PYRROLOQUINOLINE QUINONE

THE COFACTOR OF QUINOPROTEINS
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PROEFSCHRIFT

ter verkrijging van de graad van doctor
aan de Technische Universiteit Delft,
op gezag van de Rector Magnificus,
Prof. dr. J. M. Dirken,
in het openbaar te verdedigen
ten overstaan van een commissie aangewezen
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door

Johannes Frank Jzn
geboren te Delft
Scheikundig ingenieur

Krips Repro Meppel
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Dit proefschrift is goedgekeurd door de promotor
Prof. dr. ir. J.A. Duine
Un chercheur qui renonce à perdre du temps, est perdu pour la recherche.

Jean Aubray

Aan mijn ouders

Voor Rose-Marie en Laurence
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## Abbreviations

<table>
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<th>Abbreviation</th>
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<tbody>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>E₆</td>
<td>midpoint potential at pH 7.0</td>
</tr>
<tr>
<td>Eₐm</td>
<td>midpoint potential at a specified pH</td>
</tr>
<tr>
<td>ESR</td>
<td>electron spin resonance</td>
</tr>
<tr>
<td>FAD</td>
<td>flavin adenine dinucleotide</td>
</tr>
<tr>
<td>FMN</td>
<td>flavin mononucleotide</td>
</tr>
<tr>
<td>HPLC</td>
<td>high performance liquid chromatography</td>
</tr>
<tr>
<td>NAD⁺</td>
<td>nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>NMR</td>
<td>nuclear magnetic resonance</td>
</tr>
<tr>
<td>NADP⁺</td>
<td>nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>MDH</td>
<td>methanol dehydrogenase</td>
</tr>
<tr>
<td>MDHox₂, MDHox</td>
<td>the oxidized form of methanol dehydrogenase</td>
</tr>
<tr>
<td>MDHred</td>
<td>the reduced form of methanol dehydrogenase</td>
</tr>
<tr>
<td>MDHsem</td>
<td>the semiquinone form of methanol dehydrogenase</td>
</tr>
<tr>
<td>PQQ</td>
<td>2,4,7-tricarboxy-1H-pyrrolo[2,3-f]quinoline-4,5-dione</td>
</tr>
<tr>
<td>PQQH₂</td>
<td>2,4,7-tricarboxy-1H-pyrrolo[2,3-f]quinoline-4,5-diol</td>
</tr>
<tr>
<td>PQQH⁺</td>
<td>the semiquinone of PQQ</td>
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Chapter I

Introduction

Cofactors and coenzymes involved in substrate redox reactions

All living cells need energy for their maintenance and growth and to that end they all produce enzymes capable of carrying out energy generating redox reactions. In contrast to the large variety of substrates and corresponding enzymes which are used for that purpose, the number of cofactors required for the functioning of redox enzymes is rather limited. In this introductory section a brief overview of the cofactors participating in redox reactions at the substrate level will be given.

Soon after the discovery that proteins, in the form of enzymes, were responsible for cellular metabolism, it was found that the catalysis of redox reactions depended on the presence of additional non-peptide substances. In fact, at the beginning of this century Harden and Young (1905, 1906) performed their classical studies leading to the discovery of the coenzyme of alcoholic fermentation. It took, however, more than twenty years before it was realized that this coenzyme was actually a mixture of ATP, thiamine, Mg²⁺-ions, NAD⁺ and NADP⁺, of which only the latter two were found to be involved in electron transfer (Warburg and Christian 1936). The chemical structure of NADP⁺ was elucidated by Warburg and coworkers (Warburg et al. 1935), while that of NAD⁺ was established independently in the laboratories of Warburg and von Euler (Warburg and Christian 1936, Stenck and von Euler 1936).

Another coenzyme involved in electron transfer, flavin mononucleotide (FMN), was isolated from the "old yellow enzyme" by Theorell in 1935 (Theorell 1935) and was identified as the phosphorylated derivative of the previously characterized riboflavin (Kuhn et al. 1933, Ellinger and Koshara 1933). The discovery and structure elucidation of the closely related flavin adenine dinucleotide (FAD) as the cofactor of D-amino acid oxidase by Warburg and Christian (1938) rapidly followed. In addition, the recognition of the dependence of enzyme activity on derivatives of riboflavin, already known as vitamin B₂, threw new light on the functioning of vitamins.

In 1957 a third hydrogen carrier, the lipid-soluble Coenzyme Q, was isolated from ox-heart mitochondria and found to be a component of the electron transfer chain, rather than belonging to a particular enzyme (Crane et al. 1957).

In addition to these coenzymes and cofactors, which are present in all living cells, other components have occasionally been found to be involved in redox reactions. As long as half a century ago, Bortels (1930) described the involvement of molybdenum in nitrogen fixation, i.e. the reduction of nitrogen to ammonia. A molybdenum and iron cofactor is now considered to be involved in the process of nitrogen reduction (Eady 1986). More recently, nitrogenases in which molybdenum is replaced by vanadium have been isolated (Robson et al. 1986, Hales et al. 1986). Vanadium might also be involved in processes other than nitrogen reduction, as is indicated by the recently reported isolation of a vanadium containing haloperoxidase (de Boer et al. 1986).

The role of molybdenum is not only confined to nitrogenases. The relationship

![Fig. 1. The structure of molybdopterin isolated from sulfite oxidase (Kramer et. al. 1987).](image-url)
between molybdenum and xanthine oxidation was described by Westerveld and Richert in 1953. In 1964, Pateman and coworkers indicated that xanthine dehydrogenase and nitrate reductase required the same molybdenum containing cofactor. The structure of this unstable molybdopterin (Fig. 1) was only recently resolved (Kramer et al. 1987). The molybdenum cofactor extracted from carbon monoxide dehydrogenase appears to contain an additional phosphate group, attached to an unidentified residue. The compound is called bacopterin to distinguish it from molybdopterin (Meyer et al. 1986). It has been shown that in some cases the molybdenum in formate dehydrogenase is replaced by selenium (Ljungdahl and Andreesen 1975) or tungsten (Wagner and Andreesen 1977).

A particularly rich source of novel cofactors are the methanogens, members of the Archaebacteria. So far, six unusual structures have been determined, of which at least two are directly involved in substrate redox reactions. One unusual flavin derivative (5-deazaflavin) attracted attention because of its remarkable fluorescence. It is now presumed to be the electron acceptor for the hydrogenase occurring in methanogenic bacteria, and to function as an electron donor in several steps of the complex reaction cycle in which carbon dioxide is reduced to methane. A structure for 5-deazaflavin was proposed in 1978 (Eirich et al. 1978, Fig. 2). It has recently been reported that in the ultimate formation of methane, 2-(methylthio)-ethanesulfonic acid is reduced by a stoichiometric amount of N-7-mercaptopheptanoyl-phosphothreonine (Fig. 3) in the presence of a purified reductase (Hauska 1988). The role of F₄₃₀, a unique nickel tetrapyrrole (Pfaltz et al. 1982, Fig. 4), in

![Fig. 2. F₄₃₀, the N-(N-L-lactyl-γ-L-glutamyl)-L-glutamic acid phosphodiester of 7,8-didemethyl-8-hydroxy-5-deazariboflavin 5'-phosphate. (Eirich et al. 1978)]

![Fig. 3. N-7-mercaptopheptanoyl-O-phosphothreonine (HS-HTP) and its involvement in the reduction of methyl-Coenzyme M (Hauska 1988)]

![Fig. 4. The nickel tetrapyrrole F₄₃₀ after methanogenesis of the natural compound (Pfaltz et al. 1982)]

this process is not yet clear.

Nickel has also been found to play a role in the oxidation of carbon monoxide by anaerobic bacteria (Drake et al. 1980). This carbon monoxide dehydrogenase is different from the bacopterin containing enzymes found in aerobic bacteria and contains 1 Zn, 2 Ni, 11 Fe and 14 S atoms. The involvement of nickel in the reaction has been deduced from the unusual ESR spectrum, which indicates an iron-nickel-carbon complex (Ragdale et al. 1985).

An unusual cofactor has been found to be involved in the hydroxylation of phenylalanine to tyrosine (Kaufman 1958). This cofactor was found to be identical with
biopterin (Fig. 5), a pteridine occurring in the eyes of Drosophila flies (Kaufman 1962), and whose structure was elucidated a few years earlier by Forrest and Mitchell (1955).

Concerning the terminology employed to indicate the substances described above, the literature seems to be confusing as the terms cofactor, coenzyme and prosthetic group are often used as synonyms. Many authors consider a prosthetic group to be a cofactor that remains attached to a protein during dialysis (Karlson 1967, Lehninger 1975, Stryer 1981). Less clear is the difference between cofactor and coenzyme. If these terms must be distinguished, this should preferably be based on function. Thus a cofactor, although participating in the redox reaction, is only required in catalytic amounts since it is regenerated in the active site, whereas a coenzyme is consumed in stoichiometric amounts and can therefore also be considered as a cosubstrate (Karlson 1967, White et al. 1973), especially when its Km-value is comparable to that of substrates.

Discovery of PQQ

The first indication for the possible existence of a cofactor other than NAD(P)+ or flavin emerged in 1953 when Wood and Schwerdt (1953) studied the oxidation of glucose by Pseudomonas fluorescens. Glucose oxidation by extracts of this organism did not require the presence of NAD or NADP. However, no formazan formation was observed upon addition of tetrazolium salts. This was considered, at that time, to be an indication of the absence of flavins in an oxidoreductase. The appearance of an absorption peak at 565 nm in the extract after the addition of glucose pointed to the involvement of cytochrome b. Considering the substantial difference in redox potential between cytochrome b (reported to be -40 mV, which is much lower than the value nowadays expected for such a cytochrome b) and glucose-gluconate (-440 mV), the authors postulated the presence of another electron carrier with an intermediate redox potential. Dalby and Blackwood (1955), working with a cell-free extract of Aerobacter aerogenes, made similar observations and, in addition, they found that upon treatment with EDTA followed by dialysis, the activity was completely lost. Mg2+ and Ca2+ ions were able to restore most, but not all, of the activity. More specific indications of a novel cofactor were reported by Szymona and Douderoff (1960). The glucose (aldose) dehydrogenase they isolated from Rhodopseudomonas spheroides was shown to be dependent on the presence of Mg2+-ions and an unidentified cofactor present in boiled cell extract. This heat-stable substance could be removed by anion exchange resins, indicating that it was an acid, and it could not be replaced by a variety of inorganic ions or by the common coenzymes and cofactors. Likewise, Hauge noted that acid treatment of glucose dehydrogenase from Bacterium anitratum reduced its activity by 90 % and NAD and NADP failed to reactivate the enzyme (Hauge, 1961). Two years later it was found that complete reactivation of apoglucose dehydrogenase occurred when a boiled or a neutralized perchloric acid extract of the holoenzyme was used (Hauge 1963). Hauge also observed that acid extraction of the enzyme caused the disappearance of the absorption band at 337 nm (the absorbance maximum found for the enzyme in the presence of glucose). In a subsequent publication, the relationship between the extracted substance and the enzyme absorption band was established by showing that upon reduction the same spectral changes occurred in both, although the absorption maxima of the reduced and oxidized substance exhibited a blue-shift of some 20–30 nm compared to the corresponding maxima of the enzyme (Hauge 1964). Additional studies on the nature of the cofactor confirmed that pyridine nucleotides were not involved. Growing the organism in the presence of 32P-labelled phosphate led to the incorporation of only 2% of the expected amount. Also very low carbon
incorporation was obtained when $^{14}$C-labelled niacin and a niacin-negative mutant were used (Hauge and Muerer, 1963, Hauge and Muerer, 1964). The first evidence for the cofactor isolated from different organisms was indeed one and the same substance was obtained by Niederpruem and Doudoroff (1965) who showed that a boiled extract of Bacterium anitratum (provided by Hauge) was able to reconstitute the activity of glucose dehydrogenase apoenzyme isolated from Rhodopseudomonas spheroides. Aware of this result, Hauge (1964) speculated that all of the particle-bound glucose dehydrogenases described so far were of the same type, and that other microorganisms might be better suited for obtaining the large amounts of cofactor required for the resolution of its structure. At this point research on the cofactor of glucose dehydrogenase was apparently stopped, since no reports on this topic appeared until 1979.

However, as we now know, research on the nature of the cofactor continued in a quite different field. Anthony and Zatman reported in 1964 on the properties of an unusual dye-linked alcohol dehydrogenase, which had been isolated from Pseudomonas M27, and which oxidized methanol as rapidly as ethanol (Anthony and Zatman 1964). Its spectrum did not show any evidence for the presence of cytochrome, haem or a cobalamin derivative, and enzyme activity was completely independent of the addition of nicotinamide nucleotides. The chromophore of this alcohol dehydrogenase could be liberated by boiling or treatment with acid and, concomitant with the loss of enzymatic activity, the appearance of a greenish fluorescent substance was observed (Anthony and Zatman 1967). Several observations pointed to the unusual nature of the prosthetic group. It was not able to replace riboflavin in a vitamin assay and its absorption and fluorescence spectra did not resemble any of the commonly known coenzymes involved in redox reactions. Unfortunately Anthony and Zatman did not succeed in preparing a reconstitutable apo-alcohol dehydrogenase. This would have been of great help in monitoring purification schemes of the cofactor or testing alternative substances.

Based on its spectral properties and the fact that folates are involved in methyl group transfer, the authors concluded that the prosthetic group was likely to be a pteridine derivative. Anthony and Zatman, as well as other groups working on this type of alcohol dehydrogenase, were not aware of the results obtained with the cofactor of glucose dehydrogenase (C. Anthony and H.S. Forrest, personal communication) and this remained so until the late seventies.

Elucidation of the structure

Although, as already mentioned, the initial characterization of the new cofactor was carried out on material obtained from glucose dehydrogenase, this work was never completed and the final elucidation of the structure took place with material isolated from methylotrophic bacteria.

The absorption maxima of the cofactor at 248 and 330 nm and the changes occurring upon titration, indicating $pK$ values of 1.7 and 7.4, suggested to Hauge that it might be a naphthoquinone substituted with carboxyl groups. Although this would account for its solubility in water, the high value for the absorption coefficient at 330 nm ($13600 \text{ M}^{-1}.\text{cm}^{-1}$, Hauge 1964) compared to maximally 3000 $\text{M}^{-1}.\text{cm}^{-1}$ for the known naphthoquinone derivatives) remained difficult to reconcile with this hypothesis. Interestingly, based on the adsorption behaviour on Sephadex G25, Hauge considered also the possibility of a nitrogen-containing heterocyclic molecule. However, although spectral changes occurred around pH 2 which could be attributed to protonation of a nitrogen atom, no absorption to a cation-exchange resin was observed, even at pH 1.5.

The original proposal by Anthony and Zatman of a pteridine structure attracted the attention of Forrest and coworkers and the

![Fig. 6. Neopterine 2':3'-phosphate, postulated to be the cofactor of methanol dehydrogenase (Urushibara 1971)](image)

Fig. 6. Neopterine 2':3'-phosphate, postulated to be the cofactor of methanol dehydrogenase (Urushibara 1971)
group at Delft, which had both access to expertise in the field of pteridine chemistry. Accordingly, the group of Forrest (who had previously discovered biopterin) studied the pteridines produced by *Methylococcus capsulatus*. Amongst others they identified neopterin-2':3'-phosphate (Fig. 6) in this organism, and also tentatively identified this cyclic phosphate in a very pure preparation of methanol dehydrogenase from a *Pseudomonas* sp (Urushibara et al. 1971). However, evidence was presented in a later paper in favour of the involvement of an unknown lumazine derivative (Sperl 1973a, Sperl 1973b, Sperl et al. 1974, Fig. 7). The pteridine hypothesis was once more reinforced by the observation of the appearance of a fluorescent, pteridine like pigment in the culture medium when *Pseudomonas* J26 was transferred from citrate to methanol. The fluorescence spectra of this pigment were identical with those of the fluorescent substance which could be extracted from methanol dehydrogenase (Budohoski et al. 1978).

The peculiar properties of methanol dehydrogenase and the presumed pteridine-like nature of its fluorescent chromophore, encouraged us to initiate studies on the nature of this substance using methanol dehydrogenase purified from *Hyphomicrobium X*, in order to gain more insight into this enzyme. Failure to observe the formation of 2-amino-6-carboxy-4-hydroxypteridine when the fluorescent substance was oxidized with alkaline potassium permanganate, as would be expected for a pteridine, led us to consider alternative hypotheses. An important clue was obtained when, while using ESR-spectroscopy to look for paramagnetic metals in the enzyme, the signal of an organic free radical was detected. The g-value and line width of this signal indicated the presence of a quinone radical (chapter II). It was subsequently shown that the fluorescent compound readily oxidized reduced methylene blue, indicating the high redox potential

### TABLE 1

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<th>Mass (m/e)</th>
<th>Δ (ppm)</th>
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<td><strong>PQQ-Trimethylester</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>374, 0750</td>
<td>0.00044</td>
<td>C₁₂H₁₀N₁₄O₁₉</td>
</tr>
<tr>
<td></td>
<td>0.00313</td>
<td>C₁₄H₁₄N₈O₁₁</td>
</tr>
<tr>
<td></td>
<td>0.00005</td>
<td>C₁₇H₁₄N₂O₈</td>
</tr>
<tr>
<td>372, 0607</td>
<td>0.00180</td>
<td>C₈H₁₂N₈O₁₁</td>
</tr>
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<td></td>
<td>0.00137</td>
<td>C₁₇H₁₂N₂O₈</td>
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<td></td>
<td>0.00034</td>
<td>C₃H₁₆N₄O₁₅</td>
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<td>342, 0472</td>
<td>0.00252</td>
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<td></td>
<td>0.00138</td>
<td>C₁₆H₁₀N₄O₇</td>
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<td></td>
<td>0.00667</td>
<td>C₆H₁₀N₆O₁₁</td>
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<td><strong>PQQH₄-Trimethylester</strong></td>
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</tr>
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<td>0.000402</td>
<td>C₁₂H₁₀N₁₄O₉</td>
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<td></td>
<td>0.000078</td>
<td>C₁₇H₁₆N₂O₈</td>
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</table>
expected for a quinone. A reaction was also observed with 3,4-dimethoxyaniline, which is claimed to be specific for inner-ring orthoquinones (Sawicki and Elbert 1960). By reducing the isolated chromophore a free radical could be generated with the same ESR properties as the free radical in methanol dehydrogenase, thus proving the cofactor identity of the fluorescent compound. In addition, an ESR spectrum displaying hyperfine structure was obtained.

Interpretation of this spectrum indicated that it originated from a quinone containing two nitrogen atoms and three hydrogen atoms (Chapter IV). High resolution mass spectroscopy of the trimethyl ester of the cofactor and of a tetrahydro derivative enabled us to establish the elemental composition of the trimethyl ester as C$_7$H$_{12}$N$_2$O$_6$ (Duine, Frank and Jongejan 1987, Table I). After subtracting the methyl hydrogens, the three hydrogen and two nitrogen atoms which were found with ESR remained. Together with the $^1$H-NMR data obtained for the cofactor and its derivatives, and the finding that the quinone group was of the ortho inner-ring type (Chapter III), the number of possible structures was now considerably reduced (Chapter V). Meanwhile, Forrest and coworkers crystallized what they called "a degradation product" of what they presumed to be the cofactor. This compound was obtained by treating an acid extract of wet cell paste of Pseudomonas TPI with acetone (Forrest et al. 1981). The structure of the product was resolved by X-ray diffraction analysis and found to be a heterocyclic quinone derivative instead of the expected pteridine (Salisbury et al. 1979, Fig. 8). Arguments for a clear relationship between this compound and the cofactor were not provided by these authors, although they could have been aware of previous reports on the quinone nature of the isolated cofactor and the prosthetic group in methanol dehydrogenase (Duine et al. 1977, Westerling et al. 1979). Nevertheless, the quinone structure proposed for the cofactor itself was consistent with all our data (Fig. 8). Meanwhile, we had investigated other enzymes for the presence of the cofactor. In the absence of a suitable apo-methanol dehydrogenase, the physiological activity of the cofactor extracted from methanol dehydrogenase was shown by its ability to reconstitute the apo-glucose dehydrogenase from Acinetobacter calcoaceticus (Duine et al. 1979). Independently, a Japanese research group also demonstrated that the cofactors of the two dehydrogenases were identical (Imanaga et al. 1979).

The trivial name of the new cofactor has been the subject of controversy (Forrest 1981, Duine 1981, Anthony 1981). The group of Forrest proposed methoxatin in view of the methylotrophic origin of the cofactor. However, our demonstration of its occurrence in non-methylotrophic bacteria as well,

