this inhibition. Further, the esterase activity increased 1.5-fold in the presence of only dithiothreitol.

**DISCUSSION**

The gene adjacent to the gene encoding ECAO from *E. coli* [22,23] has been cloned.
Overexpression, purification and characterization of the corresponding enzyme were three major approaches utilized in this investigation to determine the identity and role of this enzyme. Overexpression of the enzyme was needed as no detectable expression was observed in E. coli TG2 cells lacking plasmid pBCP511. The nucleotide sequence and derived amino acid sequence of the cloned gene already displayed homology with sequences of NAD-AldHs from both bacterial and eukaryotic organisms.

Table 5. Effect dithiothreitol or/and inhibitor on dehydrogenase and esterase activity of PAD. Pure enzyme was incubated for 1 min with dithiothreitol or inhibitor in the assay mixture, as has been described for the dehydrogenase or esterase activity measurements in “Materials & Methods”. The reaction was started by adding phenylacetaldehyde (0.1 mM) or p-nitrophenylacetate (35 μM), respectively. To study the reversal of inhibition, the enzyme was first incubated for 1 min with inhibitor and subsequently for 5 min with dithiothreitol.

<table>
<thead>
<tr>
<th>Dithiothreitol/ inhibitor</th>
<th>Concentration (mM)</th>
<th>Dehydrogenase activity (%)</th>
<th>Concentration (mM)</th>
<th>Esterase activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td></td>
<td>100</td>
<td></td>
<td>100</td>
</tr>
<tr>
<td>Dithiothreitol</td>
<td>10</td>
<td>238</td>
<td>2</td>
<td>150</td>
</tr>
<tr>
<td>Bromoacetophenone</td>
<td>0.1</td>
<td>3</td>
<td>0.1</td>
<td>0</td>
</tr>
<tr>
<td>Bromoacetophenone + dithiothreitol</td>
<td>23</td>
<td></td>
<td></td>
<td>37</td>
</tr>
<tr>
<td>PMSF</td>
<td>1</td>
<td>9</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>PMSF + dithiothreitol</td>
<td></td>
<td></td>
<td>118</td>
<td>62</td>
</tr>
<tr>
<td>Hg²⁺</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Hg²⁺ + dithiothreitol</td>
<td>106</td>
<td></td>
<td></td>
<td>50</td>
</tr>
</tbody>
</table>

*For dehydrogenase activity 100 % = 32.0 U/mg
*For esterase activity 100 % = 0.64 U/mg

The purification of the enzyme (Table 1) yielded a homogeneous preparation as one protein band was visualized by native as well as SDS-PAGE. In addition the elution pattern obtained by applying gel filtration showed one peak and the determination of the N-terminal amino acid sequence revealed a sequence which was uniform and identical to the derived amino acid sequence [23]. Although the purification factor of the ammonium sulfate precipitation step
was low, it was necessary to obtain pure enzyme. When this step was omitted, the final product revealed minor protein bands on native and SDS-PAGE. The yield of the purification procedure was rather low. This is probably due to the instability of the PAD, which is a notable property of many NAD-AIDHs [9]. Although the presence of 0.1-0.5 M salt [10], ethanol, Triton or a reducing agent [9] during the purification should contribute to a higher stability of the enzyme, these agents have not been used here to prevent possible side effects. PAD appeared to be rather stable by purifying the enzyme at pH 7.0 and storing it at the same pH and at -80 °C. This pH value has also been found to be the most favourable condition for storage of other NAD-AIDHs, like the AIDH from *Pseudomonas aeruginosa* [9]. Storing the PAD for a few days at room temperature did not only result in a lower enzyme activity but also revealed, as was visualized by native-PAGE, a sharper protein band corresponding to a lower molecular mass (not shown). This may be due to conformational changes of the enzyme or proteolytic attack.