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\begin{figure}[h]
\centering
\includegraphics[width=0.8\textwidth]{fig8.png}
\caption{2,7,9-tricarboxy-1N-pyrrolo[2,3-f]-quinoline-4,5-dione (a) and its acetone adduct (b).}
\end{figure}
```
prompted us to propose the more descriptive semi-systematic name pyrroloquinoline quinone, which can be abbreviated to PQQ in conformity with commonly employed abbreviations such as FMN, FAO, NAD etc. In analogy with flavoproteins, the associated PQQ-dependent enzymes can be designated as quinoproteins. Nowadays, it seems that this latter nomenclature is becoming accepted among biochemists.

Isolation and production

Although much of the work on the elucidation of the structure of PQQ has been performed with material extracted from purified enzyme, this is, for obvious reasons, not the most suitable source for the preparation of large quantities of the cofactor. This also appears to apply to the use of whole cells. Forrest isolated the acetone derivative of PQQ with a yield 5 mg per kilogram of wet Pseudomonas TP1 cells (Forrest et al. 1981). Dried cells of Methylophilus methylotrophus, produced by ICI (Billingham, UK) and sold as single cell protein (Pruteen), is an even poorer source, since only 32 microgram of PQQ can be extracted from one kilogram. The best biological source for PQQ is, beyond any doubt, the culture fluid of certain microorganisms, especially methanol-grown Hyphomicrobium X and Pseudomonas species, as they produce up to 2 mg of PQQ per liter (Duine et al. 1985, Ameyama et al. 1984). Progress in the early days of our research on PQQ owed much to the harvesting of 1.36 grams of PQQ from 1100 liters of culture supernatant of Hyphomicrobium X in 1980 (unpublished data). Processing was performed using methods developed previously for the large scale extraction of Pruteen (Chapter III). The use of the macroporous ion exchange resin Amberlyst A-21 is crucial in such large scale operations since (i) it has a large capacity for PQQ (ii) it can easily be recovered from large volumes of culture fluids and (iii) the methanol-containing solvents necessary for adequate elution can be used without problems, permitting elution in a small volume.

In spite of the ease with which PQQ can be produced by microbial fermentation, chemical synthesis has so far been used for producing large amounts of it. Surprisingly soon after the elucidation of the structure of PQQ, its total synthesis in 10 steps and an overall yield of 10-15% was reported (Corey and Tramontano 1981) and the product commercialized by FLUKA AG, Buchs, Switzerland is actually made in this way. Several alternative syntheses were subsequently reported. These varied widely in approach, complexity and yield (Gainor and Weinreb 1981, Hendrickson and de Vries 1982, Mackenzie et al. 1983, 1986, Buchi et al. 1985) and offered interesting possibilities for the preparation of PQQ analogues and derivatives (Itoh et al. 1987). PQQ is also commercially produced by fermentation and sold by Ube Industries, Ltd, and Mitsubishi Chemical Industries in Japan.

Physical properties

The sodium salt of PQQ crystallizes from NaCl containing solutions as tiny, brick-red needles. At pH 7, the solubility of the sodium salt in water is at least 20 g/l (20 °C).

The absorption spectrum at pH 7 is characterized by maxima at 249, 325 and 475 nm (ε = 22496, 9898 and 742 M⁻¹.cm⁻¹, respectively at 20 °C). The latter, low intensity band originates from a forbidden n → π* transition of the lone electron pair on the quinone carbonyls. α-diketons usually exhibit a second absorption band of this type at shorter wavelengths, but this is probably masked in PQQ by the two π → π* transitions (Chapters III and V). The molar absorption coefficient determined recently (Duine et al. 1987) is higher than that reported in Chapter V, the inaccuracy being due to the small amount of PQQ available at that time. pH-induced changes in the absorption spectrum (Chapter III) are related to (i) pseudobase formation (apparent pKₐ approximately 10, Sleath et al. 1985, Rodriguez et al. 1987), and (ii) deprotonation of the three carboxyl groups and protonation of the pyridine nitrogen. Comparison with the 7,9-didecarboxy- and 9-decarboxy analogues of PQQ (Sleath et al. 1985, Noar et al. 1985) shows that the carboxyl group at C₇ should have a pKₐ between 2 and 3 and the carboxyl at C₇ a pKₐ below -2. A pKₐ = -1.6 was found
for protonation of the pyridine-N in 9-decarboxy-PQQ. Between pH 2 and 3 two protons are titrated in PQQ, indicating that the pKₐ of the 9-carboxyl group is also between 2 and 3 (J.A. Jongejan, unpublished data).

Solutions of PQQ are fluorescent between pH 1 and 11. At pH 7 the fluorescence excitation spectrum differs significantly from the absorbance spectrum. This is caused by the fact that PQQ in water is actually a mixture of PQQ and its hydrate (PQQ·H₂O) at the C₂-carbonyl, and only the latter compound is fluorescent (Chapter VIII). The absorption spectrum on the other hand, is the sum of the contributions of PQQ·H₂O and PQQ. Lowering the temperature results in a shift of the hydration equilibrium toward the hydrate, the absorption spectrum changes accordingly and the intensity of the fluorescence increases. Dehydration is observed in the presence of sodium dodecyl sulphate (Chapter IX) or at high ionic strength.

The most salient feature of the ¹H-NMR spectra of PQQ and derivatives is the low field position of the pyrrolo NH signal, usually found between 10 and 16 ppm (Chapters V, VI and X), caused by the strong interaction between this hydrogen and the carbonyl group at C₆. The C₃-H signal, a doublet due to coupling with the N₂-H, appears at approximately 7.4 ppm. The sharp singlet signal of C₆-H is generally found at 8.6 ppm. ¹³C-NMR data of PQQ were obtained as early as 1981 (Chapter VI), but most of the signals could be assigned only recently when 99 % enriched ¹³C-PQQ was investigated (Chapter X). Signals were found at δ 113.9 (C₁), 122.9 (C₃s), 126.1 (C₈s), 127.7 (C₂), 130.7 (C₄s), 137.7 (C₆s), 144.6 (C₉s), 146.4 (C₇), 147.2 (C₇s), 152.1 (C₂-COOH), 156.7 (C₇-COOH), 166.6 (C₈-COOH), 173.4 (C₄) and 179.9 ppm (C₅).

The high redox potential of PQQ, as predicted by its ability to oxidize reduced methylene blue, was confirmed by analyzing the potentiometric titration curves obtained at pH 2.0 (Em = +419 mV) and pH 7.0 (Em = +90 mV) (Chapter VI). With cyclic voltammetry at pH 2.98 and pH 5.60, midpoint potentials of +340 and +150 mV, respectively, were found (Eckert et al. 1982). After extrapolation (60 mV/pH) these values become +400 and +66 mV at pH 2.0 and 7.0, respectively, and are in reasonable agreement with our values. At pH 13.0 the intermediate formation of the semiquinone PQQ⁺ led to an aberrant titration curve and redox potentials of -218 and -242 mV at this pH could be derived for the PQQ/PQQ⁺ and PQQ⁺/PQQH₂ couples, respectively (Chapter VI). Recently, based on pulse radiolysis experiments, an Em = -114 mV was determined for the PQQ/PQQ⁺ couple at pH 7.3 (Faraggi et al. 1986). The difference in the midpoint potentials obtained at these pH-values can only be explained on the basis of a pKₐ between 8 and 9 for the phenolic -OH of the semiquinone. This would be in agreement with a pKₐ = 8.54 measured for the phenolic -OH of 7.9-decarboxy-PQQH₂ (Sleath et al. 1985), a pKₐ = 9.31 for 9-decarboxy-PQQ (Rodriguez et al. 1987) and pKₐ = 9.0 for PQQH₂ (Itoh et al. 1985).

Some features of the absorption spectrum of PQQ⁺ were obtained by mixing equal amounts of PQQ and PQQH₂ at pH 12.0. Absorption maxima, not observed for either PQQ or PQQH₂, appeared at 340, 458 and 600 (broad) nm in the presence of lithium salts. When sodium or potassium salts were used, a rapid decrease in the absorption at 458 nm, a concomitant decrease in the absorbance at 600 nm and the formation of a dark green precipitate were observed. This is ascribed to the formation of a less soluble sandwich complex between PQQ⁺ and the cation (Chapter VI). Similar results were obtained with pulse radiolysis at pH 7.3 (Faraggi et al. 1986).

Chemical reactivity

The early observation that PQQ is resistant to concentrated H₂SO₄, I M NaOH and irradiation with ultraviolet light, pointed to the remarkable stability of the molecule compared to labile coenzymes such as NAD⁺ and flavines. Yet PQQ has considerable reactivity, residing, as expected, mainly in the quinone carbonyls. The C₂-carbonyl is the target of nucleophilic agents resulting in the formation of more or less stable adducts (Chapters III and VII).

PQQ is easily reduced by a variety of reducing agents such as hydrazines, NaBH₄, dithiothreitol and β-mercaptoethanol (Chapter VI, Itoh et al. 1986). The absorption spectrum of the non-fluorescent, reduced form of PQQ (PQQH₂) is characterized by a maximum at 302 nm (pH 7.0, ε = 30484 M⁻¹cm⁻¹), which shifts to 317 nm at pH 9.5 and 315 nm at pH 1.5 (Chapter VI, unpublished results).
Autoxidation of PQOH₂ readily occurs in water containing solvents at pH values above 4. Only 50% of the expected amount of H₂O₂ was found after completion of the reaction, and it was shown that H₂O₂ reacts more rapidly with PQOH₂ than O₂. The increase in reaction rate with increasing pH suggests that PQOH⁻, a deprotonated form of PQOH₂, is the reactive species (Itoh et al. 1986b).

PQQ is reduced by several primary amines (Eckert et al. 1982). In the presence of oxygen an efficient conversion to the corresponding aldehydes, NH₃ and H₂O₂ can be achieved with catalytic amounts of PQQ (Ohshiro et al. 1983, Itoh et al. 1984). PQQ is progressively lost in the reaction, however, due to formation of oxazoles and other non-identified products (Scheme 2).

A more drastic attack, resulting in ring opening is also possible. Reduction of PQQ with NaBH₄ in the presence of oxygen leads to PQH₂ (Chapter V) most probably following the mechanism described by Platt and Oesch (1982) for the reduction of polycyclic orthoquinones (Scheme 1). Upon oxidation of the vicinal diol group with NaIO₄, a highly fluorescent compound in which the C₄-C₅ bond is broken is formed (Scheme 1). A comparable product is formed when PQQ is treated with hydrogen peroxide (O. Ghisalba, personal communication). Some indications have been obtained that the pyrrole ring can be opened by treatment with a mixture of HNO₃ and H₂SO₄, but thus far no reactions are known in which the pyridine ring is degraded, possibly because this ring is stabilized by the two carboxyl groups.

**Qualitative and quantitative analysis**

During the initial studies on the new cofactor two strategies were developed for its detection and determination (Chapters III and VII). Due to the polar character of PQQ, reversed phase chromatography on octadecyl silica is the HPLC method of choice. Retention on such columns is achieved by ion-suppression, using eluants (mixtures of water and organic solvents, so-called organic modifiers) acidified to pH 2 with phosphoric acid or trifluoroacetic acid. The peaks of PQQ and some of its derivatives are rather broad, most probably because of the difference in retention of the reversible
adducts that are formed with the components of the eluant. In an attempt to optimize the peak shape of PQQ, acetonitrile, tetrahydrofuran, dioxan, ethanol and methanol were tested as organic modifiers. Methanol was the most satisfactory. An alternative approach to achieve retention is ion-pairing with a hydrophobic counter-ion. Using tetrabutylammonium salts, retention was obtained at pH values between 3 and 7 and significantly sharper peaks were observed. No further improvement was obtained by replacing tetrabutylammonium with tetramethylammonium salts or cetrimide (Chapter VII). Although an excellent analytical tool, ion-pairing chromatography is less convenient for semi-preparative purposes, due to the inherent difficulties in removing the ion-pair reagent.

Sensitive detection of PQQ and derivatives is possible using ultraviolet absorption spectrophotometry or, when possible, by fluorescence detection which is generally more sensitive and selective. Particularly informative is HPLC coupled to photodiode array detection, permitting identification and additional resolution, with chemometric techniques such as multi-component analysis and curve resolution (e.g. Chapter IX). Identification of PQQ can also be achieved by reaction of the samples with acetone (Chapter III), butyraldehyde or NaBH₄/NaIO₄ (Chapter VII) which results in products with characteristic retention and fluorescence properties.

Particular sensitive is the biological assay developed using the glucose dehydrogenase apoenzyme from Pseudomonas aeruginosa (Chapter VII) and Acinetobacter calcoaceticus (Duine et al. 1979) or with PQQ-mutants of Acinetobacter calcoaceticus (van Kleef et al. 1987). In addition, many microorganisms gratuitously produce apoenzymes (Duine et al. 1986), Pseudomonas testosterone being a convenient source of alcohol dehydrogenase apoenzyme which permits the accurate determination of 0.05 pmol PQQ (Groen et al. 1986).

Calibration of solutions of pure PQQ is most conveniently done by spectrophotometry at pH 7.0, using the molar absorption coefficients determined for the isobestic points observed in the hydration equilibrium, i.e. 15896 M⁻¹.cm⁻¹ at 236 nm, 19122 M⁻¹.cm⁻¹ at 257 nm and 9620 M⁻¹.cm⁻¹ at 342 nm (Duine et al. 1987).

Sample preparation is of crucial importance in both qualitative and quantitative analysis. Incomplete extraction of PQQ from methanol dehydrogenase with acid methanol has been sometimes observed, while more reliable results were obtained with 1% sodium dodecylsulphate at pH 7.0 (Chapter IX, unpublished results). Contamination with PQQ becomes particularly problematic in conjunction with the very sensitive apoenzyme test. To avoid this problem, heating of the glassware at 500 °C for 1 hour, the use of fresh column material and treatment of solutions with Amberlyst A21 ion exchanger is recommended in critical applications (van Kleef et al. 1987).

Determination of covalently bound PQQ poses special problems. Proteolytic degradation of enzymes in order to liberate PQQ, also generates large amounts of amino acids which react with PQQ giving a large number of products. Protection of PQQ in these cases has been successfully achieved by derivatization in situ with hydrazines (Lobenstein-Verbeek et al. 1984). Other approaches to obtain some indication for the presence of covalently bound PQQ are based on Resonance Raman spectroscopy of the intact enzyme after reaction with phylopyridazine (Moog et al. 1986, Williamson et al. 1986) and fluorescence spectroscopy after complete hydrolysis (Ameyama et al. 1985b, 1986).

Occurrence

Although the number of quinoproteins is still far from being comparable to that of the flavoproteins and NAD(P)-dependent enzymes, their diversity and occurrence are not less interesting. At present, a large number of alcohol and aldose dehydrogenases isolated from prokaryotes is known (for a complete list the reader is referred to recent reviews (Duine et al. 1986, Duine et al. 1987)).

Methylamine dehydrogenase from Pseudomonas AM1 was the first enzyme for which evidence was obtained that it contains covalently bound PQQ (de Beer et al. 1980). Only recently it could be definitely proven that the cofactor is indeed covalently bound PQQ (van der Meer et al. 1987) and not a decarboxylated derivative as proposed by McIntire (1986).
Most interestingly, the occurrence of PQQ is not restricted to prokaryotes. In several amine oxidases of eukaryotic origin, including a lysyl oxidase isolated from human placenta, PQQ has been detected (van der Meer et al. 1986, van der Meer and Duine 1986).

Other substrates which are presumably oxidized by quinoproteins are lactate (Duine and Frank 1981), tryptophane, aldehydes, nitroalkanes (Duine et al. 1987 and references therein) and choline (Ameyama et al. 1985b). The quinoprotein nature of the enzymes oxidizing polyethylene glycol (Kawai et al. 1985), glycerol (Ameyama et al. 1985a), polyvinyl alcohol (Shimao et al. 1986) and quinic acid (M.A.G. van Kleef, unpublished results) has now been established.

There are indications that PQQ is not only involved in oxidations, but also in hydratations since it has been suggested that the nitrile hydratases from Pseudomonas chlorographis B23 and Brevibacterium R312, catalyzing the hydration of nitriles to the corresponding amides (Nagawasa and Yamada 1987) contain PQQ.

PQQ has also been found to be the prosthetic group of the enzyme dopamine-ß-hydroxylase which hydroxylates dopamine to the neurotransmitter noradrenaline (R. van der Meer, unpublished data).

Several microorganisms synthesize, either constitutively or under certain growth conditions, the apoenzyme of glucose dehydrogenase (van Schie et al. 1984, Neijssel et al. 1983, Hommes et al. 1984). In addition it has been shown that PQQ is essential for the growth of a bacterial symbiont on polyvinyl alcohol (Shimao et al. 1984). A Pseudomonas sp. produces the apoenzyme of polyvinyl alcohol dehydrogenase, while the PQQ required for its activity is produced by Pseudomonas putida VM15A.

Likewise, the growth rate of Pseudomonas testosterone on butanol is strongly enhanced by the addition of PQQ to the culture medium (Groen et al. 1986).
Mechanism of action

The spontaneous reduction of PQQ by amines and certain amino acids has not only been observed in our laboratory (J. Frank, unpublished results), but also attracted attention from other groups for use as a possible model system when it appeared that copper-containing amine oxidases are quinoproteins. Cationic micelles were found to significantly enhance the reaction rate and efficient oxidation of cyclohexyl amine was thus achieved (Ohshiro et al. 1983). Kinetic investigations into the oxidation of amines by the related phenanthrolinequinones and the 7,9- and 9-decarboxy analogues of PQQ revealed that an electron transfer mechanism is unlikely since primary amines are much better substrates than secondary and tertiary amines. In view of the relatively low redox potential of PQQ compared to some high potential quinones, hydride transfer also seems unlikely. In fact, such a mechanism could be excluded because of the observed general base catalysis. Thus it was proposed that the reaction proceeds via the covalent addition of the amine to the quinone, followed by general base catalyzed α-proton removal (Eckert and Bruice 1983, Scheme 2). In contrast to the group of Bruce (Sleath et al. 1985), Ohshiro and coworkers (Itoh et al. 1986a), used micellar systems and they found a large deuterium isotope effect with deuterated amines for this reaction, although they agreed upon the α-proton abstraction mechanism. Likewise, a comparable isotope effect was observed in the oxidation of deuterated methylamine by methylamine dehydrogenase (J.E. van Wielink, unpublished results), indicating that α-proton removal in the latter two cases is the rate-limiting step. Apparently, both the active site and the micelle environment favour the formation of the intermediate carbaminol (Scheme 2) to the point that this energetically unfavorable step is no longer rate limiting, possibly by stabilizing the twisted PQQ molecule.

A proton abstraction mechanism, based on the observation of a rate enhancement by electron withdrawing substituents, has been proposed for the oxidation of amines by bovine plasma amine oxidase (Hartmann and Klinman 1986). Release of benzaldehyde was found to precede that of NH₃, the latter occurring concomitantly with the reoxidation of the enzyme (Ruis et al. 1984), suggesting the formation of a covalent enzyme-substrate complex. Evidence for the formation of a Schiff base intermediate during the oxidation of benzylamine by bovine plasma amine oxidase has been reported recently (Hartmann and Klinman 1987).

Although in methylamine dehydrogenase the presence of PQQ semiquinone has been demonstrated (de Beer et al. 1980), its formation upon reduction of oxidized enzyme with methylamine has not been observed (Kenney and McIntire 1983, Husain et al. 1987). It is unlikely, therefore, that the semiquinone is an intermediate in the oxidation of methylamine, and thus it is conceivable that in the enzyme a similar α-proton abstraction mechanism in the oxidation of amine substrates is operative, as described above. In this respect it may not be fortuitous that quinoprotein amine dehydrogenases catalyze almost exclusively the oxidation of primary amines.

Oxidation of alcohols by PQQ does not occur spontaneously, and is not accelerated by ammonia (unpublished results), an activator in the methanol dehydrogenase mediated oxidation of alcohols. High oxidation rates could be achieved in the presence of aluminum t-butoxide or aluminum chloride (Itoh et al. 1985a), and on the basis of those results it was postulated that in the enzyme the reaction proceeds by concerted general acid-base catalysis (Scheme 3). More insight into the mechanism of action

![Scheme 3](image-url)
of methanol dehydrogenase is obtained from studies of its kinetic and spectral properties and the products formed during inhibition. Abeles and coworkers have shown that cyclopropanol behaves as an irreversible inhibitor for this enzyme (Mincey et al. 1981). The inhibition was found to be due to modification of the prosthetic group (Chapter IX) and the structure of the PQQ derivative could be established (Chapter X, Scheme 4). Since the absorption spectrum of the cyclopropanol-inactivated enzyme is almost identical with that of methanol dehydrogenase which has been oxidized in the presence of potassium cyanide (MDHox2, Dutine and Frank 1980), it is plausible that in the latter enzyme form PQQ is present as the C₅-HCN adduct (Scheme 4). During the reaction of methanol dehydrogenase with excess electron acceptor, in the presence of methanol, the formation of a transient enzyme species was observed. Most interestingly this species had the same spectrum as the cyclopropanol-inactivated enzyme (Frank et al. 1988). The spectral decay of the intermediate followed first order kinetics and it could be shown that formaldehyde was produced concomitantly. This is consistent with the concept that the intermediate enzyme form is the oxidized enzyme-substrate complex containing the C₅-methanol adduct of PQQ, in which methanol is oxidized by PQQ in a similar way as are amines (Scheme 4). In the absence of ammonium salts (which act as activators for this enzyme) the α-proton abstraction is very slow \( k = 0.06 \text{ s}^{-1} \) and characterized by a large isotope effect. Addition of activator relieves this bottleneck and a \( k_{cat} = 23 \text{ s}^{-1} \) is observed. Glucose oxidation by the glucose dehydrogenase from *Acinetobacter calcoaceticus* has also been found to exhibit an deuterium isotope effect (Hauge 1968) so that the same mechanism might be operative.

The occurrence of stable PQH⁺ in both quinoprotein amine- and alcohol dehydrogenases reflects the suitability of these enzymes to function as one-electron donors, channelling the two electrons abstracted from the substrate one at a time into the respiratory chain via the one-electron acceptors amicyanine and/or cytochromes. Thus far the presence of PQH⁺ has not been demonstrated in quinoprotein glucose dehydrogenase. However, since the enzyme is active with the one-electron acceptors cytochrome b and Wurster’s blue, it can be anticipated that here too a semiquinone intermediate must play a role.
Bioenergetics

Quinoprotein dehydrogenases donate the electrons abstracted from their substrates to the respiratory chain, hence part of the energy contained in the substrates is conserved. In view of the high redox potential of PQ, it can be anticipated that quinoprotein dehydrogenases are coupled to the respiratory chain at a relatively high level of redox potential. Indeed, it has been found that ferripyrroloquinoline cytochrome c₅₅₅ is an electron acceptor for methanol dehydrogenase in vivo (Quine et al. 1979, Anthony 1982) although it has been established only recently that, at least in vitro, the reduced form of methanol dehydrogenase (MDHred) is rapidly oxidized by its physiological electron acceptor cytochrome c₁ to the semiquinone form (MDHsem, Dijkstra et al. 1988). Similarly, methylamine dehydrogenase (E₅₅₅ = +100 mV, Husain et al. 1987) has been shown to be coupled to cytochrome c via the copper containing amicyanin (Tobari and Harada 1981). Finally, one of the quinoprotein glucose dehydrogenases from Acinetobacter calcoaceticus (E₅₅₅ = +50 mV) is linked to the respiratory chain via a specific cytochrome b which is different from the membrane bound cytochromes b (Dokter 1987).

Perspective

As already mentioned, the large variety of substrate redox reactions is, in fact, based on a small number of elementary reactions requiring only a few cofactors. Hence, the flexibility to cope with the different substrates resides mainly in the protein part of the oxidoreductases. Nevertheless, diversity among the cofactors is also seen. For example, for the oxidation of glucose, flavoproteins, NAD(P)+-dependent enzymes and quinoproteins are all found in nature. Sometimes two types of enzyme even occur simultaneously in the same organism, exemplified by the presence of both NAD+-dependent and quinoprotein alcohol dehydrogenases in Pseudomonas aeruginosa (Groeneveld et al. 1984).

Some arguments for the use of both NAD(P)+ and flavin-dependent enzymes may be derived from the following facts. NAD(P)H, in contrast to reduced flavins, is not oxidized by oxygen. Thus reducing equivalents obtained from dehydrogenation reactions in the cytoplasm can be easily transferred to the respiratory chain. On the other hand, flavins not only have a higher redox potential than the pyridine nucleotides (E₅₅₅ = -219 versus -320 mV), which in principle extends the range of substrates that can be oxidized, but more importantly, they can be oxidized in one-electron steps facilitating electron transfer to the one-electron accepting components in the respiratory chain. Flavoproteins, therefore, are generally attached to the respiratory chain, while NAD(P)+-dependent dehydrogenases are found in the cytoplasm. The successful complementation of the two classes of enzymes is witnessed by the fact that they are involved in the vast majority of substrate redox reactions. Hence, the need for alternative cofactors does not seem obvious at first sight. In some respects, quinoprotein dehydrogenases resemble their flavoprotein counterparts. PQH₂ is easily converted to PQH⁺ by the natural electron acceptors of quinoproteins, and in the isolated form it is also rapidly oxidized by oxygen to PQ. So far, all quinoprotein dehydrogenases found in Gram-negative bacteria are located at the periplasmic side of the cytoplasmic membrane. The obvious question is now whether quinoproteins have a particular advantage over flavoproteins in certain circumstances, or whether they are simply satisfactorily functioning relics of ancient times. A clue in addressing this question might be the high redox potential of PQ compared to flavins. However, for the oxidation of succinate to fumarate, (a redox couple with a rather high redox potential (+30 mV)), FAD appears to function satisfactorily. Likewise, methylamine is oxidized by a quinoprotein, but dimethyl amine and trimethyl amine which have higher redox potentials, are oxidized by flavoproteins. Apparently, the redox potential of PQ has not been a decisive factor in its selection, the latter example rather suggests that mechanistic considerations play a more important role. Perhaps this is also one of the reasons that PQ has been conserved during evolution, and also occurs in mammalian systems. Here PQ not only functions as the prosthetic group of several oxidases, but might also have a regulatory function as its structure is closely related to potent inhibitors of
prolyl-4-hydroxylase (Hanauske-Abel 1987), although much research is still required to prove this.

Biosynthesis of PQQ in Acinetobacter calcoaceticus requires four genes (Goosen et al. 1987). It is not known whether similar genes are still present in mammals, or whether the need for PQQ in mammals requires uptake in the food of PQQ or a precursor-like vitamin (Jongejan et al. 1986), as is the case for several other cofactors. Clearly much research remains to be done on PQQ before these questions can be answered.

In view of the fact that PQQ was discovered only recently in mammalian enzymes, which have been studied very intensively and for such a long time, it is likely that with the methods of analysis now available new surprises will follow in the near future. If this is the case, PQQ may well prove to be a more general cofactor than is presently assumed.

References


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25
Chapter II

Purification and properties of methanol dehydrogenase from
*Hyphomicrobium X*

Summary

(1) A method for the isolation of methanol dehydrogenase (alcohol:(acceptor) oxidoreductase, EC 1.1.99.8) from *Hyphomicrobium X* is described. The purified enzyme was resolved by polyacrylamide gel electrophoresis into one main and two minor active bands. Iron and manganese were the only detected metals in the enzyme preparation.

(2) The substrate, methanol, was oxidized to formic acid by a stoichiometric amount of artificial electron acceptor. During the reaction, no free formaldehyde could be detected. Other primary alcohols were oxidized to the corresponding aldehydes and the aldehydes were a poor substrate or no substrate at all.

(3) Some new and efficient one-electron acceptors were found. With these electron acceptors, the enzyme had a high pH optimum and ammonia was still required in the assay system.

(4) ESR spectroscopy showed the presence of an enzyme-bound organic free radical. With X-band ESR the signal had a peak-to-peak linewidth of about 0.7 mT. The signal was further resolved by Q-band ESR and the values $g_s = 2.0024$ and $g_i = 2.0056$ were derived.

(5) Under denaturing conditions the ESR signal and enzymatic activity disappeared at the same time as fluorescence appeared. Enzymatic activity is not restored when extracted cofactor and apoenzyme are brought together under normal conditions. Some properties of the unusual prosthetic group are presented in a preliminary form.

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Abbreviations: tricine: N-[tris(hydroxymethyl)]-methyl]-glycine; DCIP: 2,6-dichlorophenol indophenol. Compounds I and II are defined under Materials.

Introduction

Methanol dehydrogenase (alcohol:(acceptor) oxidoreductase, EC 1.1.99.8), methylamine dehydrogenase [1] and trimethylamine dehydrogenase [2] are enzymes found in methylotrophic bacteria grown on the corresponding substrate. These key enzyme reactions provide one-carbon units for assimilation and also energy by oxidation of one-carbon substrates [3]. They are NAD(P)-independent enzymes and use phenazine methosulphate as the primary electron acceptor.

However, the nature of the coenzyme, the natural electron acceptor and the mechanism of action are not known. For methanol dehydrogenase, these interesting enzymological aspects may have wider importance as this enzyme is present in bacteria, which are under investigation for their use in the production of single-cell protein or metabolites [4–6]. For instance, Hyphomicrobiun (a facultative methylotrophic bacterium) is a component of a mixed culture growing on methane [7].

Anthony and Zatman [8] were the first to isolate a methanol dehydrogenase and to describe some properties of the enzyme from Pseudomonas M 27. The enzymatic activity of this primary alcohol dehydrogenase could only be detected in the presence of ammonia. The assay system was optimal at a pH of 9 while phenazine methosulphate was the only suitable acceptor. From the fluorescence properties of a substance obtained by denaturing the enzyme, methanol dehydrogenase was classified as a pteridine containing enzyme [9]. Based on this suggestion, mechanisms for catalysis were proposed [10].

In the past decade, several similar methanol dehydrogenase have been isolated from bacteria grown on methane or methanol [11–16]. Urushibara et al. [17] reported the prosthetic group to be neopterin cyclic-phosphate but, later, owing to the spectral properties of a photodegradation product, it was concluded to be a lumazine derivative [18].

In order to test the hypotheses about the prosthetic group, we developed a method for the large scale production of enzyme. As reconstitution was not obtained earlier, it was thought important to assess the purity of the enzyme and to detect the occurrence of low molecular weight compounds. Some new properties of the enzyme are reported and it is also compared with previously described preparations.

Materials and Methods

Materials

DCIP, phenazine methosulphate, $N,N,N',N'$-tetramethyl-p-phenylenediamine, tricine, silicagel type 60 (Merck), 2,2'-azino-di-[3-ethyl benzthiazoline-6-sulphonic acid] (Boehringer), phenazine ethosulphate, Triton X-100 (Sigma), hydroxyapatite type HTP (Biorad), DEAE-cellulose 23 (Whatman), cellulose F (Baker) and Porapak Q (Waters) were commercial products.

The one-electron acceptors compound I and compound II (free radicals) were made by one-electron oxidation of 2,2'-azino-di-[3-ethyl benzthiazoline-6-sulphonic acid] and $N,N,N',N'$-tetramethyl-p-phenylenediamine, respectively. For every mmol of 2,2'-azino-di-[3-ethyl benzthiazoline-6-sulphonic acid] or
\(N,N,N',N'-\text{tetramethyl-p-phenylenediamine}, \) half a mmol of aqueous Br\(_2\) was used. Compound II was isolated as the perchlorate salt according to Michaelis et al. [19]. Compound I was precipitated with acetone. For some purposes compound II was purified further by adsorption on CM-cellulose and elution with the desired buffer.

**Growth of the organism**

*Hyphomicrobium* X was a kind gift from Dr. W. Harder. Cells were grown with aeration in batch culture at 30°C on the medium used by Attwood and Harder [20], but with some modifications. The spore solution contained (per l): 7.8 mg CuSO\(_4\) \(\cdot\) 5 H\(_2\)O, 10 mg H\(_3\)BO\(_3\), 10 mg MnSO\(_4\) \(\cdot\) 4 H\(_2\)O, 70 mg ZnSO\(_4\) and 10 mg MoO\(_3\). The calcium solution contained 0.25 mg CaCl\(_2\) \(\cdot\) 2 H\(_2\)O/l. The iron solution contained (per l): 3.5 g FeCl\(_3\) \(\cdot\) 6 H\(_2\)O and 24.2 g tricine. The mineral salt medium contained (per l): 2.28 g K\(_2\)HPO\(_4\) \(\cdot\) 3 H\(_2\)O, 1.38 g NaH\(_2\)PO\(_4\) \(\cdot\) H\(_2\)O, 0.50 g (NH\(_4\))\(_2\)SO\(_4\), 0.02 g MgSO\(_4\) \(\cdot\) 7 H\(_2\)O and was brought to pH 7.0 with a 2 N NaOH solution. The solutions were autoclaved or (for iron solution and methanol) sterilized by filtration. To 1 l of mineral salt medium were added 1 ml of the spore, calcium and iron solutions and 5 ml methanol. The modifications of the additions to the mineral salt medium were essential to obtain colonies on agar plates and a good yield in the fermentor.

In the fermentor, the pH was maintained at 7 with a 25% ammonia solution. During the large scale fermentation (2000 l) the methanol concentration in the culture fluid was measured at regular intervals and readjusted twice to 0.5%. A wet-cell yield of 25 g/l was obtained and the wet cells were frozen and stored at −20°C.

**Preparation of the cell extract**

1 kg of thawed cell-cake was suspended in 1.41 0.1 M Tris \(\cdot\) HCl/0.01 M EDTA (pH 7.0). The cells were lysed at room temperature by adding 600 mg lysozyme and the suspension was centrifuged for 10 min at 27 300 \(\times\) g. Supernatants were discarded and the pellets suspended in 1.21 0.1 M Tris \(\cdot\) HCl/0.5% Triton X-100 (pH 9.0). After standing for 30 min at room temperature the suspension was centrifuged as above and the supernatants stored at 4°C. The pellets were re-extracted in the same way. After two extractions, the enzyme activity in the pellets was negligible. The supernatants were combined and used immediately.

**Enzyme purification**

The precipitate formed from the extracts at 45–85% (NH\(_4\))\(_2\)SO\(_4\) saturation at room temperature was dissolved in a minimum of water and dialyzed overnight at 4°C against 101 0.02 M potassium phosphate pH 7.0 (0.02 M phosphate). The red solution was put on a silicagel column (4 × 20 cm) in 0.02 M phosphate. Cytochromes adhered to the silicagel and methanol dehydrogenase was eluted with 0.02 M phosphate.

Nucleic acids and other impurities were removed on a DEAE-cellulose column (4 × 15 cm) equilibrated with 0.02 M phosphate. The enzyme was eluted with 0.02 M phosphate and adsorbed on a hydroxyapatite column (4 × 10 cm) in 0.02 M phosphate. The column was washed with 0.02 M phos-
phate till no more fluorescing material was seen in the eluate and the enzyme was eluted with 0.1 M potassium phosphate (pH 7.0). The purified enzyme had an absorbance ratio \( A_{280 \text{nm}}/A_{347 \text{nm}} \) of 11 and was used immediately for extraction of the prosthetic group. For other purposes, it was dialyzed overnight against 0.02 M phosphate, concentrated by ultra filtration and stored at \(-80^\circ\text{C}\).

**Prosthetic group, extraction and modification**

Purified enzyme was mixed at room temperature with 9 vols. methanol and the denatured protein was removed by centrifugation. The supernatant was concentrated in a rotary evaporator. Oxidation with periodate and alkaline permanganate was done according to the literature [21,22]. Extraction of the prosthetic group under reducing conditions was performed by adding NaBH\(_4\) (0.1%) to the methanol and enzyme solution.

The purity of the prosthetic group and its derivatives was checked by cellulose thin-layer chromatography with n-propanol/2% ammonium acetate (1 : 1, v/v).

**Enzyme assay**

The test was based on the system developed by Anthony and Zatman [8]. A 0.1 M sodium pyrophosphate buffer (pH 9) was used as this gave twice as much activity as the original Tris buffer. Based on our experiments with ammonia (Duine, J.A. and Frank, J., unpublished results), the test buffer contained 50 mM NH\(_4\)Cl. For experiments with a pH above 9, a NaHCO\(_3\) buffer was employed.

Since phenazine methosulphate and phenazine ethosulphate are coupled to DCIP as end-acceptor, the enzymatic activity is expressed as \(\mu\text{mol reduced DCIP/min}\) at 21\(^\circ\text{C}\). The test buffer normally contained 1 mM KCN, because this partly suppressed the reoxidation of reduced DCIP. When compound I was the acceptor, KCN could not be used as this destroyed the acceptor. In the experiments with compounds I and II no end-acceptor was used and the measurements were performed at 420 and 612 nm respectively.

**Analytical procedures**

Methanol, ethanol, cinnamylalcohol, formaldehyde, acetaldehyde and cinnamaldehyde concentrations were estimated by gaschromatography on a Varian aerograph 2100 equipped with a flame ionisation detector. A Porapak Q column of 1.5 m length (internal diameter 2 mm) was used with N\(_2\) as carrier gas, flowing at 30 ml/min. Column temperature was 175\(^\circ\text{C}\), injector- and detector temperature 200\(^\circ\text{C}\). Compound II was chosen as the electron acceptor because phenazine methosulphate gave decomposition products with the same retention time as methanol. There was a linear relation between peak height and methanol concentration in the working range used. 1 \(\mu\text{l}\) aqueous samples were injected directly into the column. Methanol was detected about 1.3 min after injection. Formic acid was determined spectrophotometrically with 2-thiobarbituric acid [23]. Care was taken to prevent the reoxidation of \(N,N,N',N''\)-tetramethyl-p-phenylenediamine by O\(_2\) as this caused the disappearance of formic acid.
Polyacrylamide gel electrophoresis

The experiments were performed as described by Davis [24] in 5% polyacrylamide gels crosslinked with 0.17% bisacrylamide. In some experiments the gels were pre-electrophorized. Protein staining was done with Coomassie Brilliant Blue G 250. Active bands were detected with compound II or phenazine methosulphate/DCIP in 0.1 M sodium pyrophosphate/0.05 M \( \text{NH}_4\text{Cl} \)/0.01 M methanol (pH 9.0). The active bands appeared as white rings on a blue or green background, respectively.

Molecular weight estimation

A rough estimation by gel filtration was performed according to Andrews [25]. Protein was determined by the Lowry procedure with bovine serum albumin as a standard [26].

ESR spectra

The spectra were recorded on a Varian model E4 X-band spectrometer. Solutions and lyophilized enzyme were measured in a standard flat cell and in quartz capillary tubes (internal diameter 3 mm), respectively. Further resolution of the signal was first seen on an uncalibrated Q-band spectrometer at room temperature, but for determining the principal \( g \)-factors, a \( K_a \)-band (34.660 GHz) superheterodyne spectrometer at 4.2 K with a pill of enzyme powder was used (de Beer, R. and Van Ormondt, D., unpublished results).

Metal analysis

Enzyme was brought into a 0.05 M \( \text{NH}_4\text{Cl} / \text{NH}_3 \) (Suprapur, Merck) buffer (pH 9) via a Bio-Gel P-10 column. Total metal analysis was performed in an Ion Microprobe Mass Analyser (MI-TNO Apeldoorn) on gold and on platinum foil. Iron and manganese were quantitatively estimated by atomic absorption spectrophotometry. Appropriate blanks were taken around the column fractions where the enzyme appeared.

Results and Discussion

Enzyme purification

The procedure described was reproducible and convenient for large-scale enzyme production. The stable enzyme was obtained in good yield (Table I). An absorbance ratio \( (A_{280 \text{nm}} / A_{347 \text{nm}}) \) of 11 was found (Fig. 1), lower than the ratios estimated from published spectra [10,15,16]. The shape of the absorption spectrum is comparable to those found by others.

Although Anthony and Zatman [8] stated that methanol dehydrogenase is a soluble enzyme, our extraction conditions suggest a particle-bound enzyme, a conclusion that holds for methanol dehydrogenases from methane-grown bacteria [16,27].

From the results of polyacrylamide gel electrophoresis (Fig. 2) we deduce that the enzyme is electrophoretically pure because the bands detected with the activity test and the protein stained bands are identical and no other protein stained bands were seen. Moreover, the absorption spectrum of the main electrophoresis band (measured above 300 nm) appeared to be the same as for the purified enzyme.
### TABLE I

**PURIFICATION OF METHANOL DEHYDROGENASE FROM *HYPOMICROBIUM X***

The procedure was started with 2 kg cell-cake. Activities were determined at pH 9.0 in 0.1 M sodium pyrophosphate/0.01 M methanol/0.05 M NH₄Cl/0.5 mM phenazine methosulphate/60 μM DCIP at 21°C. A volume of 3 ml of this mixture was pipetted into a cuvette containing 75 μl enzyme solution. One unit of enzyme activity = 1 μmol DCIP reduced min⁻¹.

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Volume (l)</th>
<th>Total activity (units)</th>
<th>Total protein (g)</th>
<th>Specific activity units/mg</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Extract</td>
<td>4.80</td>
<td>97</td>
<td>21.6</td>
<td>3.1</td>
<td>100</td>
</tr>
<tr>
<td>Dialysis</td>
<td>0.87</td>
<td>66</td>
<td>21.6</td>
<td>3.1</td>
<td>68</td>
</tr>
<tr>
<td>Silica gel eluate</td>
<td>0.87</td>
<td>67</td>
<td>10.9</td>
<td>6.1</td>
<td>68</td>
</tr>
<tr>
<td>DEAE-cellulose eluate</td>
<td>0.88</td>
<td>66</td>
<td>9.2</td>
<td>7.2</td>
<td>68</td>
</tr>
<tr>
<td>Hydroxyapatite chromatography</td>
<td>0.29</td>
<td>48</td>
<td>5.9</td>
<td>8.1</td>
<td>49</td>
</tr>
</tbody>
</table>

* The first purification step was not tested because Triton X 100 disturbed the protein estimation.

The closely spaced banding pattern, consisting of one major and two minor bands, was reproducible for several batches of enzyme and different electrophoresis conditions. This means that either the enzyme occurs in different forms or it is an artifact of the separation procedure.

![Fig. 1. The absorption spectrum of purified methanol dehydrogenase in 0.02 M potassium phosphate (pH 7.0). (A) Protein concentration 0.9 mg/ml. (B) Protein concentration 8.2 mg/ml.](image)

![Fig. 2. Polyacrylamide gel electrophoresis of purified methanol dehydrogenase after staining with Coomassie Brilliant Blue G 250. An amount of 110 μg protein was added to the gel and electrophoresis was for 25 min.](image)
Summarizing we conclude that our enzyme preparation, although heterogeneous, was not contaminated by other proteins.

Properties of the enzyme

When the enzyme was subjected to gel filtration with protein markers a molecular weight of 120 000 was found. This is in accordance with values of other methanol dehydrogenases (except the one isolated from \textit{Methylosinus sporium} [16] which has a molecular weight of 60 000).

On total metal analysis of the enzyme, only the presence of iron and manganese was established. Quantitative measurements indicated that only about 1 metal atom is present per 7 enzyme molecules. The metals were also detected when 1 mM EDTA was included in the column buffer, which means that they are firmly bound to the enzyme. Therefore it was not surprising that EDTA had no influence on the enzyme test. Anthony and Zatman [8] reported that metals were absent from their enzyme preparation, although they found an inhibiting effect of EDTA and other chelators in their test [28].

Given the low metal content and the fact that all the protein bands after electrophoresis were enzymatically active we tentatively conclude that metals have no function in the enzyme activity in vitro.

Substrate oxidation in vitro

To correlate the substrate oxidation with the acceptor reduction, the methanol concentration during the reaction was estimated via gaschromatography. It appeared that, at the point where all the acceptor (compound II) was reduced, a quarter of the corresponding molar quantity of methanol was oxidized. This observation can be explained if the methanol is oxidized to formic acid. Indeed formic acid could be detected by the test with thiobarbituric acid. The reaction rate and stoichiometry in the methanol oxidation were not influenced by formaldehyde binding substances like dimedone and semicarbazide. Furthermore, because in the experiment described methanol was present in excess, it is clear that no free formaldehyde is formed and methanol is directly oxidized to formic acid. This behaviour of the enzyme can be explained by the fact that formaldehyde is a good substrate [15].

In contrast, acetaldehyde accumulated when ethanol was the substrate. At the point where all the ethanol was consumed acetaldehyde oxidation was detected but at a much slower rate.

An example of an aldehyde, that is not a substrate, is cinnamaldehyde, although cinnamyl alcohol is a better substrate than ethanol (Duine, J.A. and Frank, J., unpublished results). Sperl et al. [12] have investigated some aldehydes as substrate and concluded that only hydrated aldehydes are a substrate.

The direct oxidation of methanol to formic acid was rather unexpected. In methylotrophic bacteria several formaldehyde oxidizing enzymes are present [3]. Moreover, methanol dehydrogenase has a task to supply formaldehyde for biosynthesis. From the results described here, no explanation can be given how this is regulated in the cell.

Electron acceptors

Several of the usual electron acceptors were tested but, as was already
known, only phenazine methosulphate [28] and also phenazine ethosulphate (Fig. 3) sufficed. When phenazine methosulphate was dissolved in the test buffer a large amount of radicals was formed (as found by ESR). This is due to nucleophilic attack at high pH [29]. We therefore considered the possibility that those radicals are the acceptor, rather than phenazine methosulphate itself.

A search was made in the literature for water-soluble stable radicals with a satisfactory redox-potential. Compound II is a positively charged stable radical [19]. Its reduced form is employed as electron donor for cell respiration studies [30] but, to our knowledge, we are the first to show its use as an electron acceptor for methanol dehydrogenase. The same applies to compound I, a radical with an overall negative charge [31]. The reduced form has been advocated as an electron donor for peroxidases [32]. Like phenazine methosulphate these new electron acceptors required ammonia in the test but the enzyme had now a high pH optimum (Fig. 3). The pH optimum with phenazine methosulphate was not exactly definable because above pH 9, decomposition increased considerably and interfered with the test. Compound I and II also decompose at high pH, but not as rapidly as phenazine methosulphate thus, with appropriate blanks, measurements were possible. At pH 11, enzyme denaturation took place.

The results with the free radicals brought us to the question whether electron acceptors, which form stable radicals after one-electron reduction, are active. Indeed, chloranil (tetrachloro-\(p\)-benzoquinone) appeared to function as an acceptor. After the test mixture had reacted, the absorption spectrum showed the presence of the semiquinone form [33]. No further experiments were undertaken because chloranil is not very soluble.

Considering the results with chloranil, the activity of phenazine methosulphate can also be explained by regarding it as an acceptor with a stable one-electron reduced form. The com- and disproportionation reactions between oxidized, fully reduced and half reduced forms of phenazine methosulphate, phenazine ethosulphate and chloranil makes a statement about their func-
tioning under these conditions impossible. But it is clear that in the case of compound I and II, where the equilibrium lies strongly in favour of their radical state, only one-electron steps are possible. In this light, it is remarkable that a substance like potassium ferricyanide is not an acceptor, although its redox potential and dimensions seem acceptable. A certain degree of lipophilic or aromatic character may be necessary.

**ESR spectroscopy**

In all stages of the purification an ESR signal, with the same properties as was found for the pure enzyme, accompanied the active enzyme fractions. It appeared that the X-band signal was slightly asymmetric with a peak-to-peak linewidth of 0.7 mT.

Sometimes ESR at a higher microwave frequency, having a better resolving power, can give information about the origin of the asymmetry. From Fig. 4 it is clear that only one radical is present in the enzyme with \( g_z = 2.0024 \) and \( g_\perp = 2.0056 \) and that the asymmetry in the X-band signal is caused by overlapping \( g_z \) and \( g_\perp \) lines. From these data we can calculate that \( g_{iso} = 2.0045 \). On scanning a wide range of the spectrum at room temperature or at 4.2 K no further signals were seen.

The saturation behaviour and the absence of hyperfine structure in the spectrum suggest a protein-bound organic free radical. Methanol addition had no influence neither on the ESR signal nor on the optical absorption spectrum. As we shall prove (Duine, J.A. and Frank J., unpublished results) this phenomenon may be explained by the fact that the enzyme is in the reduced form.

Considering the linewidth and \( g_{iso} \) value, it is improbable that the signal can be attributed to a pteridine [36,37] or flavin [34,35] radical, where the spectrum width [34–36] and \( g \)-value [37,38] are larger. On the other hand these values are in excellent agreement with the data for quinone radicals [39]. In a forthcoming paper we will demonstrate that the signal can be related to the prosthetic group and the mechanism of action of the enzyme.

![Fig. 4. The ESR-signal of methanol dehydrogenase at X-band frequency. Lyophilized enzyme was compressed to a pill. The \( g \)-values were estimated at the points indicated with arrows.](image-url)
Properties of the prosthetic group

Enzymatic activity was not influenced by dialysis but was totally destroyed by heat denaturation, buffers with high or low pH and polar liquids such as methanol or dimethyl sulphoxide. In these circumstances a green fluorescence appeared and the ESR signal was lost.

The prosthetic group, isolated by methanol extraction, was chromatographically pure with a fluorescent spot at \( R_f = 0.33 \). The spot could also be detected by spraying with reduced Methylene Blue. The absorption spectrum is shown in Fig. 5 and is in the range of the spectrum of the enzyme. As the enzyme was pure and no other low molecular weight compounds were detected we consider this fluorescing compound to be the prosthetic group. On standing at room temperature for 2 days, the extracted substance had completely changed to a blue fluorescing compound with an \( R_f \) of 0.63 and a different absorption spectrum (Fig. 5). This process could not be accelerated by oxygen, \( \text{H}_2\text{O}_2 \) or light and we have no explanation for this behaviour.

After oxidation with alkaline permanganate no absorption spectrum could be detected. This shows that the cofactor is not a folate [10] or neopterine cyclic-phosphate [17], because in these cases a distinct spectrum is expected [22]. However, oxidation with periodate produced a vivid blue fluorescing substance with little change of the absorption spectrum and with consumption of periodate. We suggest that the cofactor contains a polyol or sugar moiety which can be oxidized by periodate.

Reduction with \( \text{NaBH}_4 \) under an argon atmosphere gave a non-fluorescing product with the spectrum shown in Fig. 6. Upon admission of oxygen this changed to a blue-green fluorescing substance with a spectrum different from that of the extracted prosthetic group (Fig. 6). This substance was stable and gave an \( R_f \) of 0.33 under chromatography.

Fig. 5. Absorbance spectrum of the extracted prosthetic group and its change on standing. (·······, extracted prosthetic group; ———, the same after standing for one day at room temperature).

Fig. 6. Reoxidation of the reduced prosthetic group. (·······, extracted prosthetic group; ———, reduced with \( \text{NaBH}_4 \); —······, reoxidation with \( \text{O}_2 \)).

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All the results point to a prosthetic group which in its extracted form is in the oxidized state and can be reduced by different substances. Since in the isolated enzyme the prosthetic group is in the reduced form (Duine, J.A. and Frank, J., unpublished results), it is understandable why all trials for reconstitution were unsuccessful [10]. Therefore further work is necessary to prove the nature and rôle of the extracted fluorescing substance.

Acknowledgements

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References

Chapter III

The prosthetic group of methanol dehydrogenase. Purification and some of its properties*

Methanol dehydrogenases isolated from bacteria belonging to different classes of methylotrophs contain the same prosthetic group. A procedure for its purification from whole cells is given. The reduced and oxidized form of the enzyme from *Hyphomicrobiun* X and those of the isolated prosthetic group are compared and it is concluded that the latter indeed functions in the enzyme. Further evidence is presented that the prosthetic group is not a pterine or lumazine derivative, but a water-soluble nitrogen-containing quinone.

Methanol dehydrogenase [alcohol-(acceptor) oxidoreductase, EC 1.1.99.8] is an unusual enzyme because it is neither an NAD(P)-dependent nor a flavin-containing dehydrogenase. It contains a chromophoric group, giving the enzyme an absorption maximum at 340–350 nm. Under denaturing conditions a fluorescing compound appears, which has been considered to be the prosthetic group (Anthony & Zatman, 1967b). However, the addition of methanol to the enzyme does not change its absorption spectrum. At the same time, reconstitution of the enzyme from apoenzyme and the resolved prosthetic group has failed so far.

The chemical nature of the prosthetic group is also not clear. Anthony & Zatman (1967b) extracted it from methanol dehydrogenase of *Pseudomonas* M27. Owing to its spectral properties, they suggested that it was a pteridine derivative and the enzyme was classified as such (Anthony, 1971; Walsh, 1978). In a study by Urushibara et al. (1971) it was tentatively identified as neopterin cyclic phosphate. Based on the properties of a photo-degradation product of the prosthetic group, Sperl et al. (1973) reported it to be a lumazine derivative. On the other hand, Duine et al. (1978) concluded from e.s.r. measurements of methanol dehydrogenase from *Hyphomicrobiun* X that the prosthetic group had a quinone structure.