The molecular mass of PAD was determined to be 190 kDa by native-PAGE, which showed one protein band, and 155 kDa by gel filtration chromatography, which showed one peak. Its subunit molecular mass of 50 kDa, as was determined by SDS-PAGE and resembling the molecular mass (54 kDa) as was determined from the deduced amino acid sequence, suggests a trimeric or tetrameric enzyme. The majority of eukaryotic NAD-AIDHs has a molecular mass of 230-260 kDa with subunit molecular masses of 53-58 kDa, and is known to be tetrameric [4,5]. Only a few eukaryotic NAD-AIDHs are presumed to be homodimers with molecular masses of 120-150 kDa consisting of 55-65 kDa subunits [4,6]. For prokaryotic NAD-AIDHs only tetrameric structures have been observed. In this case the molecular mass varied from 160 to 220 kDa and the subunit molecular mass from 40 to 55 kDa [7,8]. On basis of these findings and the high sequence homology with other prokaryotic and eukaryotic tetrameric NAD-AIDHs we favour a tetrameric structure for PAD from *E.coli*.

A systematic overview of NAD(P)-dependent oxidoreductases has revealed that most of these enzymes exhibit a strong preference for either NAD or NADP, in agreement with the distinct metabolic functions generally assigned to NAD (catabolic role) and NADP (anabolic role) [43]. The coenzyme selectivity of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), for example, depends on its subcellular localization [44]. The discrimination between NAD and NADP was suggested to be due to molecular determinants belonging to side chains which define the inner surface of the cofactor binding cleft [45]. PAD used either NAD or NADP as coenzyme
but showed 20-fold higher enzyme activity in presence of NAD than NADP. This suggests that in vivo NAD is likely to be the preferred coenzyme. The AIDH from *P. aeruginosa* [9] showed a similar preference for NAD. In this case a 25-fold higher enzyme activity was measured in presence of NAD compared to NADP. For this enzyme a $K_m$ value of 0.37 mM was determined for NAD with glycolaldehyde as substrate. This value is comparable with the $K_m$ value for NAD (0.18 mM) of PAD with phenylacetaldehyde as substrate.

The typical glycine box (GXGXXG), which also often indicates the binding site for the ADP moiety of NAD [46], is lacking in all amino acid sequences of the NAD-AIDHs. However, all the glycine residues at the two regions postulated as putative NAD-binding sites in PAD (positions 228-234 and 250-255) are conserved in the majority of NAD-AIDHs. Limited proteolysis [47] revealed that the nucleotide-binding domain of NAD-AIDHs should be localized to the amino-terminal half of the protein. With a threonine in place of the second glycine residue, the sequence GSTATG at indexes 250-255 in PAD provides the closest match to the GXGXXG pattern.

Although the stability of PAD is higher at pH 7.0, highest enzyme activity was measured at pH 9.0 and not at the physiological pH. Although only a few studies have been published on the effect of pH on NAD-AIDH-catalyzed reactions, this high pH optimum is comparable to those determined for several other NAD-AIDHs [4,10,48]. The optimum temperature of PAD for the reaction was in the range of 65 to 70 °C. This value is quite high compared with temperature optima of other NAD-AIDHs [9] and unexpected as *E. coli* normally grows at 37 °C. The tetrameric enzyme GAPDH from several species [49,50] shows, however, also highest enzyme activity at high temperature (85 °C).

PAD shows very low enantioselectivity towards racemic aldehydes. The reason for this is not clear since PAD exhibits a restricted substrate specificity (Table 2). Higher enzyme activities were measured as the chain length of the aliphatic aldehydes increased. This suggests a hydrophobic active site in PAD. This has also been suggested for several eukaryotic NAD-AIDHs [51,52] by probing the active site with a chromophoric reporter group (3,4-dihydro-3-methyl-6-nitro-2H-1,3-benzoxazin-2-one). The rather polar properties or small size of formaldehyde probably explain why the PAD activity is very low when using this aldehyde as substrate. The hydrophobic substrate pocket was also suggested to be barrel shaped to facilitate van der Waals contact interaction between the hydrocarbon chain of the substrate and the neutral
side groups of the lining amino acid residues in the pocket. This suggested barrel shaped active site has probably a restricted volume as increasing the chain length of an aromatic aldehyde revealed lower enzyme activities for PAD. Highest PAD activity was measured for phenylacetalddehyde. Taking into account that 2-phenylethylamine is a very good substrate for ECAO from E. coli [53] and that the gene of this latter enzyme is adjacent to the gene of PAD in E. coli [23], suggests that ECAO converts aromatic amines to aromatic aldehydes which are subsequently converted by PAD to their corresponding carboxylic acids.