Because of this disagreement it was necessary to study other methylotrophic bacteria and to see whether our previous proposal could be further substantiated. Since an oxidized form of methanol dehydrogenase has been obtained (Duine & Frank, 1980), it was possible to compare the spectral properties of the reduced and oxidized form of the prosthetic group with those of the enzyme, to ascertain the involvement of the former in the catalysis by the enzyme.

**Materials and Methods**

**Chemicals**

All chemicals were from E. Merck Nederland B.V., Amsterdam, The Netherlands, except for Amberlyst A21 (50–100 μm, analytical grade), a weakly basic macroporous anion exchanger, which was from Serva, Heidelberg, Germany, and Seppak C18-silica cartridges, which were from Waters Associates, Etten-Leur, The Netherlands.

Before use, Amberlyst was pretreated. After removal of fines, it was washed with 3 vol. of 4 M NaCl followed by 2 vol. of water. The ion-exchanger was stored in methanol/water (9:1, v/v).

**Growth of the organisms**

*Methylobacterium organophilum* strain xx, LMD 78.41, was obtained from the Laboratory of Microbiology Culture Collection, Delft University of Technology, and was grown in the same way as *Hyphomicrobiun* X (Duine et al., 1978). *Rhodopseudomonas acidophila* 10050 was provided by Professor J. R. Quayle, Department of Microbiology, University of Sheffield, Sheffield, U.K., and was cultured as described by Sahm et al. (1976). Wet cells of *Paracoccus denitrificans* LMD 22.21, grown on methanol, were a gift from Mr. W. Hazou, Laboratory of Microbiology, Delft University of Technology. *Methylphilus methylotrophus* was obtained in the form of Pruteen and was a gift from

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ICI, Billingham, U.K. It is noteworthy that this last mentioned material was only used for extraction of the prosthetic group because it no longer contains active methanol dehydrogenase.

**Purification of the methanol dehydrogenases**

The enzymes were purified by a reported procedure (Duine et al., 1978), which was modified in the case of *Rh. acidophila* and *Methylobacterium organophilum* (J. A. Duine & J. Frank, Jr., unpublished work). Purity was checked by polyacrylamide-gel electrophoresis and, in some cases, the prosthetic group was extracted directly from the active band.

**Purification of the prosthetic group**

*From purified enzyme*. A methanol dehydrogenase solution (100ml) containing 14 mg of protein/ml was mixed with 900 ml of methanol. The resulting suspension was centrifuged and the supernatant was passed through a column (2.5 x 1.2 cm) of 5 g of pretreated Amberlyst. The column was washed with 10 ml of methanol/water (9:1, v/v) and eluted with 15 ml of a solution of methanol/water (1:1, v/v) saturated with NaCl (at room temperature). The eluate was freed from methanol in a rotary evaporator under reduced pressure. The remaining solution was stored at 0°C and, usually after one night, the prosthetic group separated from the fluid as a brick-red precipitate.

*From whole cells*. Wet cells were extracted with 9 vol. of methanol and 1 litre of supernatant was passed through a column (2.5 x 1.2 cm) of 5 g of pretreated Amberlyst. The column was washed with 10 bed volumes of 0.5 M NaCl in methanol/water (1:1, v/v) to remove flavins and other impurities. After that, the prosthetic group was eluted with 15 ml of a solution of methanol/water (1:1, v/v) saturated with NaCl. Fractions were analysed by h.p.l.c., pooled and passed through a Seppak C18-silica cartridge to remove impurities. When the cartridge had become completely brown it was replaced by a new one. Methanol was removed from the filtrate as described above and the remaining fluid was adjusted to pH 2.0 with conc. HCl. The solution was passed through a clean Seppak C18-silica cartridge, which now retained the prosthetic group. The yellow–orange band was eluted from the cartridge with 12.5 mm-KH₂PO₄ (adjusted with conc. H₃PO₄ to pH 2.0)/methanol (8:3, v/v). Methanol was evaporated from the eluate and the remaining solution was passed through a new cartridge. After washing with dilute HCl, pH 2.0, the prosthetic group was eluted with 2 ml of methanol/water (7:3, v/v). The methanol was removed, the solution saturated with NaCl at room temperature and stored at 0°C. The precipitate was dissolved in a small amount of water, the solution adjusted to pH 2.0 with dilute HCl and subjected to preparative h.p.l.c. by injecting up to 200 µl at a time. The prosthetic group-containing fractions were pooled and freed from phosphate by using a cartridge as already described. The purified prosthetic group was stored in methanol/water (7:3, v/v) at -20°C.

**Reduction of the prosthetic group**

A solution of the prosthetic group in 500 µl of 0.01 M tetrascidium pyrophosphate, brought to pH 9.0 with conc. HCl, was transferred to a cuvette and flushed with N₂ for 15 min. After recording the u.v.–vis spectrum, 5 µl of 0.05% (v/v) 2-mercaptoethanol in water was added. The cuvette was stoppered and the absorption at 310 nm was followed with respect to time. When the absorption showed no further increase, the reduction was considered complete. At that point, the fluorescence of the solution had almost disappeared.

**Reaction of the prosthetic group with aldehydes/ketones**

A solution of the prosthetic group in water was adjusted to pH 9.0 with dilute ammonia and aldehyde or ketone was added to 5% (v/v) final concentration. The prosthetic group was completely converted in about 30 min, as judged by h.p.l.c.

**H.p.l.c. analysis**

This was performed with a Waters model 6000 A pump, equipped with an U6K injection block and an analytical µBondapak C18 column, using 12.5 mm-KH₂PO₄ (adjusted to pH 2.0 with conc. H₃PO₄)/methanol (8:3, v/v) as the eluent. The flow rate was 1.5 ml/min at a pressure of 8 MPa. The effluent was monitored with a model 440 absorption detector, operating at 254 nm.

**Analytical methods**

The spectra for u.v. and visible absorption were recorded on a Beckman UV 5260 spectrophotometer. E.s.r. measurements were done under the conditions described by Westerling et al. (1979). The n.m.r. spectrum of the prosthetic group (0.5 mg) in D₂O with 3-(trimethylsilyl)propanesulphonic acid sodium salt as an internal standard was run on a Varian SC 300 spectrometer operating at 300 MHz. The i.r. spectrum of 0.3 mg of prosthetic group, pressed with KBr to a disc, was recorded with a Hilger and Watts Infrascan spectrometer. Phosphate was determined by the method of Eibl & Lands (1969). Colour tests for quinones were done by the method of Sawicki & Elbert (1960) and Druy & Schmidt (1950).
Results and Discussion

Extraction and purification of the prosthetic group

Although the prosthetic group can be extracted from the enzyme with methanol in a chromatographically pure form, it is unstable on storage (Duine et al., 1978). However, after treatment of the extract with Amberlyst, a stable preparation was obtained. Furthermore, as it was found that Amberlyst has a high adsorption capacity for the prosthetic group and material adsorbed to it remained stable, this isolation step is very well suited for a large-scale extraction from whole cells.

H.p.l.c. was not only used to analyse prosthetic group-containing fractions, but also for the final purification of material extracted from whole cells. Pretreatment of the samples for h.p.l.c. by means of C18-silica cartridges and the precipitation with NaCl protected the h.p.l.c. column from heavy contamination and provided an excellent pre-purification. The chromatographically (by h.p.l.c.) pure preparations were also judged pure by n.m.r.

Comparison of the prosthetic groups from methanol dehydrogenases of different organisms

In general, the absorption spectra of methanol dehydrogenases from different methylotrophic bacteria have the same appearance. Although this suggests that the prosthetic groups are also the same, this point was further checked. Some methylotrophs with a normal dehydrogenase (showing the high pH optimum and need for ammonia, as was first described by Anthony & Zatman (1967a)), but differing widely in growth habitat and morphology, were chosen. *Hyphomicrobium X* is a facultative methylotrophic budding bacterium and the enzyme has been described previously (Duine et al., 1978). *P. denitrificans* is a facultative chemolithotroph, able to grow autotrophically on H₂ or methanol (Bamforth & Quayle, 1978a). *Methyliphilus methylo trophus* is an obligate methylotrophic organism growing on methanol (Ghosh & Quayle, 1978).

Besides these organisms, those with a more or less diverging enzyme were also tested. Methanol dehydrogenase from *Rh. acidophila* (a phototroph growing on methanol) with a normal absorption spectrum has somewhat different properties (Bamforth & Quayle, 1978b). It was reported that although the absorption spectrum of the enzyme from *Methylobacterium organophilum* (a facultative methylotroph, also growing on multi-carbon substrates like monosaccharides) was normal, the fluorescence characteristics were different from those reported for the prosthetic group by Anthony & Zatman (1967b). This led the authors to the conclusion that the prosthetic group may have a different structure (Wolf & Hanson, 1978). It is noteworthy, however, that the fluorescence excitation and emission maxima they give for the enzyme (285–290 and 355–360 nm respectively) are characteristic of aromatic amino acids in a protein.

Analysis by h.p.l.c. of the prosthetic groups of methanol dehydrogenase from these organisms revealed that they all co-chromatographed with the purified prosthetic group from *Hyphomicrobium X* (Fig. 1a). Moreover, they gave the same products after reaction with carbonyl compounds, as judged by the characteristic change in retention time after the reaction with, for example, acetone (Fig. 1b).

For the product from Pruteen, additional confirmation was obtained from the e.s.r. spectrum, which was found to be identical with the known hyperfine-structure spectrum of the prosthetic group from *Hyphomicrobium X* enzyme (Westerling et al., 1979). Comparison of the n.m.r. spectra led to the same conclusion. From these results we conclude that there are no reasons to believe that methanol dehydrogenases have different prosthetic groups.

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**Fig. 1.** H.p.l.c. chromatograms of the prosthetic group from *Hyphomicrobium X* enzyme and its acetone derivative

(a) A prosthetic group-containing fraction of an extract from whole cells after the Amberlyst purification step. The prosthetic group (10 μl injected) has a retention time of 6.8 min. (b) After reaction of the sample, shown in Fig. 1(a), with acetone. A solution of the prosthetic group in water was adjusted to pH 9.0 with dilute ammonia and acetone was added to 5% (v/v) final concentration. After 30 min, 10 μl of the solution was injected. The acetone derivative has a retention time of 4.2 min.
Therefore, the conflict in the literature must have other causes.

Anthony & Zatman (1967b) purified the prosthetic group from the enzyme of *Pseudomonas* M27 by TEAE-cellulose chromatography. The absorption spectrum of the material they obtained gave a maximum at 250 nm, a shoulder at 275 nm and a low broad maximum around 350 nm. However, the fluorescence-excitation maxima were situated at 255 and 365 nm.

Fig. 2 shows the absorption spectrum of the prosthetic group extracted from *Hyphomicrobium* X. As mentioned previously, this preparation no longer exhibits a ‘spontaneous’ change on storage. Several carbonyl compounds like acetaldehyde, propionaldehyde and acetone were found to react with the prosthetic group. Each time an absorption spectrum (Fig. 3) was obtained identical with the one found after the ‘spontaneous’ change (Duine et al., 1978). Analysis by h.p.l.c. showed, however, that this spectrum was displayed by chromatographically different products. The retention time of the product with acetone is 4.2 min (Fig. 1b), whereas the products with acetaldehyde and propionaldehyde have retention times of 3.8 and 9.8 min respectively (results not shown). After the reaction, the green fluorescence of the prosthetic group was changed to a more intense blue. The increase in intensity depended on the carbonyl compound used, propionaldehyde being the most effective. These observations suggest an addition type of reaction.

The differences between the maxima of the absorption spectrum of the prosthetic group described in the present paper and the excitation maxima of the one isolated from *Pseudomonas* M27 (Anthony & Zatman, 1967b) might be explained by assuming that in the latter case a partial transformation of the type described above had occurred. The reported fluorescence-excitation maxima are then most likely due to the product of transformation, being the dominant fluorescing compound in the mixture. On the other hand, from arguments given below, we consider the substance giving an absorption spectrum as shown in Fig. 2 and a retention time of 6.8 min in Fig. 1(a) as the real prosthetic group.

The function in methanol dehydrogenase

Reaction of 2-mercaptoethanol with the prosthetic group results in the disappearance of the green fluorescence and at the same time a product is formed with a different spectrum (Fig. 4). Admission of *O₂* reverses this spectral change and restores the green fluorescence. These spectral features can

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![Fig. 2. Absorption spectra of the purified prosthetic group from *Hyphomicrobium X* enzyme at different pH values](image)

---

![Fig. 3. Absorption spectrum of an acetone derivative](image)
Fig. 4. Reduction of the purified prosthetic group from *Hyphomicrobium X* enzyme with 2-mercaptoethanol

--- Spectrum of the prosthetic group in 0.01 M-tetrasodium pyrophosphate, pH 9.0; ---, spectrum after the addition of 2-mercaptoethanol to 0.005% (v/v) final concentration.

be expected for a quinone. For instance, 1,4-naphthoquinone has an absorption maximum at 250 and 330 nm, coupled with increased absorption at about 330 nm in the reduced state and decreased absorption below 300 nm.

Owing to the presence of 2-mercaptoethanol, the decrease in the region below 300 nm cannot be measured (Fig. 4), but it can be seen on reduction with NaBH₄ (Duine et al., 1978). In the latter case, however, some irreversible reduction also occurs.

There is a difference (25 nm) between the absorption maximum of the reduced prosthetic group and the methanol dehydrogenase reduced by substrate, and a still larger shift (about 50 nm) is found for the maximum of an oxidized form of methanol dehydrogenase (Duine & Frank, 1980) and the prosthetic group (Figs. 2 and 4). So the question arises whether there is any relationship between the chromophore in the enzyme and the isolated prosthetic group, although exceptionally large shifts have been reported in the literature e.g. for pyridoxal phosphate (Snell et al., 1968). But on denaturing the oxidized form of methanol dehydrogenase by heat, the normal prosthetic group was obtained and no further compound absorbing above 300 nm was found. Therefore the shift of the maximum to about 400 nm may be due to an interaction of the quinone part of the molecule with amino groups in the protein, an interaction that has been studied with model compounds like chloranil and amino acids by Birks & Silfkin (1963). Of course, other interactions may play a role because a change in the pH (Fig. 2) or the addition of carbonyl compounds (Fig. 3) also shifts the maximum to higher wavelengths.

It is also possible that the prosthetic group has become modified during the extraction and purification. However, reconstitution of activity has been found for an apoenzyme of glucose dehydrogenase (containing the same prosthetic group) from *Acinetobacter calcoaceticus* and the purified prosthetic group (Duine et al., 1980). Moreover, the hyperfine structure of the e.s.r. spectrum of the free radical removed from the enzyme is exactly the same as that induced in the purified prosthetic group by reduction (Westerling et al., 1979).

As no other low-molecular-weight compounds were detected in the enzyme we conclude that the extracted fluorescing compound described in the present paper is in fact the prosthetic group, which functions in the enzyme as the primary hydrogen acceptor.

**The nature of the prosthetic group**

On the basis of e.s.r. measurements, the prosthetic group is not a pterine or lumazine, but probably a quinone derivative (Duine et al., 1978; Westerling et al., 1979). Strong absorptions in the i.r. spectrum at 1678 and 1710 cm⁻¹, indicating the presence of carbonyl groups, are compatible with such a concept. The n.m.r. spectrum of the prosthetic group in D₂O shows two singlets at 7.14 and 8.16 p.p.m., indicating the presence of two aromatic protons not coupled to each other. The latter result is in agreement with e.s.r. measurements of the prosthetic group (Westerling et al., 1979) and e.n.d.o.r. measurements of the enzyme (De Beer et al., 1979), indicating the presence of three protons (one of which is exchangeable) and two nitrogen atoms in the molecule.

The two colour tests for quinones were positive. Even more can be deduced from the tests, because the 3,4-dimethoxyaniline reagent is claimed (Sawicki & Elbert, 1960) to be specific for inner-ring quinones.

The fact that the prosthetic group is strongly adsorbed to a weakly basic ion-exchanger means that it must be a fairly strong acid. Indeed a pKₐ of 2.5 can be estimated from the spectral changes occurring around pH 2.5 (Fig. 2). As no phosphate could be detected, one or more carboxy groups are most probably responsible for this acid character. Around pH 10 another spectral change occurs (Fig. 2), as expected for a nitrogen-containing substance.

From the properties described in the present paper, it can be concluded that the prosthetic group of methanol dehydrogenase is different from any other known cofactor. The presence of nitrogen
atoms and carboxy group(s) can explain its solubility in water. Clearly this compound is not directly comparable with the known quinones active in the electron-transport chain. As large-scale extraction of whole cells is now possible, an important drawback in the elucidation of the structure of the prosthetic group has been removed.

We thank the Central Laboratory TNO, Delft, for help with n.m.r. measurements.

References

Chapter IV

The prosthetic group of methanol dehydrogenase from *Hyphomicrobium X*. Electron spin resonance evidence for a quinone structure*

**SUMMARY:** Partial reduction of the isolated prosthetic group of methanol dehydrogenase yields a free radical with the same characteristics as the one contained in the enzyme. The Electron Spin Resonance spectrum in alkaline aqueous solution displays hyperfine structure and is interpreted in terms of an isotropic g-value, hyperfine coupling constants and nuclear spins. The magnitudes of these parameters indicate that the prosthetic group is a quinone containing two nitrogen atoms.

Methanol dehydrogenase (alcohol: (acceptor) oxidoreductase, EC 1.1.99.8) is an enzyme that is found in methylotrophic bacteria grown on C1-carbon substrates. It is a NAD(P)-independent enzyme. Several artificial one-electron acceptors can be used to detect its methanol-oxidation activity (1). As all the reported methanol dehydrogenases from methylotrophic bacteria have an identical absorption spectrum, the prosthetic group is probably the same for all of them. Although the nature of the prosthetic group is unknown, there is a general belief that it is a pteridine derivative (2,3,4). However, recently it was found that the methanol dehydrogenase from *Hyphomicrobium X* contains a free radical with characteristics that are incompatible with a pteridine derivative (1). The purpose of the present study is to show that the radical is derived from the prosthetic group and that Electron Spin Resonance can be used as a tool in the structure-elucidation of the prosthetic group.

Abbreviation: ESR - Electron Spin Resonance.

MATERIALS AND METHODS: The purification of methanol dehydrogenase and the isolation of the prosthetic group were described previously (1).

ESR spectra were recorded at room temperature (20-22°C) with a Varian E-4 X-band spectrometer. For g-value measurements the ESR spectrometer was calibrated with an anaerobic solution of Wurster's blue in ethanol (g=2.00305) (5).

The ESR spectrum depicted in Fig. 2a was interpreted in terms of hyperfine coupling constants and nuclear spins using the "pattern-search" method described earlier (6,7). Gaussian lineshapes were employed throughout. The final simulation (Fig. 2b) was performed with a top-to-top linewidth of 0.012 mT.

Preparation of samples for ESR:
a. Lyophilized methanol dehydrogenase,
40 mg of lyophilized enzyme was placed in a cylindrical quartz tube of 2 mm internal diameter and the ESR spectrum of Fig. 1 was recorded.
b. Methanol dehydrogenase in neutral aqueous solution.
40 mg of lyophilized enzyme was dissolved in 1 ml of 0.02 M potassium phosphate buffer (pH=7.1). The solution was transferred to a standard flat ESR sample-cell.
c. Methanol dehydrogenase in alkaline salicylate buffer.
0.5 ml of a freshly prepared 4M sodium salicylate (pK_a2=13.4) solution was mixed with 0.5 ml of 2M sodium hydroxide. This solution and 40 mg of lyophilized enzyme were deoxygenated separately by flushing with argon for 15 minutes. The enzyme was dissolved in the alkaline salicylate buffer and transferred under argon to a standard flat sample-cell. The ESR spectrum of Fig. 2a was then recorded.
d. The partially reduced prosthetic group in alkaline salicylate buffer.
0.5 ml of a freshly prepared 4M sodium salicylate solution was mixed with 0.5 ml of 2M sodium hydroxide. The resulting solution was deoxygenated by flushing with argon for 15 minutes. A solution of 10 μg of the prosthetic group in 20 μl of water and 20 μl of a 1% solution of mercaptoethanol in water were added rapidly to the alkaline salicylate buffer. The solution was flushed with argon for 5 minutes and transferred anaerobically to a standard flat sample-cell.
e. The partially reduced prosthetic group in deuterated alkaline salicylate buffer.
The experiment was carried out in the same way as experiment d, but using deuterium oxide instead of water and NaOD instead of sodium hydroxide.

RESULTS AND DISCUSSION.

The X-band ESR spectrum of lyophilized methanol dehydrogenase from Hyphomicrobium X is shown in Fig. 1. The slightly unsymmetrical signal has a top-to-top linewidth of 0.7 mT.

The g-value (g=2) and linewidth of the spectrum indicate that it originates from an organic free radical. The same "powder-spectrum" is observed when the lyophilized enzyme is dissolved in water. This behaviour is quite normal for a radical that is tightly linked to a high molecular-weight protein. The anisotropy cannot
Fig. 1. The X-band ESR spectrum of methanol dehydrogenase.

average out as it does when a low molecular-weight organic radical is rotating freely in a solution.

The main disadvantage of powder-spectra of organic radicals is that the hyperfine structure is often obscured. Indeed no hyperfine structure is visible in the ESR spectra of lyophilized enzyme and enzyme solution. In order to observe hyperfine interactions we must disconnect the radical from the enzyme, so that it can rotate freely in solution. We found that this can be accomplished by dissolving lyophilized enzyme in a concentrated alkaline salicylate buffer under anaerobic conditions. The ESR spectrum of the resulting solution is shown in Fig. 2a.

As expected the ESR spectrum (Fig. 2a) has hyperfine structure. The isotropic g-value of the radical in salicylate buffer \( (g=2.0046) \) is essentially the same as the one of the enzyme-linked radical \( (1,8) \).

In order to prove that the radical of methanol dehydrogenase is derived from the prosthetic group, an experiment with the isolated prosthetic group \( (1) \) was carried out.
Fig. 2. a) X-band ESR spectrum of the free radical after release from the enzyme. b) Simulation.

The isolated prosthetic group is in the oxidized state and must be reduced in order to form the radical-state which can be detected by ESR. The ESR spectrum obtained after reduction of the prosthetic group in alkaline salicylate buffer turns out to be identical with the spectrum in Fig. 2a. Extraction of the enzyme-bound radical with alkaline salicylate buffer and reduction of the isolated prosthetic group in alkaline salicylate buffer both result in the same ESR spectrum (Fig. 2a). This means that the enzyme-bound radical is derived from the prosthetic group.

The spectrum shown in Fig. 2a has a number of interesting features which shed light on the chemical structure of the radical.
The g-value (2.0046) is too large for a pterin or lumazine radical, but it is quite compatible with a quinone radical (1).

The total width between the outer lines of the spectrum is 0.69 mT. The widths of the ESR spectra of those pterin and lumazine radicals that are known range from 3 to 10 mT (6,7,9,10,11). For quinone radicals the widths range from 0.6 to 2 mT (12-18). As in the case of the g-value, the width of the spectrum (Fig. 2a) is not compatible with a pterin or lumazine structure, but it is in excellent agreement with a quinone structure.

The experimental spectrum (Fig. 2a) was interpreted in terms of hyperfine coupling constants and nuclear spins with which the spectrum could be simulated (Fig. 2b) accurately. A detailed description of the methods used for ESR spectrum interpretation has been published (6,7). Their reliability has been demonstrated in the interpretation of ESR spectra of pterin and lumazine radicals.

Two nuclei with spin=1 (\(^{14}\)N) and three nuclei with spin=1/2 (\(^1\)H) have interaction with the unpaired electron. The corresponding hyperfine coupling constants are:

\[ a_{\text{N1}} = 0.060, \quad a_{\text{N2}} = 0.081, \quad a_{\text{H1}} = 0.097, \quad a_{\text{H2}} = 0.126 \quad \text{and} \quad a_{\text{H3}} = 0.184 \quad (\text{mT}). \]

The proton with the smallest hyperfine coupling constant (H1) is replaced by deuterium if the ESR experiment is carried out with a deuterated solvent.

A complete elucidation of the structure of the radical by ESR is not possible at this stage.

The semiquinone radical that is described in this study can be generated by reduction of the isolated prosthetic group. We therefore propose that the prosthetic group of the methanol dehydrogenase from *Hyphomicrobium X* is a quinone containing two nitrogen atoms. Further investigations on the structure of the prosthetic group are in progress.
ACKNOWLEDGEMENT: We thank Dr. R. de Beer and Dr. D. van Ormondt for placing at our disposal prior to publication the results of $\kappa_\alpha$-band ESR measurements on methanol dehydrogenase. We also thank Dr. G. Kohn for valuable advice.

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Chapter V

Structure and activity of the prosthetic group of methanol dehydrogenase*

The reconstitutive ability of the isolated prosthetic group of methanol dehydrogenase with the apoenzyme of glucose dehydrogenase and the results of electron spin resonance measurements suggest that the prosthetic group has not been modified during the isolation. This result, and the properties of the directly isolated prosthetic group and derivatives, confirm the suggestion that its structure is 2,7,9-tricarboxy-1H-pyrrolo[2,3-f]quinoline-4,5-dione.

From the activity shown by derivatives of the prosthetic group and of structural analogues in the apoenzyme test it is concluded that the o-quinone structure is essential for activity. Hence the trivial name pyrrolo-quinoline quinone would be appropriate. The testing of the analogues also shows that the pyrrolo ring and the 9-carboxylic acid group are not essential for activity as they can be replaced by a pyridinol ring and a 9-hydroxy group respectively.

The determination of the molar absorption coefficient of the prosthetic group (18,400 M\(^{-1}\) cm\(^{-1}\) at 249 nm) enables its quantitative analysis. Thus it could be established that methanol dehydrogenase contains one prosthetic group per enzyme molecule. The consequences of this result in relation to already known properties of this ‘quinoprotein’ dehydrogenase are discussed.

As was previously shown, the prosthetic group of methanol dehydrogenase is a novel, nitrogen-containing o-quinone [1–4]. Recently, based on X-ray diffraction analysis of a degradation product, a chemical structure was proposed [5]. However, no evidence was presented for the presence of the o-quinone group in the proposed structure of the prosthetic group. Moreover, the question of whether the proposed structure represents that of the prosthetic group in situ remained unanswered.

In this paper, the structure of the directly isolated prosthetic group is described. Furthermore, some chemical and physical properties which are valuable for its characterization and estimation, are reported.

The reconstitutive ability of the prosthetic group could be investigated as we found that glucose dehydrogenase from *Acinetobacter calcoaceticus* contains the same prosthetic group and a suitable apoenzyme could be prepared [6]. Derivatives of the prosthetic group and structural analogues were tested with the apoenzyme system in order to see which structural element of the prosthetic group is essential for its functioning or for its binding to the apoenzyme.

**MATERIALS AND METHODS**

**Materials**

As the prosthetic groups of different methanol dehydrogenases are the same [4], experiments were performed with the purified prosthetic group from *Hyphomicrobium X* and *Methylphilus methylotrophus* (the latter provided in the form of Pruteen by ICI, Great Britain) [4]. 1,10-Phenanthroline-5,6-dione was from K&K. The synthesis of the other phenanthroline diones will be described elsewhere. Seppak C18 cartridges were from Waters, proteinase K from Boehringer, silicagel 60F\(_{254}\) thin-layer chromatography plates from Merck and cellulose thin-layer chromatography plates from Baker.

**Preparation of Derivatives of the Prosthetic Group**

Reduction with Sodium Borohydride. Approximately 1 mg prosthetic group was dissolved in 1 ml water and 10 mg NaBH\(_4\) was added. After 30 min, the solution was diluted 20-fold with water and adjusted to pH 2.0 with 2 M HCl. The reduced compound was

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*Abbreviations.* ESR, electron spin resonance; NMR, nuclear magnetic resonance; compound la, 2,7,9-tricarboxy-1H-pyrrolo[2,3-f] quinoline-4,5-dione; compound Ia, 2,7,9-tricarboxy-1H-pyrrolo[2,3-f] quinoline-4,5-dihydro-4,5-diol.

*Enzymes.* Methanol dehydrogenase (EC 1.1.99.8); glucose dehydrogenase (EC 1.1.99.–).

adsorbed to a Seppak C18 cartridge, washed with dilute HCl, pH 2.0, and eluted with methanol/water (7/3, v/v). The resulting product (after drying) was methylated without further purification. For other purposes, the solution was subjected to semi-preparative high-performance liquid chromatography.

Methylation with Dimethyl Sulphate. Approximately 0.5 mg of the compound to be methylated was dried for 20 h in vacuo over P2O5 and dissolved in 200 μl dry N,N-dimethyl formamide. Then 5 mg anhydrous K2CO3 and 10 μl dry dimethyl sulphate were added under continuous stirring. The course of the reaction was followed by silicagel thin-layer chromatography with chloroform/ethanol (95/5, v/v). If necessary, more K2CO3 and dimethyl sulphate were added. The reaction mixture was diluted 20-fold with water and passed through a Seppak C18 cartridge. The cartridge was washed with 10 ml water and the compound eluted with methanol. Finally, it was purified by semi-preparative high-performance liquid chromatography.

Oxidation with Sodium Periodate

Approximately 0.1 mg compound IIa was dissolved in 1 ml 0.2 M KH2PO4, the pH adjusted to 5.0 and 50 μl 1 mM NaIO4 added. The course of the reaction was followed by high-performance liquid chromatography or on cellulose thin-layer chromatography plates with propanol/2% ammonium acetate (1/1, v/v).

Analytical and Semi-Preparative High-Performance Liquid Chromatography

The prosthetic group was analysed on a μ Bondapak C18 column (0.38 x 30 cm) in methanol/12.5 mM KH2PO4, adjusted to pH 2.0 with conc. H3PO4 (27/73, v/v) and the effluent monitored at 254 nm. In the case of derivatives with a small retention time, the methanol content was lowered to 21% (v/v). The methylated prosthetic group and derivatives were analysed with methanol/water (45/55, v/v) and 1,10-phenanthroline-5,6-dione (III) with methanol/water (27/73, v/v). For semi-preparative high-performance liquid chromatography, the compounds were dissolved in the solvent used for their analysis. Each injection (up to 500 μl) contained such an amount of material that the separation could still be monitored by the detector operating at its lowest sensitivity. The flow rate was in all cases 1.5 ml/min.

Molar Absorption Coefficient of the Prosthetic Group

Approximately 3 mg prosthetic group was dissolved in 400 μl of 2H2O and a 300-MHz NMR spectrum was recorded. The integrals of the proton signals were compared with those of a known concentration of external tetramethylsilane.

Estimation of Prosthetic Group
in Methanol Dehydrogenase

Methanol dehydrogenase from Hyphomicrobium X was purified as previously described [1]. The A280/A345 and A345/A280 ratios were 9.6 and 2.6 respectively. The pH of 1 ml methanol dehydrogenase solution, containing a known amount of protein, was lowered to 2.0 by adding a known volume of 2 M HCl. The denatured protein was removed by centrifugation (5 min at 12000 × g). The sediment was washed with 1 ml dilute HCl pH 2.0 and suspended in 1 ml 0.05 M potassium phosphate pH 7.0. Proteinase K was added (0.1 mg/mg protein) and after incubation at 37°C until the solution became clear, an absorption spectrum was recorded. The quantity of prosthetic group in the supernatants was estimated by comparing the peak area obtained by high-performance liquid chromatography with that of a known amount of prosthetic group. Protein concentrations were determined by the Lowry procedure [7], using bovine serum albumin as a standard.

Test for Biological Activity

Glucose dehydrogenase from Acinetobacter calcoaceticus and its apoenzyme were prepared as described [6]. Samples to be tested were mixed with an equal volume of apoenzyme solution in 0.02 M potassium phosphate pH 7.0. After incubation for 30 min at room temperature, 0.8 ml 0.2 M Tris/HCl pH 8.0 containing 40 mM glucose and 0.8 ml 0.2 mM Wurster’s blue (referred to as Compound II in [1]) in water, were added. After mixing, the decrease in absorbance was recorded at 600 nm. Enzymatic activity is expressed as the rate of reduction of Wurster’s blue: 1 unit corresponds to 1 nmol reduced/min, using a molar absorption coefficient of 9 x 10^3 M⁻¹ cm⁻¹ [4].

1H NMR and Mass Spectrometry

Mass spectra were taken with a Varian MAT 311A spectrometer. Field desorption mass spectrometry was performed with activated carbon emitter wires. 1H NMR spectra were recorded at 30°C on a Varian SC 300 spectrometer operating at 300 MHz using pulse Fourier transform mode (max. 23 K data table of 32 bits). Internal references were 2,2-dimethyl-2-silapentane-5-sulphonate for 2H2O and tetramethylsilane for C2D3Cl3 solutions.
RESULTS

Reduction with Sodium Borohydride

Previous work [1] had shown that reduction of the prosthetic group with NaBH₄ and subsequent oxidation with O₂ leads to a product (IIa) that differs from it in several respects. Thus compound IIa no longer oxidizes reduced methylene blue [1] and its retention time on high-performance liquid chromatography is 5.4 min while this is 11.5 min for the prosthetic group Ia, indicating an increase in polarity. Comparison of Fig.1 and 2 shows that the absorption spectrum has also changed. The increased number of protons in compound IIa compared to Ia made it especially suitable for analysis by NMR spectroscopy.

The reduction to an aliphatic vicinal diol seemed to be unusual as we could find no precedent in the literature. However, when the model compound 1,10-phenanthroline-5,6-dione (III) was treated in the same way, it appeared that the product 1,10-phenanthroline-5,6-dihydro-5,6-diol (IV) had also lost its quinone nature as it no longer oxidized reduced methylene blue. Also the retention time on high-performance liquid chromatography had decreased from 5.5 min for III to 3.0 min for IV.

Methylation

Methylation of compounds Ia and IIa resulted in highly fluorescent compounds that were soluble in chloroform. This was found to be essential for a detailed analysis by NMR and mass spectrometry. Thin-layer chromatography on silicagel with chloroform/ethanol (95/5, v/v) revealed RF values of 0.54 and 0.64 respectively, while partially and non-methylated material remained at the start position. As expected, reversed-phase high-performance liquid chromatography of the methylated compounds no longer required acidified solvents and furthermore, due to their decreased polarity, the methanol content had to be raised to 45% (v/v) (Fig.3). The trimethyl esters Ib and IIb had retention times in this system of 9.0 and 6.0 min respectively. Material eluting without retention appeared to be partially methylated, as was demonstrated by the fact that it had an absorption spectrum differing from the starting material, while further methylation converted it to the methylated compounds described above, with absorption spectra as depicted in Fig.1 and 2.

NMR Spectra

The ¹H-NMR spectrum of the prosthetic group Ia in D₂O (pH 8) only showed two aromatic signals at 7.14 ppm (broadened singlet, 3-H) and 8.16 ppm (broad, 8-H). The spectrum of its trimethyl ester (Ib) in C₂D₃Cl₃ indicated that the compound was methylated at three positions (methyl signals at 3.98, 4.08 and 4.18 ppm) while a fourth labile hydrogen was not
Fig. 3. High-performance liquid chromatogram of methylated compounds. A mixture of compounds Ia and IIa was methylated. The products were adsorbed to a Seppak C18 cartridge and eluted with 1 ml methanol. 5 µl of this solution was chromatographed on a µBondapak C18 column (0.38 × 30 cm) with methanol/water (45/55, v/v) as the solvent at a flow rate of 1.5 ml/min. The absorbance of the effluent was recorded at 254 nm

methylated (13.0 ppm, broad, 1-H). The aromatic hydrogens were observed at 7.49 ppm (3-H, doublet 2 Hz due to spin-coupling with 1-H) and 8.90 ppm (8-H).

Only aromatic hydrogens were found in the 1H NMR spectrum of the reduction product IIa in 2H2O (singlet at 6.9 ppm, broad signal at 8.0 ppm). However, the H2HO signal obscures some other signals of IIa as became apparent from the spectrum of the methylation product IIb in C2HCl3. Those four one-proton signals in the 1H NMR spectrum of IIb that do not correspond to signals found for compound Ib constitute a —CHOH—CHOH-grouping (Fig. 4). From the fact that besides these four signals no major changes had occurred, it is clear that this aliphatic diol structure can only arise from a quinone structure. The other signals of compound IIb were found at 3.93, 4.03 and 4.08 ppm (three methyl groups), 7.12 ppm doublet 2 Hz, broadened, 3-H), 8.60 ppm (broadened singlet, 8-H) and 11.9 ppm (broad 1-H). Small coupling constants of 0.5 – 1 Hz each found between 3-H and 4-H and between 5-H and 8-H are in agreement with the structure of the trimethyl ester IIb, as well as the coupling constant of 2 Hz between 1-H and 3-H. The large coupling constant between 4-H and 5-H indicates that these hydrogens are anti-opposed.

The low-field positions of 1-H and 8-H in the trimethyl esters Ib and IIb both point to a hydrogen bond between 1-H and 9-C=O.

The reduction of a quinone to an aliphatic diol was also found when reducing 1,10-phenanthroline-5,6-dione III. The 1H NMR spectra of the reduced product in C2HCl3 are in full agreement with the structure of 1,10-phenanthroline-5,6-dihydro-5,6-diol (IV), showing in particular a two-proton singlet at 4.96 ppm (5-H and 6-H) and a broadened singlet at 2.7 ppm (OH; signal exchanges with 2H2O).

Mass Spectrometry

The mass spectrum of the trimethyl ester Ib showed a M+ ion at m/e 374 (69%, relative intensity) and fragments at m/e 372 (15), 344 (32), 342 (94), 318 (26), 314 (41), 286 (47), 282 (72), 254 (100), 239 (11) and 228 (23). However, the field desorption mass spectrum showed a M+ ion at m/e = 372. This difference can be explained by assuming that the compound is reduced by the residual water vapour in the inlet system, generating a (M + 2)+ peak. This behaviour is common to many quinones, especially o-quinones [8].
The mass spectrum of the trimethyl ester IIb showed a $M^+$ ion at $m/e$ 376 (19) and fragments at $m/e$ 358 (67), 347 (64), 326 (50), 315 (49), 298 (37), 283 (23), 266 (25), 255 (24) and 43 (100). Here the field desorption method showed equally a $M^+$ ion at $m/e$ 376, in agreement with the fact that this compound is no longer a quinone.

Mass spectral analysis of 1,10-phenanthroline-5,6-dione (III) and 1,10-phenanthroline-5,6-dihydro-5,6-diol (IV) revealed $M^+$ ions at $m/e$ 210 and 214 respectively. These results clearly confirm the observation that reduction of the diones Ia and 1,10-phenanthroline-5,6-dione (III) with NaBH₄ under the conditions employed, introduces four hydrogen atoms.

Oxidation of Compound IIa with Sodium Periodate

Reaction of compound IIa with acid periodate leads to a strongly violet-fluorescing product [1], showing a different $R_f$ on cellulose thin-layer chromatography (0.56 versus 0.33 for IIa itself) and an increased retention time on high-performance liquid chromatography (6.4 min versus 5.4 min for IIa).

In view of the fact that compound IIa contains an aliphatic vicinal diol structure, this reaction is not unexpected. Although the product has not yet been identified, its chromatographic properties and its failure to oxidize reduced methylene blue, exclude that it is the dione Ia or a closely related quinone. As compound Ia fails to react with periodate under these conditions this confirms that the vicinal diol group of compound IIa must have its origin in the quinone group of the prosthetic group compound Ia.

Molar Absorption Coefficient of the Prosthetic Group

A 311-fold dilution of a 16.8 mM (estimated by NMR) solution of the prosthetic group Ia in 0.05 M potassium phosphate pH 7.0 had an absorbance at 249 nm of 0.995. Hence the molar absorption coefficient at that wavelength is $18400 \text{ M}^{-1} \text{ cm}^{-1}$.

The Prosthetic Group Content of Methanol Dehydrogenase

As estimated by high-performance liquid chromatography, 30.6 nmol prosthetic group Ia could be extracted from 3.74 mg methanol dehydrogenase (31.2 nmol assuming $M_r = 120000$ for this enzyme [1]). As the precipitated protein solubilized with proteinase K showed no absorbance above 300 nm (indicating that the prosthetic group had been completely extracted), 0.98 prosthetic group per enzyme molecule is found.

Biological Activity

Table 1 summarizes the biological activity exhibited by the prosthetic group, some of its derivatives and synthetic analogues with the apoenzyme from glucose dehydrogenase. The choice of the phenanthroline-diones Va, Vb and Vc as structural analogues of the prosthetic group was stipulated by their relative ease of preparation. Owing to different starting materials employed for their synthesis the compounds have different ester groups, which excludes a direct comparison of their biological activity with that of prosthetic group.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration</th>
<th>Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prosthetic group Ia</td>
<td>0.2</td>
<td>53.7</td>
</tr>
<tr>
<td>Compound IIa</td>
<td>100</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>Compound IIa after NaIO₄ oxidation</td>
<td>100</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>Phenanthroline-dione Va</td>
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</tr>
<tr>
<td>Phenanthroline-dione Vb</td>
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<td>Phenanthroline-dione Vc</td>
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<td>12.9</td>
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<tr>
<td>Water</td>
<td>-</td>
<td>&lt;0.1</td>
</tr>
</tbody>
</table>

DISCUSSION

The results obtained by NMR and mass spectral analysis clearly indicate that the directly isolated prosthetic group of methanol dehydrogenase is the quinone Ia. The question can be raised whether the proposed structure of Ia is in fact the structure of the prosthetic group in the enzyme. However, the detached free radical form of the prosthetic group from the enzyme (which is functional in the enzyme catalysis [4]) has the same hyperfine ESR spectrum as that
induced in the isolated prosthetic group [2]. Therefore, it is concluded that no modification has occurred during the isolation, a view which is supported by the fact that the isolated prosthetic group is active in the apoenzyme test.

As mentioned above, the free radical form is functional in the enzyme catalysis and it has the properties of a quinone free radical [2]. This implies that the quinone group of the molecule is participating in the redox cycle of the enzyme. 2-Mercapto-ethanol is able to reduce the prosthetic group to the 'semiquinone' form (Ia⁻) [2] or to the 'quinol' form, the corresponding 4,5-diol [4] which can be converted with oxygen to the original oxidized form, Ia [4]. On the other hand, NaBH₄ reduces it to the aliphatic 'diol' form, compound Ila, which is no longer active in the apoenzyme test (Table 1). Obviously, modification of the quinone structure results in complete loss of activity, which is not the case for certain alterations in other parts of the molecule (Table 1). Therefore we conclude that the quinone part of the molecule is the essential group participating in the different redox forms of the enzyme.

The functionality of the structural analogues of the prosthetic group in the apoenzyme test offers an opportunity to extend our knowledge concerning the chemistry of the prosthetic group and the mechanism of enzyme catalysis. The synthesis of other, similar analogues is in progress.

As the occurrence of the prosthetic group is not restricted to methanol dehydrogenase [6,9], the introduction of an appropriate trivial name seems justified. In view of its chemical structure and the relevant part of the molecule, we propose the name of pyrroloquinolone quinone. In analogy with distinct groups like haemoproteins and flavoproteins, we suggest classifying this novel group of dehydrogenases as 'quinoproteins'.

The conclusion that methanol dehydrogenase contains only one prosthetic group is somewhat surprising in the light of the other properties of the enzyme. For instance, this result implies that only two electrons can be accepted per enzyme molecule, although methanol can be oxidized to formic acid by the enzyme and no evidence was found for the presence of formaldehyde as intermediate [1]. In any case, the possibility that one enzyme molecule oxidizes methanol directly to formic acid is excluded.

Although most of the methanol dehydrogenases have $M_r = 120000$, others have been reported which have $M_r = 60000$ [10]. As the *Hyphomicrobium* enzyme ($M_r = 120000$) contains one prosthetic group, it would be interesting to compare the enzymological properties of both groups of enzymes.

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Note Added in Proof: After this paper had been accepted for publication, it appeared that an extract from methanol dehydrogenase contains also reduced prosthetic group, 2,7,9-tricarboxy-1H-pyrrolo[2,3-f]quinoline-4,5-diol, which is surprisingly stable against oxidation under these conditions. This compound can not be detected in the described chromatographic procedure because it shows practically no absorbance at 254 nm and an unfavourable polarity for this system. Ultimately, it appears now that the enzyme contains two prosthetic groups of which one is detected as the reduced- and the other as the oxidized-form of the prosthetic group.
Characterization of the second prosthetic group in methanol dehydrogenase from *Hyphomicrobium X*

Procedures are described for preparing 2,7,9-tricarboxy-1H-pyrrolo[2,3-f]quinoline-4,5-diol (pyrrolo-quinoline quinol) from 2,7,9-tricarboxy-1H-pyrrolo[2,3-f]quinoline-4,5-dione (pyrrolo-quinoline quinone).

When methanol dehydrogenase is denatured, two compounds are liberated which have the same properties as the quinone and quinol mentioned above. On analysing the extract by high-performance liquid chromatography, one molecule of the quinone and one molecule of the quinol per enzyme molecule are found.

Mixtures of pyrrolo-quinoline quinone and pyrrolo-quinoline quinol at high pH produce the semiquinone form and, under certain conditions, a diamagnetic complex. Since electron spin resonance (ESR) shows that methanol dehydrogenase contains the semiquinone and the absorption spectrum suggests the presence of a diamagnetic dimer, it is tentatively concluded that the two prosthetic group molecules in the enzyme interact with each other.

NMR experiments of pyrrolo-quinoline quinone in $^2$H$_2$O demonstrate that it is partly hydrated, most probably at the C-5 position. Although methanol adds in the same way, it is still questionable whether the product of this addition plays a role in the mechanism of the enzymatic reaction.

Potentiometric titrations show a midpoint potential of the quinone/quinol couple of $+90$ mV at pH 7.0 and the formation of the semiquinone as an intermediate in the titration at pH 13.0.

Methanol dehydrogenase (EC 1.1.99.8) has 2,7,9-tricarboxy-1H-pyrrolo[2,3-f]quinoline-4,5-dione (pyrrolo-quinoline quinone) as a prosthetic group and high-performance liquid chromatography analysis after denaturation demonstrated one molecule of pyrrolo-quinoline quinone per enzyme molecule in the extract [1].

![Pyrrolo-quinoline quinone and quinol](image)

It was later found, however, that the extract contains yet another compound which appears to be pyrrolo-quinoline quinol. This compound is relatively resistant to autoxidation under the extraction and chromatography conditions described. In addition, its properties are unsuitable for detection in the chromatography system which was used for the quinone, which is why it was missed in our earlier investigation.

In this paper, the characterization of pyrrolo-quinoline quinol and its estimation in the extract of the denatured enzyme are reported. Since one quinone and one quinol molecule per enzyme molecule are found, the properties of the enzyme are compared to those of mixtures of the compounds in order to see whether the prosthetic groups in the enzyme are able to interact with each other.

**MATERIALS AND METHODS**

**Materials**

Methanol dehydrogenase, pyrrolo-quinoline quinone and its trimethyl ester were prepared and purified as previously described [1,2]. Seppak C$_{18}$ cartridges and the $\mu$Bondapak C$_{18}$ column were from Waters Associates. All N$_2$ gas used was purified from traces of oxygen by treatment with catalyst R 3–11 (a kind gift from BASF).

**Reduction of Pyrrolo-quinoline Quinone**

a) A solution of the quinone (2 mg/50 ml) in 2 M HCl was made anaerobic by flushing with N$_2$ and a slight excess of phenylhydrazine was added. The course of the reaction was followed by observing the increase in absorbance at 310 nm. The solution was passed through a Seppak C$_{18}$ cartridge and the cartridge washed with an HCl solution of pH 1.5. The product was eluted with methanol and stored under N$_2$ at $-20^\circ$C.

b) A solution of the quinone (30 mg/2 ml) in water was adjusted to pH 7.0 with 2 M NaOH and 2 mg of PtO$_2$ was added. H$_2$ was passed through until the reaction was complete, as judged by high-performance liquid chromatography. The catalyst was removed by filtration. After acidification with 2 M HCl, the precipitated product was collected by
For WNR measurements the sediment was dried in vacuo over P₂O₅.

**Reduction of Pyrrolo-quinoline Quinone Trimethylester**

30 mg pyrrolo-quinoline trimethylester was dissolved in 2 ml trifluoroacetic acid. After the addition of 5 mg PtO₂, the solution was flushed with H₂ until the reaction was complete, as was established by high-performance liquid chromatography [1] in methanol/water (70/30, v/v) and detection at 365 nm. The catalyst was removed by filtration. For NMR measurements, trifluoroacetic acid was removed with a stream of N₂ and the product dissolved in nitrogen-flushed (⁴H)pyridine.

**Potentiometric Titrations**

The midpoint potentials at different pH values were determined by potentiometric titration of 0.1 mM pyrrolo-quinoline quinone solutions, using a Metrohm EA 234 combined micro platinum reference electrode (Ag/AgCl, 3 M KCl) at 20 °C. At pH 2.0 (0.1 M KH₂PO₄ titrated with concn H₂PO₄), I⁻ was used as the oxidant. At pH 7.0 (0.1 M potassium phosphate buffer), 5 µM phenmethazole methanol was included as a mediator in the mixture and I⁻ or K₃Fe(CN)₆ were used as oxidants. The same oxidants could be used at pH 13.0 (2 M sodium salicylate titrated with concn NaOH).

The titration curves were analyzed using the extended version of the Reed-Berkson method [3]. The value of the semiquinone formation constant was determined according to Clark [4].

**Analysis of Prosthetic Group in Methanol Dehydrogenase**

25 µl methanol dehydrogenase in 0.02 M potassium phosphate buffer pH 7.0 was mixed with 25 µl 1 M NaH₂PO₄, pH 1.0 (with concn HCl) and 100 µl methanol was added. After centrifugation (5 min at 12000 × g), the supernatant was analyzed for prosthetic group by high-performance liquid chromatography [1] using methanol/water/85% H₃PO₄ (45/54.5/0.5, v/v/v) as the solvent and detection at 313 nm. The total amount of prosthetic group in the extract was determined by repeating the analysis after the quinol was oxidized to the quinone by an excess of 2,6-dichloroindophenol.

**NMR Spectroscopy**

¹H and ¹³C NMR spectra were recorded with a Varian SC 300 spectrometer operating at 300 and 75.5 MHz respectively, using pulse Fourier-transform mode (max. 32 K data table of 32 bits). The internal reference was 2,2-dimethyl-2-silapentane-5-sulphonate for ¹H NMR of (C₂H₅)₂SO solutions and tetramethylsilane for C₂HCl₅ solutions. For ¹³C spectra, the solvent signal of (C₂H₅)₂SO at 39.6 ppm was used as reference.

**RESULTS**

**Reduction of Pyrrolo-quinoline Quinone and Characterization of the Product**

The product obtained after the reduction of pyrrolo-quinoline quinone with phenylhydrazine or catalytic dehydrogenation shows an absorption spectrum (Fig. 1) which is quite different from that of the quinone; it has a molar absorption coefficient of 25050 M⁻¹ cm⁻¹ at 302 nm (based on a molar absorption coefficient of 18400 M⁻¹ cm⁻¹ for the quinone at 249 nm [1]). The direction of the shift of the wavelength maxima is in accordance with that which is normally observed for the conversion of a quinone into a quinol [5].

Further information on the nature of the product was obtained from ¹H NMR spectra of pyrrolo-quinoline quinone, its trimethyl ester and their reduced products. The quinone in (C₂H₅)₂SO showed signals at 7.24 ppm (doublet 2 Hz, 3-H), 8.63 ppm (8-H) and 13.3 ppm (broad, 1-H), while its reduced product gave signals at 7.42 ppm (doublet 2 Hz, 3-H), 8.61 ppm (8-H), 9.33 ppm (broad, OH) and 12.4 ppm (broad, 1-H). For the quinone trimethyl ester in C₂HCl₅, signals were found at 3.98, 4.08 and 4.18 ppm (methyl groups), 7.49 ppm (doublet 2 Hz, 3-H), 8.90 ppm (8-H) and 13.0 ppm (broad, 1-H) and for the reduced compound at 4.01, 4.09 and 4.17 ppm (methyl groups), 6.00 ppm (broad, OH + H₂O), 7.54 ppm (doublet 2 Hz, 3-H), 8.80 ppm (8-H) and 12.4 ppm (broad, 1-H). It is likely, therefore, that the pyrrole and pyrrole rings were not involved in the reduction. Evidence that the carbonyl groups of the quinone become reduced was obtained by comparing the ¹³C NMR spectra in (C₂H₅)₂SO of the quinone: 113.8 (C-3), 124.8, 126.8, 127.9, 129.3 (C-8), 134.5, 136.3, 147.2, 149.1 (nine aromatic carbon atoms), 161.2, 165.3, 169.0 (three COOH groups), 173.7 and 178.4 ppm (two 'quinone' carbon atoms) and its reduced product: 105.6 (C-3), 111.0, 119.2 (C-8), 122.9, 123.7, 128.1, 131.2, 134.5, 137.9, 140.9, 142.5 (eleven aromatic carbon atoms), 162.2, 165.4 and 170.1 ppm (three COOH groups). Similarly, pyrrolo-quinoline quinone trimethyl ester showed 'quinone' carbon signals (173.9 and 177.5 ppm), whereas its reduced product showed two aromatic carbon signals instead. It is concluded, therefore, that the reduced product is the diol form of pyrrolo-quinoline quinone, referred to as pyrrolo-quinoline quinol.