During and after formation of a ternary complex (NAD-enzyme-substrate), revealed by initially velocity studies (Fig. 2), a cysteine and a glutamate [14,15,21,54-56] could be involved in the catalysis. Comparison of amino acid sequences of many NAD-AIDHs showed that a cysteine and glutamate, at positions 302 and 268, respectively, were both conserved residues in the molecule. The proximity of this glutamate to the cysteine is evident from the fact that both residues are the only two residues derivatized [57] by bromoacetophenone, a well-characterized affinity reagent [58]. Since the cysteine and glutamate residues are also conserved in PAD, at position 306 and 272, respectively - the same 34 amino acid residue distance - and the enzyme is also inhibited by bromoacetophenone, suggests a similar catalytic cycle for PAD. The proposed role for the glutamate residue is that of ionization of the cysteine and consequent facilitation of formation of a hemiacetal from an aldehyde [59]. The hemiacetal is subsequently oxidized, via hydride transfer to NAD, to a thioacetyl intermediate which was proposed to be hydrolyzed by the action of general base catalysis [17,60,61].

The fact that formate esters were not substrates for PAD (no dehydrogenase activity) is not clear since certain molybdoprotein aldehyde dehydrogenases, like those from Comamonas testosteroni (to be published elsewhere) and Amycolatopsis methanolica [62], for example, do show enzyme activity for formate esters (although a different reaction mechanism is proposed for these latter enzymes).

PAD did show esterase activity by catalyzing the hydrolysis of p-nitrophenyl acetate. Such a dual reaction specificity (dehydrogenase and esterase activity) was first reported for GAPDH in 1961 [18] and subsequently observed for several other eukaryotic NAD-AIDHs. The finding that these enzymes catalyze ester hydrolysis strongly supported the hypothesis that an enzyme-thioester is an important intermediate in the dehydrogenase reaction pathway [17]. Competitive behaviour between the aldehyde and ester (Fig. 3) and inhibition of the
dehydrogenase as well as the esterase activity by bromoacetophenone is in agreement with this suggestion indicating also that the dehydrogenase and esterase reactions occur at the same active site. This was also shown for several other NAD-AIDHs by mutational analyses [15,16].

The coenzyme for the dehydrogenase reaction, NAD, exerted a modifying influence on the esterase activity, producing a 1.5-fold enhancement of the rate of nitrophenyl ester hydrolysis. NADH exerted even a 17-fold stimulation of the esterase reaction. In spite of this, the maximal rate of dehydrogenation was 50 times greater than the maximal rate of the esterase reaction without NAD(P)(H). NADH could not force the esterase reaction (17-fold) to proceed at a rate faster than the dehydrogenation reaction. This characteristic stimulation of esterase activity by NAD and NADH has been documented for NAD-AIDHs from various sources [17,63-69]. These stimulations varied, however, from 2 to 11-fold. The presence of NAD is proposed to increase the nucleophilicity of the active site residue, cysteine 302 [16,66] and hence increases the velocity of the attack of the nucleophile on the ester carbonyl-group to form a covalent intermediate. Both esterase and dehydrogenase reactions have a common acyl intermediate, acetyl-enzyme-NADH, formed from either the oxidation of aldehyde or the hydrolysis of a nitrophenyl ester in the presence of NADH. The fact that the stimulated esterase reaction was still slower than the dehydrogenation reaction suggests that the rate-limiting step in esterase reaction occurs prior to the deacylation step. If deacylation were rate-limiting, the $V_{\text{max}}$ for nitrophenyl ester hydrolysis would have been equal to or greater than that found for the dehydrogenase reaction. Such a conclusion was also reached for human and horse liver NAD-AIDH [17,63].