As can be expected, pyrrolo-quinoline quinol is relatively resistant to autoxidation at low pH, but is rapidly oxidized at high pH. For instance (Fig. 1), after flushing a cuvette containing a neutral solution of the quinol with air, the absorption spectrum rapidly turned into that of the quinone.
Fig. 2. High-performance liquid chromatography of an extract of methanol dehydrogenase. Methanol dehydrogenase in 0.02 M potassium phosphate pH 7.0 was extracted with an equal volume of 1 M potassium phosphate, adjusted to pH 1.0 with concn HCl. After the addition of 2 vol. methanol and centrifugation (5 min at 10000 × g), 10 μl of the supernatant was chromatographed on a β-Bondapak C18 column (0.38 × 30 cm) with methanol/water/85% H3PO4 (45/54.5/0.5, v/v/v) as the solvent at a flow rate of 1.5 ml/min. The absorbance of the effluent was monitored at 313 nm.

The polarity of the quinol compared to that of the quinone is distinctly lower as it precipitates at low pH and its retention time during reversed phase chromatography (Fig. 2) is much longer. Similarly, the trimethyl ester of the quinol has a higher retention time (6.2 min) than the trimethyl ester of the quinone (3.4 min).

Prosthetic Groups of Methanol Dehydrogenase

In a typical experiment, 3.3 nmol methanol dehydrogenase (assuming a molar absorption coefficient of 175400 M⁻¹ cm⁻¹ at 280 nm for a pure preparation [1]) yielded 3.12 nmol pyrrolo-quinoline quinone on extraction. In addition to the peak of the quinone, a larger peak with the same retention time as the quinol was seen (Fig. 2). After oxidation, only the quinone was found in a quantity of 6.93 nmol.

Potentiometric Titration of Pyrrolo-quinoline Quinol

At pH 2 and 7, the titration curves of the quinol did not significantly differ from those expected for a two-electron transfer. The calculated midpoint potentials for the quinone/quinol couple at pH 2.0 and 7.0 were +419 mV and +90 mV, respectively.

At pH 13.0, a titration curve was obtained which deviated from the theoretical n = 2 curve (E° = 25 mV). This deviation did not change after a tenfold increase or decrease of the quinol concentration. Thus, this deviation may be ascribed to the formation of an intermediate semiquinone [4], a view substantiated by ESR measurements which show that the semiquinone is stable in sodium salicylate buffer pH 13.0 [6]. The semiquinone formation constant was determined to be 2.54. This means that the maximum amount of the semiquinone formed during the titration in this system is 44% of the total amount of pyrrolo-quinoline quinone. Accordingly, the midpoint potentials of the semiquinone/quinol and quinone/semiquinone couples at pH 13.0 are -242 mV and -218 mV respectively. During and after the titration, absorption spectra were measured in order to check that the semiquinone and the quinone were indeed formed.

Spectral Properties of Pyrrolo-quinoline Quinone and Pyrrolo-quinoline Quinol

¹H-NMR of the quinone in D2O at pH 6.0 showed that this solution contained two components in a molar ratio of 2:1 with signals at 7.17 ppm (s) and 8.16 ppm (broadened) for the minor and at 7.15 ppm (s) and 8.26 ppm (broadened) for the main component. ¹3C-NMR spectra of the same solution showed signals for the minor component, amongst others, at 92.5 ppm and 193.3 ppm which can be attributed to a >C(OH)2 and >C=O structure, respectively. In principle, each of the carbonyl groups can become hydrated but we prefer the C-3 position because the hydrated compound shows an upfield shift for 8-H of 0.10 ppm and a downfield shift for 3-H of 0.02 ppm.

A similar phenomenon of addition was observed when methanol was added to a solution of pyrrolo-quinoline quinone trimethylster in C₅H₇Cl₂. ¹H-NMR spectra after the addition of methanol showed signals at 3.37, 3.96, 4.04, 4.14 (CH₃ groups), 7.44 (d, 2 Hz, 3-H) and 8.75 (s, 8-H) ppm. Compared with the values for the quinone trimethylster, the signal at 3.37 ppm points to >C(OH)OCH₃ group and again, the larger shift for 8-H (0.15 ppm) compared to that for 3-H (0.05 ppm) points to elimination of the carbonyl group at C-5.

Methanol dehydrogenase contains free radical [6] and it was, therefore, attempted to obtain the absorption spectrum of pyrrolo-quinoline semiquinone. Since this semiquinone had been obtained earlier [6] in sodium salicylate (pH 13.0), attempts were made to measure its absorption spectrum in this solvent. Although an absorption maximum was detected at 458 nm, further analysis was impossible due to the high background absorbance at lower wavelengths. Accordingly, other solvents were tried.

After mixing equimolar amounts of the quinone and quinol anaerobically in a methanol-containing LiOH solution (pH 12.7), ESR measurements showed the same hyperfine spectrum as was described earlier [6]. Comparison of the absorption spectra before (Fig. 3) and after (Fig. 4) mixing shows the presence of a new compound in the mixture with an absorption maximum at 458 nm.

When the same experiment was performed with NaOH/NaCl-containing solvents (pH 13.0), the absorption spectrum of the mixture (Fig. 4) was quite different. CsOH/CsCl-containing solvents caused an even larger shift to higher wavelengths. In both cases a small ESR signal was observed without hyperfine structure but with the characteristics of an immobilized free radical, as is found for methanol dehydrogenase [6]. After standing at low temperature, the compounds precipitated. When the precipitates were dissolved in an HCl solution pH 2.0, equimolar concentrations of the quinone and quinol were found.

As all these different compounds show absorption maxima above 400 nm, a careful inspection of the spectrum of methanol dehydrogenase (Fig. 5) was necessary in order to look for the presence of these compounds in the enzyme.
Fig. 3. Absorption spectra of pyrrolo-quinoline quinone and pyrrolo-quinoline quinol in a LiOH-containing solvent. Pyrrolo-quinoline quinol, obtained by reduction of the quinone with $\text{H}_2$, was diluted with an $\text{N}_2$-flushed solution of methanol/water (50/50, v/v) saturated (0.35 M) with LiOH (pH 12.7). After recording the spectrum (---), air was admitted to the cuvette. The spectrum was measured again (-----) and it appeared that the spectrum was identical to that of pyrrolo-quinoline quinone under these conditions.

Fig. 4. Absorption spectra of mixtures of equimolar concentrations of pyrrolo-quinoline quinone and pyrrolo-quinoline quinol in alkaline solvents, 100 μl of a 1.8 mM pyrrolo-quinoline quinol solution, obtained by reduction of the quinone in methanol/water (50/50, v/v) with $\text{H}_2$, was diluted anaerobically and another 100 μl diluted anaerobically with 2 ml methanol/water (50/50, v/v) saturated with LiOH (0.35 M, pH 12.7). After flushing the solutions with $\text{N}_2$ for 20 min, the solutions were mixed anaerobically and the absorption spectrum recorded (---). From a potentiometric titration under similar conditions it could be estimated that the concentration of the semiquinone is about 50% of the theoretical amount. A similar experiment was performed using a methanol/water (50/50, v/v) solvent (pH 13.0) containing 0.27 M NaCl and 0.08 M NaOH (-----).

DISCUSSION

Because pyrrolo-quinoline quinone is a complicated molecule, several reduction products of it can be expected. The only product hitherto found is the stable dihydro-quinol form [1] which is obtained by reduction with excess NaBH₄.

The reduction conditions employed here give, however, a product which is the quinol form of pyrrolo-quinoline quinone, as is clearly shown by NMR. This compound seems biologically significant since it is present in an extract from isolated methanol dehydrogenase. High-performance liquid chromatography analysis shows that the extract contains one molecule of the quinone and, in addition, one molecule of the quinol per enzyme molecule.

The potentiometric titrations at pH 2.0 and 7.0 confirm that the quinol is the two-electron reduced product of pyrrolo-quinoline quinone, consistent with the two additional protons found in this compound by NMR. The corrected midpoint potentials for the quinone/quinol couple are +419 mV and +90 mV respectively, values normally found for a quinone [7]. The behaviour at pH 13.0 shows, as can be expected, semiquinone formation.

The results suggest that quinoprotein dehydrogenases will have a higher midpoint potential than flavoprotein and NAD(P)-dependent dehydrogenases. Although the value for methanol dehydrogenase is still unknown, the fact that the enzyme is coupled to the respiratory chain at the level of cytochrome $c$ [8] points in this direction.

NMR experiments show that pyrrolo-quinoline quinone in water-containing solutions is partly hydrated, probably at C-5, a position where reactions with acetone [9] and acetaldehyde [10] also take place. Addition of acetone or acetaldehyde does not change the absorption spectrum of the enzyme and since it is unknown how pyrrolo-quinoline quinone is bound to the enzyme, it is impossible to discuss the reactivity of C-5 in the enzyme. The same difficulty is met when judging the significance of the reaction between pyrrolo-quinoline quinone and methanol as a model system for the enzymatic reaction, but, as cinnamyl alcohol added to an oxidized form of methanol dehydrogenase is converted immediately to cinnamaldehyde [11], a mechanism involving a quinone-alcohol adduct which is subsequently oxidized by electron acceptor, is excluded.

The absorption spectrum of the semiquinone is shown in Fig. 4. It should be noted, however, that this is an anionic form and the semiquinone in the enzyme may be a neutral form in which, for example, the nitrogen atoms are protonated or bound to the protein. These different situations are probably reflected in the different values of the proton coupling constants of the semiquinone in solution [6] and in the enzyme [12], and the absence of a proton signal in the latter case. But, as the absorption spectrum of the enzyme shows a maximum in the same region as the semiquinone and oxida-
tion of the enzyme results in a decline of the absorbance at this wavelength (Fig. 5), accompanied by a concomitant decrease of the ESR signal [10], it is concluded that the shoulder at 475 nm in the absorption spectrum of the enzyme is related to the semiquinone.

Spectral measurements of mixtures of pyrrolo-quinoline quinone and pyrroloquinoline quinol in solutions with alkali hydroxides from Li to Cs having equal cation concentrations, show a shift of the absorption maximum to higher wavelengths (Fig. 4) and a decrease of the ESR signal with a change of the hyperfine structure spectrum to that of a 'powder spectrum'. A similar behaviour has been reported for several o-quinones and the shift to higher wavelengths has been attributed to the formation of a diamagnetic radical complex in which the alkali-metal ion is included as a counterion [13].

On close inspection of the absorption spectrum of the isolated enzyme (Fig. 5), a broad shoulder at 600 nm which disappears on oxidation of the enzyme is observed. This may indicate that the enzyme also contains a diamagnetic complex, implying that the two prosthetic groups in the enzyme have some interaction with each other according to equilibria such as: quinone + quinol ⇌ 2 semiquinone ≡ diamagnetic complex. The interaction between the two prosthetic groups may also be responsible for the complex absorption spectrum in the 300-400-nm region, resulting from the summation of the absorption spectra of the different species.

The question whether the conclusions are valid for all methanol dehydrogenases cannot be definitively answered at the moment. However, the similar absorption spectra and the free radical in the enzyme from Rhodopseudomonas acidophila [14], suggest so.

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Chapter VII

Detection and determination of pyrroloquinoline quinone, the coenzyme of quinoproteins*

A convenient determination of pyrroloquinoline quinone (PQQ) in extracts of purified enzymes is possible with ion-pair chromatography on a HPLC reverse-phase column and with uv detection. However, when culture supernatants have to be analyzed, a fluorescence detection system is more appropriate. Proof for the presence of PQQ can be obtained by treating such a sample with butyraldehyde, which converts the coenzyme into a stable adduct having a suitable retention time in the system. The sensitivity and selectivity of the analysis can be further enhanced by reducing the sample with NaBH₄, which produces the dihydrodiol form of the coenzyme (PQQH₂) and oxidizing PQQH₂ with NaIO₄ to a strongly fluorescing compound. A procedure is described for the easy preparation of an apoenzyme from the quinoprotein glucose dehydrogenase of Pseudomonas aeruginosa strains. With this biological test system, very low amounts of PQQ can be detected. However, when PQQ is present in the form of adducts, the analysis has to be performed via reduction to PQQH₄, oxidation with NaIO₄, and HPLC.

Key Words: pyrroloquinoline quinone; PQQH₄; glucose dehydrogenase; quinoprotein; apoenzyme; HPLC.

Pyrroloquinoline quinone (PQQ)² is the semisystematic name for 2,7,9-tricarboxy-1H-pyrrolo[2,3-f]quinoline-4,5-dione (1,2). It is the coenzyme of quinoproteins, a recently discovered class of dehydrogenases which occurs in many bacteria (3).

In contrast to the well-known quinones functioning as carriers in the respiratory chain, PQQ is hydrophilic. Furthermore, it is an ω-quinone instead of a p-quinone. Therefore, special methods had to be developed for the analysis of this novel coenzyme.

Due to the strongly acid group(s), PQQ behaves as an anion under neutral or slightly acid conditions (4). The C-5 carbonyl group reacts easily with aldehydes and ketones, yielding fluorescing adducts (5). Covalent addition of water or alcohols also occurs in this position but, in this case, pH and temperature have a strong influence on the equilibrium, as shown by the variations in fluorescence intensity (PQQ itself is nonfluorescing) with changes in these parameters (5). A stable, strongly fluorescing derivative of PQQ can be obtained, however, by reducing PQQ to its dihydrodiol form (PQQH₂) and oxidizing this PQQH₂ with periodate to an unknown (probably a ring-fission) product (2).

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² Abbreviations used: PQQ, pyrroloquinoline quinone (2,7,9-tricarboxy-1H-pyrrolo[2,3-f]quinoline-4,5-dione); PQQH₄, the dihydrodiol form of PQQ, 2,7,9-tricarboxy-1H-pyrrolo[2,3-f]quinoline-4,5-dihydro-4,5-diol.

Originally, the PQQ determination in purified enzymes was performed by ion-suppression chromatography on a C₁₈ HPLC reverse-phase column (4). PQQ shows a rather broad peak in this chromatographic system, however, probably due to the formation of adducts with solvent molecules. Therefore, knowing the meanwhile discovered properties of the coenzyme, better chromatographic systems were developed. These are compared with a biological test system which uses an easily obtainable apoenzyme of a quinoprotein glucose dehydrogenase (EC 1.1.99.17) from Pseudomonas aeruginosa strains.

MATERIALS AND METHODS

Materials. The PQQ used was prepared by both biological (4) as well as synthetic methods (6). A standardized procedure was developed for converting PQQ into the fluorescing compound. A solution containing PQQ (2 ml) was mixed with 0.2 ml of an alkaline NaBH₄ solution (44 mg NaBH₄ in 1 ml 1 M NaOH) and the mixture put into a tray or beaker. Since O₂ participates in the reaction (a full account of the preparation of PQQH₄ will be given elsewhere), it is essential that the height of the fluid layer is less than 3 mm. The conversion into PQQH₄ was complete after 2.5 h. To prepare the fluorescing compound, 450 µl 1 M Na-acetate, pH 4.7, and 300 µl 150 mM NaIO₄ (a large excess) were added to this solution. The fluorescing compound was formed immediately and remained stable for at least 16 h.

The butyraldehyde adduct of PQQ was prepared by mixing 200 µl of sample with 100 µl 0.2 M Na₂B₄O₇ buffer, adjusted to pH 8.0 with concentrated HCl, and 90 µl 0.5% (v/v) butyraldehyde solution. Complete conversion was achieved in 20 min.

Chemicals were obtained from E. Merck, except NaIO₄ which was from BDH and butyraldehyde which was from Hopkin & Williams Ltd, Chadwell Heath, Sussex, England. Methanol dehydrogenase (EC 1.1.99.8 (7)) and culture supernatants (4) were prepared as described previously.

HPLC analysis. The HPLC system consisted of a Waters Model 6000 A pump equipped with a U6K injection block, a Model 720 system controller, a Model 710B WISP automatic sample injector, and a Model 730 data module. Chromatography was performed with an RCM 100 module containing a 10-µm C₁₈ RCM cartridge. The effluent was monitored with an M 480 uv detector. For fluorescence monitoring, excitation was performed at 360 nm with a F4 T5 BL uv lamp and UG1 excitation filter (Schott), and detection with a KV 418 filter (>425 nm) and a M 420 AC detector. The eluant for ion-suppression chromatography consists of concentrated H₃PO₄/H₂O/CH₃OH (0.4/72.6/27, v/v/v) but a slightly higher methanol concentration (32%) was used for the chromatography of the PQQ–butyraldehyde adduct. For ion-pair chromatography, 15 mM tetrabutylhydrogen sulfate (adjusted to pH 3.5 with concentrated H₃PO₄)/methanol (60/40, v/v) was used.

Apoenzyme assay. P. aeruginosa strains LMD 76.39 and LMD 79.58 were provided by the Laboratory of Microbiology Culture collection, Delft. The first strain was originally obtained as ATCC 10145. The second strain was kindly provided by Professor Dawes (8). The bacteria were cultured aerobically at 30°C in a mineral medium (9) containing 0.03 M sodium gluconate. Cells were harvested in the stationary phase and stored at −20°C. Frozen cell cake (10 g) was suspended in 10 ml 0.02 M potassium phosphate buffer, pH 7.2. The bacteria were disrupted in a French pressure cell at 110 MPa. A few milligrams of DNase were added and, after the suspension was incubated for 5 min at room temperature, it was centrifuged (48,000g) at 4°C for 20 min. The supernatant was dialyzed at 4°C against 0.20 M Tris/HCl, pH 8.0, containing 0.01 M EDTA and a few drops of toluene, for 2 days with one change of buffer. The preparation was further dialyzed for 1 day against 0.2 M Tris/HCl, pH 8.0, containing 0.01 M MgCl₂, yielding the apoenzyme preparation which was stored at −80°C.
The test procedure for PQQ is as follows: 20 μl apoenzyme preparation is incubated with 30 μl sample at room temperature for 30 min in a spectrophotometric plastic microcuvette, followed by 30 μl 0.15 M KCN in 0.02 M potassium phosphate, adjusted to pH 8.0 with concentrated HCl, and 500 μl 0.04 M D-glucose in 0.2 M Tris/HCl, pH 8.0. The reaction was started by the addition of 500 μl 200 μM Wurster’s blue solution (7). The rate of discoloration was measured at 612 nm, at room temperature, with the recorder at 0.1 absorbance units full scale and a chart speed of 240 mm/min.

A molar absorption coefficient of 12.4 \(10^3\) M\(^{-1}\) cm\(^{-1}\) at 612 nm was used in the calculations of initial rates of disappearance of Wurster’s blue.

RESULTS AND DISCUSSION

Chromatographic Elution Conditions

In the ion-suppression chromatographic system, the peak corresponding to PQQ in the chromatogram is unusually broad (\(N = 917\) at \(k' = 5\)). Since this results in a low selectivity and sensitivity, other eluants were tried. When the methanol in the system was changed for tetrahydrofuran or acetonitrile, the results were even worse, and, therefore, ion-pairing systems were tried. Tetrabutylammoniumhydrogen sulfate, in particular, gave satisfactory results, since a much sharper peak was obtained for PQQ (\(N = 4210\) at \(k' = 6\)). Ion-suppression chromatography, however, worked satisfactorily for the analysis of the butyraldehyde adduct and the fluorescing compound.

Chromatographic Detection and Determination Methods

Detection and determination of PQQ in relatively clean samples (e.g., extracts of purified quinoproteins) can be performed using ion-pair chromatography with uv detection at 254 nm. In order to obtain further proof of its presence, the preparation of a suitable adduct was attempted. Of the compounds tested, butyraldehyde gave an easily preparable adduct which appeared to have the best retention time (11.2 min compared to 6.8 min for PQQ) in these chromatographic systems with these types of samples.

When complex samples have to be tested, a more selective detection method becomes necessary. Hydrated PQQ fluoresces and since this is not a common property, fluorescence detection seemed more appropriate. The improvement in selectivity is demonstrated by the chromatograms of a sample of bacterial culture fluid produced by \textit{Hypnomicrobiun X}, as found with uv detection at 254 nm (Fig. 1a) and fluorescence detection (Fig. 1b). Since it is known that PQQH₄ can be converted with NaIO₄ into a highly fluorescing com-

![Fig. 1. HPLC with the ion-pairing system on a 10-μm C18 RCM cartridge of a bacterial culture fluid, obtained from \textit{Hypnomicrobiun X} grown on methanol: (a) uv detection at 254 nm, detection level 0.005 AUFS; (b) fluorescence detection (sensitivity level 8×); and (c) after conversion into the fluorescing compound and fluorescence detection (sensitivity level 4×).]
pound (2), the sample was treated in this way in order to further improve the sensitivity of detection. Figure 1c shows that not only the sensitivity but also the selectivity is improved by this treatment. On addition of PQQ to the sample (concentrations ranging from 0.25 to 5 μM), it appeared that there was a linear relationship between the amount added and the product formed. An additional attractive feature of the method is that adducts of PQQ, which can easily form in a culture supernatant, are quantitatively converted into the fluorescing product (as tested with the acetaldehyde adduct).

**Biological Test**

The first biological test described using the apoenzyme of glucose dehydrogenase which was purified from *Acinetobacter calcoaceticus* (10). However, on several occasions it appeared that, during the conversion of holoenzyme into apoenzyme, all activity was lost. Since it was found by accident that PQQ can be easily removed from the quinoprotein glucose dehydrogenase obtained from *P. aeruginosa* and *Klebsiella aerogenes* strains (unpublished results) by a dialysis procedure (which does not work for the *A. calcoaceticus* enzyme), an attempt was made to develop a biological test with this novel type of apoenzyme. All batches of *P. aeruginosa* cells tested gave an active apoenzyme with about 5% holoenzyme activity. Although a procedure for purification of holoenzyme from *P. fluorescens* is known (11), this is not necessary since the apoenzyme in the cell-free extract works adequately in the biological test. However, to prevent the oxidation of reduced Wurster’s blue with oxygen (via cytochromes in the preparation) it is absolutely necessary to include KCN in the system. As is apparent from Fig. 2, there is a linear relationship between the reaction velocity and the amount of PQQ in the sample. The test is specific for PQQ since no effect was found on addition of FAD, FMN, or yeast extract.

**Comparison of the Test Systems**

*P. aeruginosa* strains are readily available. With the new procedure for obtaining apoenzyme, a very easy and sensitive (2 nM PQQ is the lowest detection level for the conditions described here) biological test for PQQ is now available. However, adducts of PQQ have a very low activity in this test (to be published elsewhere). So when adduct formation can be expected in a sample, conversion into the fluorescing compound and analysis via HPLC is necessary. Using fluorescence detection, however, this method is also relatively sensitive (using a sample of 30 μl, the lowest detection level is 80 nM at a signal-to-noise ratio of 10).

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Chapter VIII

Covalent addition of H$_2$O, enzyme substrates and activators to pyrroloquinoline quinone, the coenzyme of quinoproteins*

The fluorescence excitation and the absorption spectrum of 2,7,9-tricarboxy-1H-pyrrolo[2,3-f]quinoline-4,5-dione (pyrroloquinoline quinone, PQQ), measured in the pH range 7.0–10.0, are quite different. However, when the temperature of the solution is lowered, the shape and maxima of these spectra become more similar. $^1$H-NMR in $^2$H$_2$O revealed a temperature-dependent equilibrium between PQQ and a hydrated form. Evidence is presented that low temperature favours the formation of the fluorescence species which is PQQ, hydrated at the C-5 position.

Even further hydration is possible since absorption, fluorescence and NMR spectroscopy of PQQ in borate buffer pH 10.0 reveal additional hydration at the C-4 position, pointing to a dihydrate.

PQQ also reacts with quinoprotein enzyme substrates and activators. Spectroscopic measurements showed the existence of 5-alkoxy-5-hydroxy-PQQ and 5-amino-5-hydroxy-PQQ in the presence of alcohols and 2 M NH$_4$Cl, pH 9.0, respectively. In the latter case, the existence of 5-amino-PQQ could also be demonstrated. Addition compounds with amines appear to be unstable. The amines become probably oxidized because pyrrolo-quinoline quinol (PQQH$_2$) was found as the reaction product. On the other hand, an addition compound containing an imino bond could be isolated after addition of urea to a PQQ solution.

Spectral characteristics of PQQ and its addition compounds are presented since these data are necessary for the spectral analysis of quinoproteins and the quantitative estimation of coenzyme.

In the past few years it has become clear that in addition to the NAD(P)-dependent and flavoprotein dehydrogenases there is another class, the so-called quinoproteins, in which pyrroloquinoline quinone (PQQ) is involved as the coenzyme [1]. The spectroscopic characteristics of PQQ [2], pyrroloquinoline semiquinone (PQQH) [3], pyrroloquinone quinol (PQQH$_2$) [3] and pyrrolo-quinoline dihydroquinol (PQQH$_4$) [2] have been described. However, before a spectral analysis of quinoproteins can be started, some other points have to be considered.

Comparison of the absorption [2] and fluorescence excitation spectrum [4, 5] of PQQ reveals a large difference in shape and maxima which cannot be ascribed to an impurity in shape, in several chromatographic systems tested, the PQQ-containing fractions always fluoresce. Furthermore, the shape and position of the peak of PQQ are similar in chromatograms produced by absorbance or fluorescence detectors in high-performance liquid chromatography (unpublished results), suggesting a direct relationship between PQQ and the fluorescing compound. Since it was recently discovered that PQQ in water becomes partly hydrated at the C-5 position [3], the question arose whether the fluorescing and the hydrated species are identical.

Alcohols and amines are substrates for quinoprotein methanol and methylamine dehydrogenase respectively [1].

Abbreviations. NMR, nuclear magnetic resonance; pyrroloquinoline quinone (PQQ), 2,7,9-tricarboxy-1H-pyrrolo[2,3-f]quinoline-4,5-dione; pyrroloquinoline quinol (PQQH$_2$), 2,7,9-tricarboxy-1H-pyrrolo[2,3-f]quinoline-4,5-diol; pyrroloquinoline semiquinone (PQQH), the semiquinone form of PQQ; pyrroloquinone dihydroquinol (PQQH$_4$), 2,7,9-tricarboxy-1H-pyrrolo[2,3-f]quinoline-4,5-dihydro-4,5-diol.

Enzymes. Methanol dehydrogenase (EC 1.1.99.8); methylamine dehydrogenase (EC 1.4.99.3).

Furthermore, amine and ammonium salts are activators for quinoprotein alcohol dehydrogenases and PQQ possibly interacts with amino groups of the protein part of quinoproteins [6, 7]. Therefore, it was necessary to investigate whether these compounds react with PQQ and to determine the spectral characteristics of these addition compounds.

MATERIALS AND METHODS

Materials