Reducing agents such as cysteine, dithiotreitol, glutathione or β-mercaptoethanol were found to be necessary for maximal dehydrogenase activity as well as esterase activity (Table 3). These results indicate that PAD is most active when in fully reduced state although we have to realize that thiol agents can form thiolhemiketaladducts with aldehydes which could be better substrates for the enzyme. Bromoacetophenone, PMSF and Hg²⁺ were effective inhibitors (Table 4) for the dehydrogenase and esterase activity of PAD. Bromoacetophenone derivatizes the glutamate and cysteine residue in the active site [57] thereby preventing the conversion of the substrate. PMSF can react with serine as well as cysteine residues. A serine residue should be capable of affecting the coenzyme binding, thereby affecting the active site cysteine residue even in the absence of coenzyme [16]. Addition of a reducing agent after the addition of these inhibitors produced partial reversal of enzyme inhibition indicating that the enzyme requires the
presence of -SH groups (cysteine) for activity.

The physiological role of NAD-AIDHs has always been ambiguous. Although these enzymes catalyze the oxidation of a wide variety of aldehydes, acetaldehyde has been thought to be the only one of physiological significance because of the involvement in ethanol metabolism [70]. Acetaldehyde can hardly be the natural substrate for the bacterial PAD since highest activity was found for 10 mM acetaldehyde (in the concentration range from 0.01 to 100 mM) which far exceeds physiological concentrations, typically low micromolar values [71]. On the other hand phenylacetaldehyde, showing highest enzyme activity and having a $K_m$-value of 10 $\mu$M, can be the natural substrate. Betaine AIDH from E. coli, which shows high similarity to the PAD, shows highest levels of similarity to the prototype human AIDH-2, which has been implicated in the metabolism of biogenic amines, polyamines and the products of lipid peroxidation [70,72]. This similarity suggests also the involvement of PAD in the metabolism of aromatic amines in E. coli.

PAD appeared to be similar to many other NAD-AIDHs of both prokaryotic and eukaryotic origin with respect to its sequence, structure, active site and mechanism of action. It is clear that the expression, purity and stability of PAD will allow detailed investigation of the molecular properties and provide more insight into the function of the enzyme and of NAD-AIDHs in general. Mutational analyses of PAD from the genetically easily accessible E. coli should contribute to elucidate which amino acid residues of the active site are involved in catalysis.
REFERENCES


SUMMARY

One of the main topics in our research group is the oxidation of alcohols and amines by bacterial quinoprotein and nicotinoprotein oxidoreductases. Since the first oxidation step in both conversions yields aldehydes and the fate of the aldehydes is relevant in the outcome of the enzymatic kinetic resolution of racemic alcohols, it was decided in first instance to study the role of the already known molybdenopterin aldehyde dehydrogenase from Comamonas testosteroni (AlDH) in this connection. Since we found that this AlDH shows very low enantioselectivity for a variety of racemic aldehydes, it was attempted to reveal the reason for this by performing structural and mechanistic studies on this enzyme as well as on two other aldehyde dehydrogenases from Amycolatopsis methanolica (FEDH and DL-AlDH) [Chapter 2 and 3].

The three molybdenopterin aldehyde dehydrogenases (AlDHs) characterized now consist of three different subunits, have a total molecular mass of approximately 150 kDa and contain a molybdenopterin cytosine dinucleotide (MCD) [Chapter 2 and 3]. The enzymes are suggested to be members of the xanthine oxidase-family although N-terminal sequences of the subunits of the AlDHs show greatest similarity with other MCD-containing bacterial dehydrogenases. The AlDHs contain FAD, iron, acid-labile sulphide, molybdenum and CMP in a molar ratio of 1:4:4:1:1. The iron atoms and acid-labile sulphides are arranged as two nonidentical [2Fe-2S] clusters (but not in DL-AlDH since only one cluster has been detected) which show magnetic interaction with each other. In case of DL-AlDH a Mo(V) EPR signal is observed [Chapter 3]. The signal indicates possible hyperfine interaction of this molybdenum with a proton in close proximity. In addition, magnetic coupling between the Mo(V) and [2Fe-2S]-1 cluster is observed.

Since EPR of the as isolated AlDHs revealed for the first time a free radical form of the pterin moiety of the pterin molybdenum cofactor (Moco), we attempted to elucidate its structure and to answer the question whether the pterin moiety has a structural or catalytic role in the enzyme [Chapter 4]. Determination of the concentration of the g = 2.004 signal yielded 12, 15 and 2 % of the enzyme concentration for AlDH, FEDH and DL-AlDH, respectively. The values of the various hyperfine coupling constants are consistent with the properties expected for a molybdenum(VI)-trihydropterin radical in which the N5-atom is engaged in two hydrogen bonding interactions with the protein. The majority of the electron (spin) density of the radical is located at and around the N5-atom and at the proton bound to the C6-atom of the pterin ring.
The radical is not magnetically isolated as there is cross-relaxation with a nearby, rapidly relaxing, oxidized [2Fe-2S]-cluster involving its magnetic $S = 1$ excited state in this process. Preliminary experiments on the function of the radical have not given definitive answers on its role in catalysis. When the radical turns out to be a genuine catalytic intermediate, the pterin-ring system might act as a one-electron carrier, shuttling between its trihydro- and tetrahydro-redox states, mediating electron transfer between the molybdenum ion and the nearest [2Fe-2S] cluster.

The purified γ-subunit of AIDH (which has not been purified for any other molybdoprotein) contains only the plant-type [2Fe-2S] cluster and lacks the second [2Fe-2S] cluster [Chapter 2]. Deflavo-AIDH also shows the characteristics of the plant-type [2Fe-2S] cluster and lacks the second [2Fe-2S] cluster. An optical difference spectrum (deflavo-AIDH minus γ-subunit) suggests that in deflavo-AIDH the pterin-moiety is in the fully oxidized state. This oxidized state of the pterin might be due to direct exposure to oxygen or to oxidation by the [2Fe-2S] cluster during unfolding. The function (electron transfer or structural) of FAD in prokaryotic molybdoenzymes remains unclear in view of the existence of bacterial molybdoproteins lacking FAD. This includes aldehyde oxidoreductase from *Desulfovibrio gigas* which shows that FAD is dispensable for aldehyde oxidation.

In many molybdoproteins, the [2Fe-2S]-2 cluster has a more positive redox potential ($E_m$) than [2Fe-2S]-1, however, the reverse is true for AIDH [Chapter 2]. The potentials of both [2Fe-2S] clusters in AIDH are pH dependent, which is most simply explained by the presence of an acid/base group in electrostatic contact and/or close to the [2Fe-2S] cluster. Compared to xanthine oxidase and xanthine dehydrogenase, all the potentials of the redox couples in AIDH are 0.1 to 0.2 V higher, except the potential of the [2Fe-2S]-2 cluster.

Although the prokaryotic molybdoprotein AIDHs of aerobic bacteria are able to convert a wide variety of aldehydes and formate esters, their substrate specificities are different, indicating variation in the nature of their active sites [Chapter 2 and 3]. The very low enantioselectivity towards racemic aldehydes suggests that the steric conformation of the residues attached to the asymmetric C-atom is irrelevant with respect to discrimination between the enantiomers in binding as well as in turnover rates. The AIDHs, however, exhibit a restricted substrate specificity which suggests that the steric conformation of the substituents to the chain should affect the tightness of this interaction.

Studies conducted in our research group and that of Dr. Postma, University of
Amsterdam, revealed that a gene (feaB) for a NAD(P)-dependent aldehyde dehydrogenase (NAD-AlDH) was present in Escherichia coli next to the gene (maoA) of a quinoprotein aromatic amine oxidase (ECAO). Since an overexpressing strain was available, the NAD-AlDH was purified and characterized [Chapter 5].