PQQ [2, 6], PQQH$_2$ [3], PQQH$_4$ [2], PQQ-trimethyl ester [2] and the acetone addition compound of PQQ [6] were prepared and purified as previously described. The addition compound of PQQ and urea was made as follows: 10 mg PQQ was dissolved in 5 ml 0.1 M potassium phosphate, pH 7.0, 6 M urea. After standing for 1 h at room temperature, the solution was acidified with 6 M HCl to pH 2.0 and applied to a Seppak C$_8$ cartridge (Waters Ass.). The cartridge was washed with dilute HCl (pH 2.0) and the addition compound eluted with methanol/water (90:10, v/v). After a few minutes, the addition compound precipitated spontaneously from the eluate. The suspension was centrifuged and the sediment dried in vacuo over P$_2$O$_5$. The compound was dissolved in (C$_2$H$_5$)$_2$SO for $^1$H-NMR measurements.

Absorption and Fluorescence Spectroscopy

Absorption spectra were measured on a Beckman UV 5260 spectrophotometer. The temperature of the cuvette was varied with the temperature controller CTC 250. Measurements at liquid nitrogen temperature were performed on an Amino

DW 2a spectrophotometer with samples of 2-mm light path, frozen between perspex plates.

Computation of the absorption spectra of PQQ and its hydrate (PQQ-H₂O) were done in the following way: 16 mg of PQQ-Na₂ was dissolved in 1 L 0.02 M potassium phosphate, pH 7.0. Spectra were measured at 24 °C and 42 °C and absorbance values recorded at 1-nm intervals with the wavelength ranging over 225–500 nm. The Lambert-Beer’s equation for this system of two components can be written as follows:

\[ A(\lambda, t) = c_{\text{PQQ}}(\lambda) \cdot c_{\text{PQQ}}(t) \cdot l + c_{\text{PQQ-H₂O}}(\lambda) \cdot c_{\text{PQQ-H₂O}}(t) \cdot l. \]

Substitution of two temperatures (t₁ and t₂), followed by elimination of \( c_{\text{PQQ-H₂O}}(\lambda) \) from the two resulting equations gives:

\[ c_{\text{PQQ}}(\lambda) = \frac{c_{\text{PQQ-H₂O}}(t₂) \cdot A(\lambda, t₁) - c_{\text{PQQ-H₂O}}(t₁) \cdot A(\lambda, t₂)}{c_{\text{PQQ}}(t₁) \cdot c_{\text{PQQ-H₂O}}(t₁) \cdot l - c_{\text{PQQ}}(t₂) \cdot c_{\text{PQQ-H₂O}}(t₁) \cdot l}. \]

A similar elimination procedure results in an equation for \( c_{\text{PQQ-H₂O}}(\lambda) \). The relative concentrations of PQQ and PQQ-H₂O were determined by ¹H-NMR in D₂O at pH 7.0. At t₁ (24 °C), 39.7 % PQQ-D₂O and 61.3 % PQQ were found. At t₂ (42 °C), 31.9 % PQQ-D₂O and 69.9 % PQQ were found. Control experiments showed that the absorption spectra of PQQ in H₂O and in D₂O are identical.

Fluorescence measurements were carried out in quartz cells in a home-built spectrophospho-fluorimeter [8] under right-angle illumination. Excitation spectra were automatically corrected with the aid of a rhodamine B quantum counter; the emission spectra are uncorrected.

**NMR and Mass Spectroscopy**

¹H and ¹³C NMR spectra were measured at 30 °C (unless otherwise indicated) on a Varian SC 300 spectrometer, operating at 300 and 75.5 MHz respectively, using the pulse Fourier-transform mode (max 32 K data table of 32 bits). For D₂O and (CH₃)₂SO solutions, 2,2-dimethyl-2-silapentane-5-sulphonate was used as an internal reference. Mass spectra were taken with a Varian MAT 311 A spectrometer.

**RESULTS AND DISCUSSION**

**Addition of H₂O to PQQ**

Absorption spectra of PQQ solutions with varying pH show only marginal differences in the pH range 5–9 [6]. However, as is apparent from Fig. 1, temperature has a marked influence. These changes are reversible, suggesting a temperature-dependent equilibrium.

The fluorescence excitation spectra of PQQ (Fig. 2) are quite different from the absorption spectra [2]. On lowering the temperature, the intensity of the fluorescence increased significantly while an increase of the temperature had the opposite effect. This suggests that there is an equilibrium between a fluorescing and a non-fluorescing species which is shifted in favour of the fluorescing species at low temperature. Measurement of the absorption spectrum at liquid nitrogen temperature confirmed this since the absorption spectrum (in the measurable range) appeared to be similar to the fluorescence excitation spectrum presented in Fig. 2.

Previously published ¹H-NMR results of PQQ in D₂O indicated that part of this substance is hydrated at the C-5 position [3]. It is also known that acetone [9] and acetaldehyde [7] add at the C-5 position of PQQ and that these addition compounds are strongly fluorescent [6]. Since the excitation spectrum of the acetone addition compound (Fig. 3) is nearly identical to its absorption spectrum [6], this suggests that upon addition of a substance at the C-5 position, PQQ is transformed into a strongly fluorescing compound.
The similarity in shape of the excitation spectrum of the acetone addition compound and PQQ in H₂O are in accordance with the view that PQQ, hydrated at the C-5 position (PQQ-H₂O), is responsible for the fluorescence. Further confirmation for this was obtained by comparing the effect of temperature on the equilibrium as found in the absorption/fluorescence excitation spectra and on that studied by NMR (results at the higher temperatures were not useful because the NMR signals from PQQ and PQQ-H₂O for 3-H coalesced at 55 °C and those for 8-H at 75 °C). In the measurable range, lowering the temperature favoured the formation of hydrate as well as of the fluorescing species. The results of NMR and absorption spectra at varying temperatures were used to calculate the absorption spectra of PQQ and PQQ-H₂O (Fig.4). The spectrum of PQQ resembles that of other α-quinones [10] in having a quinoid n → π* transition around 475 nm (Fig.4), a characteristic which is lacking in the spectrum of PQQ-H₂O. The resemblance between the fluorescence excitation spectrum (Fig.2) and the calculated absorption spectrum of PQQ-H₂O is not perfect because the maximum at 360 nm has somewhat lower absorbance in the latter case. This imperfection may be due to the presence of a small amount of dihydrate of PQQ (see below), a compound which is fluorescent, having an excitation maximum in that region. Unfortunately, excitation spectra measured at different emission wavelengths could not confirm this because the excitation spectra were similar in shape.

It was found that absorption spectra in borate buffer pH 10.0 (Fig.5) are quite different from spectra in other buffers at a comparable pH (Fig.6 and [6]). Borate buffers at a lower pH, lower borate concentrations or higher temperatures were less effective in changing the absorption spectrum. These characteristics point to a complex of borate with vicinal diol groups. The existence of such a complex can only be explained by assuming that additional hydration of PQQ-H₂O takes place at the C-4 position, resulting in a small amount of dihydrate (PQQ-2H₂O) which reacts further with borate (Scheme 1).

The structure of PQQ-2H₂O is related to that of PQQH₄ (Scheme 1). Therefore, it is not surprising that the absorption spectrum [2] and the fluorescence excitation spectrum (Fig.3) of PQQH₄ are very similar to the absorption (Fig.5) and fluorescence excitation spectrum (results not shown) of PQQ in borate buffer. ¹H-NMR of PQQ in ³H₂O buffered with 0.15 M sodium borate, pH 10.0, showed a shift of the signals of PQQ [2] to 6.86 ppm (s, 3-H) and 7.89 ppm (s, 8-H),
values which are close to those of PQH₂ [2]. ¹³C-NMR of this solution showed signals at 103.4 and 105.6 ppm (C-4 and C-5), 112.6 ppm (C-3), 120.1 and 124.9 ppm (C-8), 125.9, 130.2, 134.0, 143.5, 152.9 and 160.6 ppm (nine aromatic carbon atoms), 171.9, 175.7 and 179.6 ppm (three COOH groups), which is in agreement with the proposed structure. The possibility of hydration at both C-4 and C-5 is confirmed by the fact that the mass spectrum of PQQ-trimethyl ester dissolved in H₂¹⁸O showed the exchange of two oxygen atoms.

Addition of Quinoprotein Substrates and Activators to PQQ

¹H-NMR results of PQQ-trimethyl ether in CD₂Cl₂ demonstrated already [3] that the addition of methanol transforms this compound into a 5-hydroxy-5-methoxy derivative. Therefore, it was interesting to find out whether this reaction also takes place between PQQ and methanol.

Fig. 7 shows the effect of temperature on the absorption spectrum of PQQ in methanol. It is clear that changes are observed comparable to those of PQQ in water (Fig. 1 and 2). However, the absorption and excitation spectra of PQQ in methanol are more similar than those of PQQ in water, suggesting that the addition of methanol occurs to a higher extent than the addition of water. This was also apparent from the absorption spectra of PQQ in methanol below 0°C. Since these spectra were identical to those measured at 0°C, this substantiates the view that methanol adds more easily to PQQ than water. Higher alcohols seem to add even better than methanol. For instance, the spectral changes of PQQ in water, induced upon additions, were reached by far lower concentrations of n-butanol than methanol.

Even in methanol, carefully dried PQQ is sparingly soluble. Moreover, on standing, the dissolved PQQ was rapidly converted into a pale-yellow precipitate. This observation has also been reported for other o-quinones and it has been concluded that two molecules of methanol add to one molecule of the o-quinone [11]. This conclusion is in agreement with the fact that the pale-yellow product of PQQ and methanol showed a strong fluorescence when dissolved in dimethylsulfoxide, having an excitation maximum comparable to that of PQH₂. Unfortunately, the methanol addition compound is unstable so that no NMR measurements could be performed. However, in view of the observations mentioned above, it is plausible that two molecules of methanol can add to one of PQQ, just as in the case of water.

Ammonium and primary amine salts are activators for quinoprotein alcohol dehydrogenases but it is unknown whether these compounds interact with the protein or with the coenzyme part of these enzymes. Furthermore, amines are also substrates for quinoprotein amine-dehydrogenases, enzymes which convert these compounds into the corresponding aldehydes and ammonia. In order to test the first aspect, an attempt was made to demonstrate a reaction of PQQ with NH₃ in buffers at pH 9.0 (the usual test conditions for quinoprotein alcohol dehydrogenases). The absorption spectrum of PQQ in NH₃ buffer pH 9.0, is different from that in pyrophosphate buffer at this pH (Fig. 6) and also from its fluorescence excitation spectrum (Fig. 8). This might be explained by assuming that, besides a C-5 addition compound, there is still another compound present. Experiments with urea also revealed a reaction with PQQ. The addition compound was isolated and it appeared to have a 5-amino-quinone structure since signals were found at 7.06 ppm, (d 2.5 Hz, 3-H), 7.61 and 7.68 ppm (broad, C=S⁻NH₃⁺), 8.47 ppm (s, 8-H) and 12.1 ppm (broadened, 1-H). In view of these results, a similar compound might be formed in the case of NH₃.

H-NMR of PQQ in D²H₃O, pH 9.0, showed that two compounds exist which are different from PQQ or PQQ-D₂O. Signals were found at 6.95 and 7.03 ppm (both 3-H, intensity ratio 28:72) and a broadened signal at 8.20 ppm (8-H). Accordingly, it is postulated that PQQ reacts with NH₃, giving a 5-amino-5-hydroxy compound and the 5-imino form of PQQ.

![Scheme 3](image)

It is worth mentioning that no reaction was observed at pH 7.0. This might be an indication that the model reaction studied here is relevant for the activation step of NH₃ in the enzymes (activation of methanol dehydrogenase is virtually absent at pH 7.0).

Amines presumably react in the same way. Absorption spectra of PQQ in ethylamine at pH 9.0 resembled those of the acetone or methanol addition compounds. However, the amine addition compounds are unstable since the spectra rapidly changed to that of PQH₂ (these experiments were performed under anaerobic conditions). Amino acids did not react with PQQ, except lysine and arginine which gave rise...
the enzyme is in a reduced form since one molecule each of PQQ and PQQH$_3$ are found per enzyme molecule in an extract of the denatured enzyme [3]. However, PQQH$_2$ is nonfluorescent. Unfortunately, the oxidized form of methanol dehydrogenase is very labile [7, 13] and it reacts instantaneously with alcohols to form products. Therefore, rapid detection techniques will be necessary to observe fluorescing transients originating from PQQ-alcohol adducts.

Spectral measurements during a long time showed that the addition compounds of PQQ and alcohols or NH$_3$ are stable. This is in contrast to the oxidized form of methanol dehydrogenase which is degraded in the presence of NH$_3$ but which can be stabilized by cyanide [12]. Cyanide appeared also to react with PQQ. Absorption and fluorescence spectra of PQQ with cyanide at pH 6.0 provide evidence for the formation of a C-5 derivative of PQQ.

It is clear that PQQ is able to react with all the substances which play a role in the enzymic reaction of methanol dehydrogenase. It is to be expected that studies of the kinetics of, for instance, the formation and dissociation of a PQQ addition compound with cyanide will explain why cyanide can function as a stabilizer [12], and activator [7] as well as a competitive inhibitor for substrates [12] and activator [13].

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Chapter IX

Inactivation of quinoprotein alcohol dehydrogenases with cyclopropane derived suicide substrates*

Quinoprotein alcohol dehydrogenases can be inactivated by cyclopropanol, cyclopropanone hydrate, and, depending on whether they can oxidize secondary alcohols, also by cyclopropanone ethyl hemiketal. Only enzyme molecules containing the oxidized coenzyme (PQQ), but not those with the coenzyme in the semiquinone form (PQQH\textsuperscript{+}), become inactivated with these compounds. The inactivation process proceeds without proton production or electron acceptor consumption and free radical is not observed in the inactivated enzyme. It could be demonstrated that a stoichiometric relationship exists between enzyme inactivation, PQQ converted, PQQ adduct formed, and cyclopropanol added. Thus the dimeric and monomeric enzyme become fully inactivated with two and one molecule of cyclopropanol, respectively, indicating that the dimeric enzyme contains two independently acting catalytic sites. Inactivation of the enzyme by cyclopropanol and cyclopropanone hydrate produces chromatographically different PQQ adducts. Since cyclopropanemethanol, cyclobutanol and cyclohexanol are not suicide substrates, the inactivation presumably proceeds via a ring opening such as proposed for the metal-ion-catalysed degradation of cyclopropane derivatives. The results are in accordance with our view on the reaction mechanism of these enzymes but not with that of others [Mincey et al. (1981) Biochemistry 20, 7502 – 7509]. The reasons why their model has to be refuted are discussed.

Bacterial alcohol oxidation frequently proceeds via quinoprotein alcohol dehydrogenases (EC 1.1.99.8), that is via respiratory-chain-linked oxidoreductases which have pyrroloquinoline quinone (PQQ) as their coenzyme [1]. Originally, these enzymes were isolated from methanol-converting bacteria, the reason why the trivial name 'methanol dehydrogenase' was assigned to them [2]. It should be realized, however, that other primary alcohols, formaldehyde, and to a lesser extent, acetaldehyde are also oxidized by these enzymes. Moreover, quinoprotein alcohol dehydrogenases also occur in bacteria which are not restricted to or cannot use methanol as a carbon or energy source. Not unexpectedly, methanol is a bad substrate or no substrate at all, while other primary alcohols but also secondary alcohols and higher aldehydes are substrates in these cases [3].

Most quinoprotein alcohol dehydrogenases have a relative molecular mass of about 120000 [2]. They are probably dimeric enzymes since denaturation produces subunits of the same size (M\textsubscript{r} 60000) and two molecules of coenzyme per enzyme molecule are found in the enzyme from Hyphomicrobium X [4]. Exceptions have been reported, however: the enzymes from two methane-grown bacteria have an M\textsubscript{r} of 60000 [5, 6]. As after denaturation the same molecular mass was found, these are monomeric enzymes.

Recently, it was reported that cyclopropanol spontaneously inactivates methanol dehydrogenase, that is, enzyme as it is isolated in the absence of electron acceptors [7]. Curiously, only an amount stoichiometrically equivalent to 14% of the enzyme was sufficient for complete inactivation. As it was found that 14% of the enzyme preparation had the coenzyme in its semiquinone form (PQQH\textsuperscript{+}), and the assumption was made that cyclopropanol behaves as a substrate, it was concluded that only those enzyme molecules which contain PQQH\textsuperscript{+} are catalytically active. This is in contradiction to our findings which indicate that enzyme molecules containing PQQH\textsuperscript{+} have to be oxidized further before they can react with substrate [1]. Nevertheless, mainly based on ESR experiments, Mincey et al. [7] postulated the involvement of a three-electron reduced form of PQQ in the enzyme cycle. Although we have already reported [8] that this model can be refuted on grounds of incorrect interpretation of the results of ESR experiments, it was felt that the behaviour of cyclopropanol required further investigations. However, using a dimeric enzyme, quite different results were obtained compared to those reported by Mincey et al. [7]. Since they used a monomeric enzyme preparation, we also extended our experiments to this enzyme form. Thus far, it is unknown how cyclopropanol inactivates the enzyme, although the involvement of the ring structure seems plausible. In order to gain insight in this, the effect of other cyclopropane and cycloalkane derivatives was also studied.

MATERIALS AND METHODS

Enzyme preparations

Methanol dehydrogenase from Hyphomicrobium X, obtained as described [9], was used for experiments with dimeric enzyme. Monomeric enzyme originated from Pseudomonas BB\textsubscript{1}, a methylotrophic bacterium which produces monomeric as well as dimeric methanol dehydrogenase. The purification of these enzyme forms will be described elsewhere.

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Oxidation of the enzyme

Isolated enzyme was dialyzed against 0.1 M sodium borate containing 20 mM NH₄Cl, pH 9.0. To 1 ml of 0.1 mM enzyme solution, 50 µl 0.2 M KCN, pH 9.0, was added. After the addition of Wurster’s blue (equimolar to the enzyme concentration), endogeneous substrate and substrate in the buffer was oxidized by adding 0.1-µl aliquots of 0.1 M K₃Fe(CN)₆ until the blue colour persisted. Enzyme concentrations were calculated from absorbance measurements at 205 nm and 280 nm [10].

Inactivation experiments

Variable amounts of suicide substrates were added to the oxidized enzyme; if necessary, more K₃Fe(CN)₆ solution was added to maintain the oxidized state of the enzyme. The progression of the inactivation process was followed by testing 25 µl of the mixture in the normal enzyme assay [9], using an excess of substrate.

Methods for determining the extent of inactivation

The effect of the suicide substrate on the enzyme was not only followed by the enzyme assay but also by analysis of the absorption spectrum of the enzyme, PQQ conversion, and product formation.

To samples of inactivated enzyme, an excess of substrate was added and the mixtures injected on an HPLC gel filtration column (Serva Si 200 polyol, 4.1 x 250 mm, flow rate 0.4 ml/min) in 0.1 M sodium phosphate buffer, pH 7.0, coupled to a Waters model 6000 A solvent delivery system. The eluting enzyme peak was checked for homogeneity by a Hewlett-Packard 1040A high-speed photodiode array detector (the shape of the spectra, measured up slope, at the top, and down slope, were compared after normalization). The absorption spectra were transferred to a Hewlett-Packard 8450A spectrophotometer with which multicomponent analysis was performed, using the absorption spectra of unmodified and fully inactivated enzyme as standards.

The chromatographed enzyme fraction was collected and concentrated via a Millipore CX 30 filter. Sodium dodecyl sulfate (0.2% w/v) was added and the mixture incubated at 50 °C for 1 min. PQQ and its adduct were freed from protein by HPLC gel filtration (conditions as above) in 0.1 M sodium phosphate, containing 0.1% (w/v) sodium dodecyl sulphate, pH 7.0. The check for homogeneity of the low-molecular-mass peak, and multicomponent analysis (using the absorption spectra of PQQ and the corresponding adduct) were performed as indicated above.

Proton production in the inactivation process

Dimeric enzyme (73 µM) was dialyzed against 1 mM sodium borate, containing 2 mM NH₄Cl and 2 mM KCN, pH 9.0. After adding Wurster’s blue (equimolar to the enzyme) and substrate or suicide substrate, 1-µl aliquots of 0.1 M K₃Fe(CN)₆ were added from a Methrom E 655 burette. The pH was measured with a Metrohm pH meter and the change in pH automatically compensated by addition of 0.01 M NaOH from a Metrohm E 655 burette. The additions were registered with a recorder. Experiments were performed in a 250-µl titration vessel with a micro-electrode, while the system was made anaerobic by blowing argon gas over the surface of the solution. Calibrations were performed with a 0.01 M HCl solution.

Chromatography of PQQ and its adducts

Inactivated enzyme was denaturated by adding 9 vol. of methanol. After centrifugation, samples of the supernatant were injected on a C₁₈ Radial PAK HPLC column as described [11] with 85% H₂PO₄/H₂O/CH₃OH (0.4/69.6/30, v/v/v) as the eluant. The effluent was monitored with absorbance detection at 250 nm (Waters model 480 detector), fluorescence detection (Waters model 420 AC, λₑxc = 360 nm, and λₑmit > 418 nm), or photodiode array detection.

Suicide substrates

Cyclobutanol and cyclohexanol were from Aldrich. Cyclopropanol was prepared by reduction of cyclopropylacetate with LiAlH₄ [12] and purification via a C₁₈ reversed-phase column with H₂O as the eluant (k’ = 3) [7], using absorbance detection at 210 nm or a refractive index detector. Purity and concentration of the collected fraction were measured by ¹H-NMR. Cyclopropylacetate was prepared [14] by oxidizing cyclopropylmethylketone.

Cyclopropanone ethyl hemiketal was synthesized [15] from 3-chloropropionic ethyl ester via a methanolation step [16]. Cyclopropanone hydrate was prepared [13] by heating a cyclopropanone ethyl hemiketal solution (pH < 7.0) at 100°C for 4 min. Purification was performed on a HPLC C₁₈ reversed-phase column with water (pH < 7.0) as the eluant and absorbance detection at 210 nm. The ethyl hemiketal and the hydrate were clearly separated in this system (k’ of 14 and 2, respectively).

RESULTS

Conditions for inactivation with the suicide substrates

Incubation of the dimeric enzyme, as it is isolated, from Hyphomicrobium X with low and high concentrations of cyclopropanol, for as long as 24 h, gave no inactivation. However, the fully oxidized dimeric enzyme stabilized with cyanide [9] was completely inactivated by cyclopropanol. Fig. 1 shows the time-dependent inactivation process in the presence of a near equimolar concentration (to PQQ) of cyclopropanol. A comparable inhibitory effect was found for cyclopropanone hydrate but the ethyl hemiketal form showed no effect (the slight inhibitory effect observed was due to hydrolysis of the hemiketal to the hydrate). On the other hand, for monomeric as well as dimeric enzyme from Pseudomonas BB1, the ethyl hemiketal and the hydrate were efficient inhibitors. Cyclopropylamine, cyclopropanemethanol, cyclobutanol and cyclohexanol had no effect on the enzyme species (cyclopropanemethanol was a normal substrate).

Monomeric enzyme behaved in a similar way as the dimeric enzyme from Hyphomicrobium X. However, since the oxidized form of the enzymes from Pseudomonas BB1 are relatively stable and the inactivation rates with cyclopropanol are much higher, the oxidation of the monomer could be performed with only Wurster’s blue as electron acceptor while cyanide could be omitted from the system. Curiously, spontaneous, partial inactivation of some monomeric enzyme preparations occurred on incubation with cyclopropanol without the oxidation step. In these cases, enzyme preparations showed an absorption spectrum different from that of isolated enzyme (MDHox) but more reminiscent to that of fully oxidized enzyme (MDHox*) [1]. Substrate addition indeed changed the absorption spectrum.

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Fig. 1. Inactivation of methanol dehydrogenase from Hyphomicrobium X by cyclopropanol. Enzyme (21 μM) in 0.1 M sodium borate buffer containing 20 mM NH₄Cl, 10 mM KCN, and 20 μM Wurster’s blue, pH 9.0, was oxidized by adding 0.1 M K₃Fe(CN)₆ in 0.2-μl steps, just until the blue colour persisted. One part of this mixture was used for the control experiment (O— O), to the other part cyclopropanol (38 μM, final concentration) was added (● — ●). The inactivation process was followed by testing 25-μl samples in the enzyme assay, using an excess of substrate

of these enzyme preparations. This anomaly was never observed for dimeric enzyme forms.

Spectral and chromatographic analysis of inactivated enzyme preparation

Oxidized, dimeric enzyme samples, treated with variable quantities of cyclopropanol, were freed from low-molecular-mass contaminants by HPLC gel filtration. The absorption spectrum of fully inactivated enzyme (Fig. 2) is very similar to that reported for MDHox, the stabilized, oxidized enzyme form that oxidizes substrate with concomitant spectral changes [1, 9]. An other similarity is that the inactivated enzyme contains no free radical as no ESR signal was observed. Despite this resemblance, no spectral change is seen after addition of substrate to the inactivated enzyme.

Chromatographic analysis of the methanol extracts from enzyme samples inactivated by cyclopropanol, cyclopropanone hydrate and ethyl hemiketal revealed that PQQ (retention time of 3.8 min) was converted into different products (retention times of 6.0 min, 3.2 min and 8.8 min, respectively). Spectral analysis in different parts of the eluted peaks demonstrated that they were homogeneous, suggesting that only one product was formed with each suicide substrate. However, as there were indications that the modified coenzyme was only partly extracted, an attempt was made to develop another method in order to achieve complete extraction.

Enzyme samples, purified as indicated above and denatured with sodium dodecyl sulphate, showed a colourless (no absorbance above 300 nm) protein peak on HPLC gel filtration in 0.1% (w/v) sodium dodecyl sulphate. Concomitantly with the extent of inactivation, a gradual spectral change of the low-molecular-mass fraction was observed (Fig. 3). The absorption spectrum of the product extracted from fully inactivated enzyme appeared to be identical to that of the product obtained by the methanol extraction procedure. This indicates that inactivation by cyclopropanol produces only one product which can be completely removed from the enzyme by the sodium dodecyl sulphate extraction procedure.

Fig. 2. Absorption spectra of methanol dehydrogenase from Hyphomicrobium X, inactivated by variable amounts of cyclopropanol. Samples of enzyme, inactivated as described in the legend of Fig. 1, were incubated with variable amounts of cyclopropanol for 1 h. After that an excess of substrate was added, followed by HPLC gel filtration in 0.1 M sodium phosphate buffer, pH 7.0. Spectra were taken by the photodiode array detector in the slope of the curve. For comparison, the spectra were normalized. The ratios of cyclopropanol to enzyme concentration were: 0 (— — —), 0.7 (— — —), 1.4 (— — —), and 46 (—— — —)

Fig. 3. Absorption spectra of the compounds extracted from cyclopropanol-inactivated enzyme. Samples of enzyme were inactivated with variable amounts of cyclopropanol and chromatographed (see the legend of Fig. 2). The collected samples were denatured while protein and low-molecular-mass compounds were separated from each other by HPLC gel filtration in 0.1 M sodium phosphate buffer containing 0.1% (w/v) sodium dodecyl sulphate, pH 7.0. The absorption spectra were taken by the photodiode array detector at the top of the curve and normalized for comparison. The spectra originate from samples inactivated in a concentration ratio of cyclopropanol to enzyme of 0 (— — —), 0.7 (— — —), 1.4 (— — —), and 46 (—— — —)

Stoichiometry of the inactivation process