The enzyme appears to be a NAD(P)-dependent phenylacetaldehyde dehydrogenase (PAD) and is a homotetramer with a molecular mass of 155-190 kDa. Enzymatic activity as determined in the assay shows a pH optimum of 9.0 and a temperature optimum between 65 and 70 °C. In contrast to several other bacterial NAD-AlDHs, PAD is not activated by potassium or phosphate ions. Steady-state activity measurements showed that the reaction of NAD and aldehyde with PAD proceeds via a sequentially ordered process. PAD prefers long-chain aliphatic aldehydes or short-chain aliphatic aldehydes containing a phenyl group as a substrate. Apparent K_m values for NAD and phenylacetaldehyde were found to be 0.18 mM and 10 μM, respectively, and activity with NAD is 20-fold higher than with NADP. These data, together with the localization of the maoA and feaB genes and the known substrate specificity of ECAO, indicate that PAD is a NAD-dependent aromatic aldehyde dehydrogenase involved in the metabolism of aromatic amines via ECAO.

Like other NAD-AlDHs, PAD shows aldehyde dehydrogenase as well as esterase activity. A 17-fold increase of esterase activity was observed in the presence of NADH. Phenylacetaldehyde and p-nitrophenylacetate, the substrate for the esterase activity assay, acted as competitive inhibitors for each other in the respective assays. This suggests that both activities derive from the same active site. The inhibition of both activities by bromoacetophenone, phenylmethanesulfonylfluoride and Hg²⁺ was alleviated upon addition of a thiol reducing agent. All these properties indicate that PAD is a normal NAD-AlDH.
Enzymologische studie aan bacteriële aldehyde dehydrogenasen

SAMENVATTING

De oxidatie van alcoholen en amines door bacteriële quinoproteïne en nicotinoproteïne oxidoreductasen is één van de hoofdonderwerpen in onze onderzoeksgroep. Omdat bij de eerste oxidatie-stap bij beide conversies aldehyde worden gevormd en het lot van deze aldehyde belangrijk is voor het resultaat van de enzymatische kinetische resolutie van racemische alcoholen, werd in eerste instantie onderzoek gedaan naar de rol van het al bekende molybdoproteïne aldehyde dehydrogenase uit Comamonas testosteroni (AIDH). Omdat dit enzym een erg lage enantioselectiviteit vertoond voor racemische aldehyde, werd getracht de rede hiervoor te achterhalen door structurele en mechanistische studies te doen aan dit enzym en aan twee andere aldehyde dehydrogenasen uit Amycolatopsis methanolica (FEDH en DL-AIDH) [Hoofdstuk 2 en 3].

De drie bovengenoemde molybdoproteïne aldehyde dehydrogenasen (AIDHs) die nu gekarakteriseerd zijn bestaan uit drie verschillende subeenheden, hebben een moleculelair gewicht van rond de 150 kDa en bevatten een molydoperine cytosine dinucleotide (MCD) [Hoofdstuk 2 en 3]. De enzymen behoren naar alle waarschijnlijkheid tot de xanthine oxidase-familie al vertonen de N-terminale sequenties van de subeenheden van de AIDHs de meeste homologie met die van andere MCD-bevattende bacteriële dehydrogenasen. De AIDHs bevatten FAD, ijzer, zuur-label zwavel, molybdeen en CMP in een molaire ratio van 1:4:4:1:1. De ijzer en zuur-labiele zwavel atomen zijn aanwezig als twee niet-identieke ijzer-zwavel [2Fe-2S] clusters die magnetische interactie met elkaar vertonen. Dit blijkt niet het geval voor DL-AIDH omdat voor dit enzym maar één cluster wordt gedetecteerd [Hoofdstuk 3]. Daarentegen wordt voor dit enzym wel een Mo(V) EPR-signaal gevonden. Het signaal geeft aan dat er mogelijk hyperfijne interactie is tussen de molybdeen en een dichtbij gelegen proton. Daarnaast is er ook magnetische koppeling geobserveerd tussen de Mo(V) en de [2Fe-2S]-1 cluster.

Omdat met EPR aan de geïsoleerde AIDHs voor het eerst een pterine radikaal (afkomstig van de pterine molybdeen cofactor (Moco)) werd geobserveerd, werd getracht zowel de structuur als de mogelijke structurele of catalytische rol van dit radikaal in het enzym te achterhalen [Hoofdstuk 4]. De concentratie van het g = 2.004 signaal betreft 12, 15 en 2 % van de


Voor veel molybdoproteïnen heeft de [2Fe-2S]-2 cluster een hogere redox potentiaal ($E_m$) dan de [2Fe-2S]-1 cluster. Het tegenovergestelde is echter waar voor ALDH [Hoofdstuk 2]. De potentialen van beide [2Fe-2S] clusters in ALDH zijn pH-afhankelijk. Dit is naar alle waarschijnlijkheid mogelijk indien een zuur/base groep aanwezig is die electrostatisch contact heeft met de cluster en/of dichtheid bij de cluster gelegen is. De potentialen van de redox centra in ALDH zijn allen 0.1-0.2 V hoger dan die in xanthine oxidase en xanthine dehydrogenase met uitzondering van de potentiaal van de [2Fe-2S]-2 cluster.

Hoewel de prokaryotische molybdoproteïne ALDHs uit aerobe bacteriën in staat zijn om
een breed scala aan aldehyden en formaat esters om te zetten, zijn de substraatspecificiteiten van de enzymen verschillend. Dit geeft aan dat de ‘active sites’ verschillend zijn. De erg lage enantioselectiviteit voor racemische aldehyden suggereert dat de sterische conformatie van de residuen, die aan het asymmetrische C-atoom zitten, niet relevant is voor de keuze tussen de enantiomeren in binden en omzettingssnelheden. De AIDHs laten echter een dermatte beperkte substraatspecificiteit zien dat de sterische conformatie van de substituентen aan de keten een effect zou moeten hebben op de sterkte van de interactie.

Studies in onze onderzoeksgroep en aan de Universiteit van Amsterdam, onder leiding van Dr. Postma, toonde aan dat een gen (feab) voor een NAD(P)-afhankelijk aldehyde dehydrogenase (NAD-AIDH) aanwezig is in Escherichia coli naast het gen (maoa) van een quinoproteïne aromatisch amine oxidase (ECAO). Omdat er een stam aanwezig was die voor overexpressie kon zorgen, werd dit NAD-AIDH gezuiverd en gekarakteriseerd [Chapter 5].

Het enzym blijkt een NAD(P)-afhankelijk fenylacetaldehyde dehydrogenase (PAD) te zijn. Het is een homotetramer met een moleculeig gewicht van 155-190 kDa. De enzymatische activiteit blijkt optimaal bij een pH van 9.0 en een temperatuur van 65-70 °C. In tegenstelling tot andere bacteriële NAD-AIDHs wordt PAD niet geactiveerd door middel van kalium- of fosfaat- ionen. Steady-state activiteitsmetingen laten zien dat de reactie van NAD en aldehyde met PAD via een opeenvolgend geordend proces gaat. PAD heeft qua substraat voorkeur voor alifatische aldehyden met een lange koolstofketen en alifatische aldehyden met een korte koolstofketen die tevens een fenyl-groep bevatten. De apparent Kₘ waarden voor NAD en fenylacetaldehyde zijn 0.18 mM en 10 μM, respectievelijk, en de enzymactiviteit met NAD is 20-maal hoger dan met NADP. Deze resultaten tezamen met de lokalisatie van de maoa en feab genen en de substraatspecificiteit van ECAO tonen aan dat PAD een NAD-AIDH afhankelijk aldehyde dehydrogenase is die betrokken is bij de omzetting van aromatische amines via ECAO.

PAD vertoont net als andere NAD-AIDHs, naast aldehyde dehydrogenase activiteit, ook esterase activiteit. In aanwezigheid van NADH wordt deze esterase activiteit 17-maal hoger. Fenylacetaldehyde en p-nitrofenylacetaat (substraat voor esterase activiteit) gedragen zich als competitieve remmers voor elkaar. Dit suggereert dat beide activiteiten plaats vinden aan dezelfde ‘active site’. Remming van beide activiteiten door bromoacetofenon, fenylmethaansulfonylfuoride en Hg²⁺ kan deels worden opgeheven door toevoeging van een thiol verbinding. Al deze eigenschappen wijzen er op dat PAD een normaal NAD-AIDH is.
List of publications

Molybdoprotein aldehyde dehydrogenases from *Comamonas testosteroni* and *Amycolatopsis methanolica*

A second molybdoprotein aldehyde dehydrogenase from *Amycolatopsis methanolica* NCIB 11946
*Archives of Biochemistry and Biophysics* **325**, 1-7 (Chapter 3)

Characterization of molybdoprotein aldehyde dehydrogenases from *Comamonas testosteroni* and *Amycolatopsis methanolica*
Submitted to *Biochemical Journal* (Chapter 2)

Molybdopterin radical in bacterial aldehyde dehydrogenases
To be published in *Biochemistry* (Chapter 4)

Cloning of the gene encoding phenylacetaldehyde dehydrogenase from *E. coli* and enzymological characterization of the overexpressed enzyme
(Chapter 5)
Curriculum vitae


Vanaf maart 1998 is ondergetekende werkzaam als post-doctoraal medewerker bij de Sectie Industriële Microbiologie (Vakgroep Voedingstechnologie) aan de Landbouwuniversiteit te Wageningen.
Dankwoord

En dan kom ik nu toe aan 't stukje proefschrift dat waarschijnlijk wel grijs gelezen zal worden (zie stelling 19). Velen zullen zich afvragen: “word ik nou wel of niet genoemd?” Ten eerste wil ik Simon de Vries noemen. Jouw inspirerende begeleiding en ongelooflijk enthousiasme voor het onderzoek zorgden ervoor dat het uiteindelijk allemaal gelukt is. Ik had het bijna voor elkaar om je als co-promotor op te laten treden (wat niet meer dan terecht zou zijn), echter de kleine lettertjes zaten ons tegen. Simon, bedankt en ik heb veel van je geleerd! Naast Simon bedank ik mijn promotor Hans Duine voor zijn adviezen, zijn kritische houding bij het schrijven van de artikelen en hoofdstukken en de mogelijkheid tot promoveren.

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dicht gaan (zie stelling 17), het bewonderen van onze eigen Franse vierwieler of menig gestruikel onderweg? Ik weet ‘t niet. En, wie heeft nu eigenlijk ‘t bonnetje? Aard, jij hebt me laten zien dat ‘s avonds werken op het Kluysterlab best wel gezellig is. Daarnaast was je altijd wel in voor speciale nevenactiviteiten zoals karaoken met Gerard Joling, port proeven uit een bierglas en zelfs schilderen zonder alcohol. Voor de nevenactiviteiten (SWAK-feestjes, borrelen, Koornbeurs, voetbal, geen Nighttown (ja, lach maar)) wil ik naast Arjen en Aard, ook Jan Feitsma (ghè), Arie Braat (m’n fiets is nog steeds niet gemaakt) en Toine Overbeek (ben benieuwd naar jouw dankwoord) bedanken.

Intussen is het zaalvoetbalteam “de Kluystercreaks” een begrip geworden in Delft. Hierbij ben ik onder leiding van ene heer Remie zelfs 3 keer gepromoveerd (van de 3e klasse naar de hoofdklasse). Helaas ging het daarna onder russische leiding en wat streover (zie stelling 14). Alle Kluystercreaks de afgelopen jaren, bedankt!

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The tide shall turn to shelter us from storm
The seas of charity shall overflow and bath us all

("It's getting late in the evening" - M. Hollis)
Ik heb 'n steen verlegd
in 'n rivier op aarde.
Nu weet ik dat ik nooit zal zijn vergeten.
Ik leverde bewijs van mijn bestaan.
Omdat door 't verleggen van die ene steen
de stroom nooit meer dezelfde weg zal gaan.

("De Steen" - B. Vermeulen)