Monomeric enzyme was completely inactivated with a stoichiometric amount of cyclopropanol. This enzyme species contains one PQQ molecule (unpublished results). Not unexpectedly, therefore, it was found that all the PQQ was modified in the fully inactivated enzyme.
Fig. 4. The relationship between the cyclopropanol added, the remaining enzyme activity, enzyme in the MDHox₁ form, and PQQ in the enzyme. Enzyme samples, treated with variable amounts of cyclopropanol, were inactivated, purified and extracted as described in the legends of Fig. 3 and 4. Enzyme activity ( ) was measured in the enzyme assay. The amount of PQQ in the samples (Δ) was calculated by multicomponent analysis of the spectra presented in Fig. 2. The amount of enzyme having the absorption spectrum of MDHox₁ (Δ) was calculated by multicomponent analysis of the spectra presented in Fig. 2.

On addition of an equimolar amount of cyclopropanol to dimeric enzyme, only half the activity was lost and about 50% PQQ modified (Fig. 4). As shown in Fig. 4, a linear relationship between the concentration of cyclopropanol used and the effects on the enzyme was found. In this experiment, full inactivation of the enzyme and complete modification of PQQ was obtained with about 1.8 molecules of cyclopropanol per dimeric enzyme molecule.

Proton production during the inactivation

The performance of the method used was tested with methanol and formaldehyde as a substrate. Since approximately 1 and 1.5 proton/electron were found, respectively, the method seemed adequate for the experiment with cyclopropanol. Protons were neither consumed nor produced and electron acceptor was not consumed (compared to an experiment without substrate or inhibitor) during the inactivation with cyclopropanol.

DISCUSSION

The cyclopropane derivatives found to be effective as inhibitor can be considered as suicide substrates since, just like normal substrates, they only react with the oxidized enzyme form. Not unexpectedly, therefore, the presence of substrate prevents or lowers the irreversible inhibition by these compounds. Even some kind of substrate specificity is apparent as both enzyme species from Pseudomonas BB₁ (secondary alcohols are substrates) are efficiently inactivated by cyclopropanone ethyl hemiketal while enzyme from Hyphomicrobium X (secondary alcohols are not substrates) is not. Since all enzyme species are inactivated by cyclopropanone hydrate, the ethyl group in the hemiketal compound is probably prohibitive for binding to the active site of the enzyme from Hyphomicrobium X.

A stoichiometric relationship exists between enzyme inactivation, cyclopropanol added, and PQQ modified. This indicates that any suicide substrate molecule which is processed transforms a PQQ molecule into product and leads to an inactivation event. The relationship found for dimeric enzyme from Hyphomicrobium X (Fig. 4) shows that the enzyme has two catalytic sites which act independently, a conclusion supported by the fact that the turnover number of dimeric enzyme from Pseudomonas BB₁ is twice as large as that of the monomeric species (unpublished results). These results suggest that the spectral anomalies of dimeric enzyme found earlier, interpreted as indications for interaction between the two coenzyme molecules [4], have to be explained in another way.

The products extracted from enzyme, inactivated by cyclopropanol and cyclopropanone, are spectrally and chromatographically different. This indicates that inactivation by cyclopropanol is not due to production of cyclopropanone, which might act as an inactivating agent. In agreement with this, neither proton production nor electron acceptor consumption is found during the inactivation process. These observations suggest that the enzyme transforms the suicide substrate into a reactive compound without net oxidation. In nature, the enzyme was the forming of the cyclopropane ring lies at hand. In this respect, the mechanism proposed for the chemical degradation of cyclopropanol [17] and cyclopropanone [18], catalysed by metal ions, is very interesting. Starting with a one-electron oxidation step, ring fission occurs, resulting in a free radical of propionaldehyde and propionic acid, respectively.

Assuming a similar reaction in the enzyme, PQQ might react with cyclopropanol to form PQQH₂ and a free radical of propionaldehyde (Scheme 1). Recombination of the two radicals could result in a stable PQQ adduct, explaining why no net oxidation or proton production is observed for the reaction and the absence of free radical in the inactivated enzyme. In agreement with this, the absorption spectrum of the extracted compounds are very similar to those of PQQ adducts [19]. The fact that cyclopropane compounds lacking an OH group in the ring or higher cycloalkanols are not suicide substrates is a further confirmation for this mechanism, since it has been reported [17] that the metal-ion-catalysed degradation of these compounds is very slow compared to that of cyclopropanol and cyclopropanone. It is expected that final proof for the proposed mechanism will be obtained when the structure of the PQQ adducts is known (in progress).

Some of the results described here differ fundamentally from those reported by Minecy et al. [7]. The most striking difference is that cyclopropanol inactivated their enzyme preparation in the absence of electron acceptor, while inactivation of our dimeric enzyme preparations always needed oxidation with electron acceptor before they could be inactivated with suicide substrate. However, some of our isolated monomeric enzyme preparations were partly in the fully oxidized state and were, indeed, partly inactivated by only cyclopropanol. As Minecy et al. [7] used monomeric enzyme, perhaps their enzyme was in the fully oxidized form. Unfortunately, this assumption cannot be verified as no absorption spectrum was given for the preparation they used in the inactivation experiment. Moreover, it should be remembered that the main argument for their view, that cyclopropanol reacts with PQQH₂ in the enzyme, is invalid since this was based on incorrect interpretation of the results from their ESR experiments [8].

Curiously, they found that an amount of cyclopropanol, stoichiometrically equivalent to 14% of the enzyme, completely inactivated it. It should be noted, however, they stated that their enzyme was very labile in the absence of substrate, although this is in contradiction with the data from their
control experiment [7]. Finally, the fact that they found, when using [1-3H]cyclopropanol, only 30–40% of the radioactivity is released on extraction with methanol, needs comment. We observed that methanol extraction of inactivated enzyme only partially released the PQQ adduct. On the other hand, quantitative extraction was obtained with the sodium dodecyl sulphate procedure. So it can be excluded that there is more than one product, that part of the suicide substrate covalently adds to the protein or that only 14% of the coenzyme reacts with cyclopropanol.

In view of the discussion above and the evidence reported already [8], there are no arguments to support the mechanism proposed by Mincey et al. [7], stating that only semiquinone forms of the coenzyme are catalytically active and that substrate oxidation leads to a three-electron reduced form of PQQ in the enzyme. On the other hand, the results described here are in full agreement with our already reported reaction scheme for methanol dehydrogenase [1, 8].

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REFERENCES


chloropropionate, respectively (Jansen, Belgium). Cyclopropane hydrate was obtained by heating an aqueous solution (pH < 7) of cyclopropanone hemiketal at 100 °C for 4 min, followed by rapid cooling in an ice bath. Complete conversion was checked by hplc on a Novapak-C18 column (Waters, USA) with water (Milli-Q grade) as the eluant (1.5 ml/min) and detection at 210 nm. The retention times of the hemiketal and the hydrate were 8.8 and 3.2 min respectively. Wurster’s blue (N,N,N',N’-tetramethylphenylenediamine) was prepared as described previously [14]. 4-Hydroxy-pyrroloquinoline was a kind gift of Prof. C.W.Rees. All other chemicals were from Merck (Darmstadt, FRG). Seppak C18-cartridges were from Waters, USA.

Extraction of the cofactor from cyclopropanol-inactivated MDH

The modified prosthetic group was extracted from the enzyme by adding 9 volumes of methanol. After centrifugation the methanol was evaporated under reduced pressure. The residue was acidified with 6 M HCl to pH 2.0 and the product was adsorbed to a C18 Seppak cartridge (Waters). After washing the cartridge with 10 ml 0.002 M HCl, the compound was eluted with 2 ml methanol. Further purification was achieved by adsorbing the product to a small column (4 x 8 mm) of Amberlyst A21 ion exchanger. After washing with 3 ml 0.5 M NaCl in 50% (v/v) methanol, the product was eluted with 1 ml 1.0 M NaCl in 90% (v/v) methanol.

Purification of methanol dehydrogenase.

Methanol dehydrogenase was purified from frozen cells of Hyphomicrobium X, using a procedure reported previously [14].

High performance liquid chromatography

Hplc on Novapak-C18 RCM-cartridges was performed with a Waters M6000 solvent delivery system controlled by a M720 system controller and equipped with a U6K injector. The eluent (at a flow rate of 1.5 ml/min) was monitored with a Hewlett-Packard HP1040A photodiode array detector. Alternatively, absorbance (λ=254 nm, Waters M440 detector) and fluorescence detectors (excitation 340-360 nm, emission >410 nm, Waters M420AC detector) were used in series.

Reaction of PQQ with cyclopropanol

500 mg PQQ was dissolved in 25 ml water and the pH was adjusted to 6.5 with 2 M NaOH. 500 mg Ag2O and 400 μl cyclopropanol were added and the mixture was degassed under vacuum in an ultrasonic bath for 3 min. The reaction was allowed to proceed under gentle stirring, and formation of product was followed by hplc by taking 20 μl aliquots at 30 min. intervals. Prior to hplc analysis the samples were diluted with 5 ml 0.02 M HNO3 and adsorbed to a Seppak C18-cartridge. After washing with 10 ml 0.002 M HNO3, the components were eluted with 1 ml methanol. When conversion was almost complete the reaction mixture was acidified to pH 2.0 with 6 M HNO3 and centrifuged (48000 x g, 10 min.). 1 ml portions of the supernatant were adsorbed to a Seppak C18-cartridge, washed with 5 ml 0.002 M HNO3 and eluted with 1 ml 70 % (v/v) methanol. The eluates were pooled and methanol was removed under reduced pressure. After acidification of the remaining aqueous solution with 6 M HCl to pH 1.0, compound 3a precipitated and was collected by filtration and dried in vacuo over P2O5 (yield 92 mg).

Preparation of MDH inactivated with cyclopropane derivatives

10 mg of cyclopropanol and 1 mg of Wurster’s blue were added to 514 nmol of MDH in 4 ml 0.1 M borate buffer, pH 9.0. The mixture was titrated with aliquots of 1 μl potassium ferricyanide solution (0.2 M in water) until the reaction mixture remained blue for at least 15 min. The inactivated enzyme was dialyzed overnight against 20 mM potassium phosphate buffer, pH 7.0. Likewise, samples containing 89 nmol of MDH and 0.2 mg of Wurster’s blue were treated with 5 mg cyclopropanone hydrate and 5 mg cyclopropanone hemiketal, respectively.
The effectiveness of CuO and ZnO as catalysts for the reaction was investigated by mixing a solution of 10 mg PQQ in 1 ml of water, adjusted to pH 6.5, 10 mg of the oxide and 10 μl cyclopropanol, following the procedure described for Ag₂O.

Reaction of PQQ with cyclopropanone hydrate and cyclopropanone hemiketal

10 mg of cyclopropanone hydrate or cyclopropanone hemiketal were added to 30 mg of PQQ dissolved in 10 ml 0.1 M NH₄Cl/NH₃ pH 9.0. After completion of the reaction (as judged by hplc analysis) the reaction mixture was brought to pH 2.0 with 6 M HCl and the PQQ adduct (2b, 2c) was adsorbed to a Seppak C₁₈-cartridge. After washing with 10 ml 0.002 M HCl, the adduct was eluted with 1 ml methanol.

Reduction of PQQ adducts with NaBH₄

Reduction of PQQ adducts 2a, 2b and 3 was performed by dissolving 5-10 mg in 2 ml water, flushing with Argon for 15 min. and adding 10 mg solid NaBH₄. Completion of the reaction was checked by hplc. After acidification of the reaction mixture to pH 2.0 with 6 M HCl, the products were recovered by adsorbing them to a Sep Pak C₁₈-cartridge, washing with 0.002 M HCl, elution with 1 ml methanol and evaporation of the solvent under reduced pressure.

Oxidation with sodium periodate

4b (2 mg) was dissolved in 2 ml 0.01 M sodium phosphate buffer pH 6.5 and 2 mg NaIO₄ was added. After standing for 1 h in the dark, the oxidation products were recovered after acidification using a Seppak C₁₈-cartridge as described above.

Dehydration of NaBH₄-reduced PQQ and PQQ-adducts

Dehydration to pyrroloquinoline derivatives was performed by dissolving 5-10 mg PQQH₄ (4d) or reduced PQQ derivative (4b-4d) in conc. H₂SO₄. Completion of the reaction was judged by hplc. 4b and 4d were completely dehydrated within 5 min at room temperature, 4a and 4c required 2 hours at 50 °C. The products were recovered as described above.

Copper complexes of pyrroloquinoline derivatives

The presence of an 8-hydroxyquinoline moiety in the pyrroloquinoline derivatives was tested by spectrophotometric titration with Cu²⁺-ions at pH 4.0, according to Schoellhammer and Hemmerich [15].

Absorbance and fluorescence spectroscopy

Absorbance spectra were measured on a Hewlett-Packard HP8450A spectrophotometer. Fluorescence spectra were obtained with a home-built spectrophospho-fluorimeter [16]. Excitation spectra were automatically corrected with the aid of a rhodamine B quantum counter; the emission spectra were uncorrected.

NMR spectroscopy

¹H- and ¹³C-NMR spectra of the synthesized and extracted PQQ adducts were recorded at 30 °C with a Varian SC300 spectrometer, operating at 300 and 75.5 MHz respectively, or with a Varian VXR 400 spectrometer at 400 and 100 MHz. The solvent was in all cases (C₆H₄)₂SO. 2,2-dimethyl-2-silapentane-5-sulphonate was used as an internal reference for ¹H-NMR. For ¹³C-NMR, the solvent signal of (C₆H₄)₂SO at 39.6 ppm served as an internal reference.

RESULTS

Properties of modified PQQ extracted from cyclopropanol-inactivated MDH

Hplc analysis of the methanolic extract of MDH inactivated by cyclopropanol revealed that PQQ was no longer present. Instead two
fluorescent products were found with retention times of 11.3 and 12.8 min. compared to 7.3 min. for PQQ (20 min. linear gradient 28.5-53 % (v/v) methanol, 0.4 % (v/v) H₃PO₄). Rechromatography of the collected fractions containing the pure components after standing for 12 h at 4 °C, revealed the presence of the other component, indicating a slow interconversion. Both components had the same absorption spectrum (Fig 1) and fluorescence excitation spectrum (not shown), closely resembling that of the acetone adduct of PQQ (2a) [8,17].

¹H-NMR of the product(s) purified from the extract showed a singlet at 8.52 ppm (8-H) and a multiplet at 5.77 ppm. These signals were accompanied by similar, but smaller (ratio about 4:1), signals at 8.47 and 5.90 ppm. The aromatic 3-H signals were observed at 7.20 ppm.

**Reaction of PQQ with cyclopropanol**

No reaction was observed when cyclopropanol was added to solutions of PQQ with a pH between 5 and 9. In the presence of Ag₂O, maximal conversion (typically 70 % of the PQQ originally present) was achieved in a few hours. Similar results were obtained with CuO and ZnO, but Ag⁺-ions were not effective. Although the reaction proceeds in the presence of oxygen, higher conversions were observed in its absence.

**Characterization of the products of the reaction of PQQ and cyclopropanol**

Hplc analysis of the products purified from the reaction mixture revealed that they coeluted with the PQQ adducts extracted from MDH. In addition the absorption spectra taken at the apex of the peaks in the individual chromatograms were found to be identical and in both chromatograms the ratio between the absorption and fluorescence signals were the same. The ¹H-NMR spectrum of the synthetic compound mixture showed signals at 5.78 (m), 7.20 (d, 2Hz), 8.54 (s) and 13.10 ppm (broad). A similar set of
signals with a lower intensity (ratio about 3:1) was found at 5.91, 7.195, 8.49 and 12.94 ppm. These results clearly showed that the synthetic and the enzymatic products were identical.

Both compounds contain a C-CH₂-CH₂-CH₀ grouping, with ¹H-NMR signals for the major component at 5.78 (d 5 Hz and d 3 Hz, CH₂-CHO) and 2.0-2.4 ppm (multiplets, CH₂-CH₂). The structure, 3b, was deduced from its ¹³C-NMR spectrum. This spectrum is very similar, with respect to shifts and (non-decoupled) signal patterns, to that of 2a, with the exception of the signals of C₅ and its substituents (Table 1). As the structure 2a has been unequivocally established by X-ray analysis [18], this indicated a keto group at C₄ and a disubstitution at C₅ in structure 3b. The ring closure to a tetrahydrofuran structure in 3b was based on the observation of a J-coupling of 5.5 Hz between the C₅ carbon and the C₅-CH₂-CH₂-CH₀ proton.

The two possible configurations of the hydroxyl-substituent in this ring accounts for the two interconverting species observed in the compound. The full assignment of the signals in the above mentioned ¹³C-NMR spectra was possible by comparison with the spectrum of 99% ¹³C-labelled PQQ (Table 1).

During ¹³C-NMR spectroscopy compound 3b partially decomposed as evidenced by the appearance of signals of PQQH₂ [19] and 2-propenal.

Reaction of PQQ and MDH with cyclopropanone hemiketal and -hydrate

At pH 9.0, PQQ readily reacts with both the hemiketal and hydrate of cyclopropanone. In both cases a single product was obtained as judged by hplc-analysis (40% methanol, 0.4% H₃PO₄, retention time 10.6 and 4.4 min. respectively). The absorption and fluorescence excitation spectra of both compounds were almost identical to those of 2a and 3b (results not shown). The ¹H- and ¹³C-NMR-spectra (Table 1) pointed to structures 2b and 2c.

¹H-NMR of 2b: δ 2.0-2.3 (m, CH₂-CH₂), 7.19 (d 2.2 Hz, 3-H), 8.53 (s, 8-H) and 13.0 ppm (broad, 1-H).

¹H-NMR of 2c: δ 1.09 (t 7 Hz, CH₃CH₃), 2.0-2.3 (m, CH₂-CH₂), 3.88 (AB-pattern, CH₂CH₃), 7.16 (d 2.2 Hz, 3-H), 8.53 (s, 8-H) and 14.2 ppm (broad, 1-H).

These data support addition of the cyclopropanone derivatives, after opening of the cyclopropane ring, at C₃ of PQQ.

MDH could be completely inactivated with cyclopropanone hydrate, while only partial inactivation occurred with the hemiketal. Hplc analysis of methanolic extracts of the two preparations revealed that in the former
TABLE 1
13C Chemical shifts of PQQ and PQQ-adducts

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<td>C5</td>
<td>179.9 (d 58, d 50, d 16)</td>
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<td>88.8</td>
<td>79.8</td>
<td>79.5</td>
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<td>CHOCH3</td>
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\(^a\) The assignments for compound 1 were deduced from HETCOR and selective proton-decoupling experiments.

\(^b\) Chemical shifts in ppm, internal reference (C\(_6\)H\(_5\))\(_2\)SO at 39.6 ppm.

\(^c\) Coupling constants in Hz with an accuracy of ± 3 Hz. All signals are broadened by further coupling, unless stated otherwise.

preparation only a product coeluting with the reaction product of PQQ and cyclopropanone hydrate (2b) was present, while in the latter preparation besides this compound also unmodified PQQ was found. Apparently, inactivation of enzyme in the latter case is also caused by cyclopropanone hydrate, this compound being formed by partial hydrolysis of cyclopropanone hemiketal under the conditions of the reaction. The absorption spectra of MDH inactivated by cyclopropanone hydrate and cyclopropanol [6] were of similar shape, but the former was blueshifted by 8 nm.

reduction of 2a, 2b and 3b

Reduction of 2a, 2b and 3b by NaBH\(_4\) led in all cases to substances with an absorption spectrum similar to that of PQH\(_4\) [9] (Fig. 2). Hplc analysis of reduced 3b revealed a main (>90%) component eluting at 12.5 min. (20 min. linear gradient, 18.5-56.5% (v/v) methanol, 0.4% (v/v) H\(_3\)PO\(_4\)). The 1\(^H\)-NMR spectrum pointed to the open structure 4b, which explains the disappearance of the stereoisomerism after reduction of 3b. The 1\(^H\)-NMR spectrum indicated the presence of a rotationally hindered C\(_5\)-CH\(_2\)-CH\(_2\)-CH\(_2\)-OH grouping as depicted in Fig. 3. Further signals were found at: 4.91 (s, 4-H), 6.85
Fig. 2. Absorption spectra of the reduced (4b) and dehydrated (5b) adduct between PQQ and cyclopropanol. Spectra were taken in the apex of the eluting peaks (in methanol/water/0.4% (v/v) H$_3$PO$_4$). (- - -) 4b and (-----) 5b.

(d, 3-H), 8.40 (s, 8-H) and 11.9 (broad, 1-H).

Reduction of 2a also led to a single component (as judged by hplc), which was subjected to the dehydration procedure (see below) without further analysis.

Reduction of 2b yielded 2 components in a 6:4 ratio (hplc: 4.7 and 5.5 min., 40% (v/v) methanol, 0.4% (v/v) H$_3$PO$_4$) with similar absorption spectra. In the $^1$H-NMR spectrum a similar mixture was seen, with signals at 1.9-2.7 (m, CH$_2$-CH$_2$), 5.08/4.87 (s, 4-H), 6.91/6.86 (d 2.2 Hz, 3-H), 8.43/8.41 (s, 8-H) and 12.0/11.9 ppm (broad, 1-H), both consistent with structure 4c. Dehydration converted both into the same component (see below) and only one of the isomers was rapidly oxidized by sodium periodate, indicating that the compounds are cis/trans isomers.

Dehydration of 4a-4d

Acid treatment of 4a-4d yielded in all cases single or main dehydration products with similar absorption spectra (Fig. 2, dashed line). Minor impurities accompanied 5b and 5c.

Fig. 3. Partial structure of 4b. $^1$H Chemical shifts are in ppm, spin-spin coupling constants are in Hz.
$^1$H-NMR of 5a: 1.58 (d 6Hz, CH$_3$), 3.18 and 3.73 (AB-pattern dd 16 Hz and 8 Hz, and dd 16 Hz and 9 Hz, CH$_2$), 5.24 (m, CH$_{2}$OH), 7.20 (d 2 Hz, 3-H), 8.64 (s, 8-H), 8.7 (broad, 4-OH) and 13.0 ppm (broad, 1-H)

$^1$H-NMR of 5b: 1.87 (m, CH$_3$), 3.09 (t, CH$_2$), 3.56 (t, CH$_2$O), 5.5 (broad, CH$_{2}$OH), 7.38 (d 2Hz, 3-H), 8.75 (s, 8-H), 9.6 (broad, 4-OH) and 12.8 ppm (broad, 1-H).

$^1$H-NMR of 5c: 2.67 (t, CH$_2$COO), 3.33 (t, CH$_2$), 7.38 (d 2Hz, 3-H), 8.78 (s, 8-H), 9.7 (broad, 4-OH) and 12.4 ppm (broad, 1-H).

The $^1$H-NMR spectrum of dehydrated 4d with signals at 7.27 (d 2 Hz, 3-H), 7.52 (s, 4-OH), 8.81 (s, 8-H), 9.8 (broad, 5-OH) and 12.5 ppm (broad, 1-H) is consistent with both structures 5d and 5e. As 5d can be distinguished chromatographically from authentic 5e (retention time 16.0 min. compared to 10.4 min. for 5e, linear gradient, 20 min., 23.5-91.5% (v/v) methanol, 0.4% H$_3$PO$_4$), it was assigned structure 5d.

Copper complexes of the dehydrated PQQ-derivatives

Titration of 5.8 nmol 5d with a stoichiometric amount of Cu$^{2+}$, resulted in the formation of a new absorption band, showing a 20 nm redshift of the 305-310 nm band (Fig. 4). No such change was observed upon addition of equimolar amounts of Cu$^{2+}$ to a solution of 5e. No red shifts were observed either upon addition of equimolar amounts of Cu$^{2+}$ to 5a or to 5b, in agreement with the location of the phenolic hydroxyl group at the C$_4$-position. Titration of 5c resulted in an unexpected red shift of 26 nm.

DISCUSSION

Inactivation of MDH by cyclopropanol is accompanied by modification of its cofactor PQQ, as evidenced by the altered chromatographic and spectrophotometric properties of the extracted cofactor [6]. Spectroscopic data reveal that the C$_5$-3-propanal adduct is formed, indicating that opening of the cyclopropane ring occurs during the reaction. The closed tetrahydrofuran structure (3b) is the preferred configuration of the adduct, in particular that of one of the stereoisomers. The striking similarity of the $^1$H- and $^{13}$C-NMR spectrum of the adduct and those of the acetone adduct indicates that addition has occurred at the C$_5$-position of PQQ. This is also supported by the absence of a 8-hydroxyquinoline moeity in the pyrroloquinoline derivatives prepared from both adducts. Cycloproppane hydrate, but not the ethyl hemiketal, is also a good inhibitor of MDH [6] and it modifies PQQ in a way analogous to that found for cyclopropanol.

Two mechanisms can be envisaged for the formation of an adduct between cyclopropanol and PQQ. In the ionic mechanism the cyclopropoxy anion is generated by a general base catalyzed proton abstraction. The cyclopropoxy anion then isomerizes under opening of the ring to the propenal carbanion, which subsequently attacks the electrophilic C$_5$-carbon of PQQ. Alternatively, the reaction proceeds according to a radical mechanism. Interaction of the cyclopropoxy anion or cyclopropanol with PQQ generates the cyclopropoxy (anion) radical and PQQH'. After ring-opening to the 3-propenal radical, which is known to occur very rapidly [20], it combines with PQQH' to form the covalent adduct in a concerted process. This is in fact the mechanism...
proposed previously [6].

The reaction of free PQQ with cyclopropanol only takes place in the presence of a suitable catalyst. A mechanism involving the direct formation of a cyclopropoxy radical does not seem likely since (i) Ag⁺-ions are not effective and (ii) CuO and ZnO are efficient catalysts, while it is known that Cu²⁺- and Zn²⁺-ions are not capable to oxidize cyclopropanol [21]. Apparently, the metal oxides generate the cyclopropoxy anion. The high reactivity of the cyclopropy carbamion formed upon ring-opening requires that it should be generated in close proximity to PQQ in order to be able to react with PQQ following one of the two mechanisms mentioned. This might be achieved in a complex formed by the simultaneous adsorption of PQQ and cyclopropanol to the metal oxide. The low reaction rate observed for the model reaction may reflect either the inefficiency of heterogeneous catalysis or may be related to the relatively slow ring-opening of the cyclopropoxy anion to the 3-propanal carbamion ($k = 1.5 \times 10^{-4} \text{ s}^{-1}$ [22]). Acetone does not react with PQQ in the presence of Ag₂O at pH 6.5, presumably because under this condition the acetone carbamion is not formed. It is interesting to note here that acetone does not react with enzyme bound PQQ either, since it is not an inhibitor for MDH, although propanol is a good substrate.

The behaviour of MDH towards cyclopropanol and its genuine substrate methanol shows some similarity. Recently it has been observed that the reaction of oxidized MDH with methanol generates a transient enzyme form, which has the same absorption spectrum as that of cyclopropano-inactive MDH [3], preceding the formation of reduced MDH and the product formaldehyde. In contrast, the semiquinone (PQQ⁻-containing) form of MDH does not react with either of these compounds [6,8]. Possibly, the redox potential of PQQ⁻ ($E_g = -112 \text{ mV at pH 7.3}$, [23]) is too low to induce a cyclopropoxy radical and the reduced electrophilicity of the C⁵-carbon in PQQ⁻ prevents nucleophilic attack of the cyclopropoxy anion. Although a radical mechanism for the reaction of cyclopropanol with PQQ in MDH has been put forward [6], an ionic mechanism cannot be excluded. From earlier studies it can be derived that the rate constant for inactivation of MDH is at least $1 \times 10^{-3} \text{ s}^{-1}$ [6]. Although this is faster than the base catalyzed ring-opening of cyclopropanol in solution, at first sight excluding the ionic mechanism, it is not known whether ring-opening of an enzyme bound cyclopropoxy anion is accelerated by the participation of the electrophilic C⁵-carbon of PQQ in the reaction.

REFERENCES

3. Frank, J., Dijkstra, M., Duine, J.A. and Balny, C., in the press
Samenvatting

Verreweg het grootste aantal van de in levende cellen voorkomende dehydrogenasen en oxidassen blijkt voor zijn werking afhankelijk te zijn van de reeds lang bekende cofactoren NAD(P)⁺ en flavines. In sommige gevallen wordt echter gebruik gemaakt van andere cofactoren. Dit proefschrift beschrijft de zuivering en karakterisering van een destijds onbekende cofactor: pyrroloquinoline quinone, afgekort tot PQQ.

In Hoofdstuk I wordt de ontdekking van PQQ beschreven en aangegeven welke plaats deze cofactor inneemt ten opzichte van vergelijkbare cofactoren. Tevens worden de thans bekende eigenschappen samengevat in samenhang met de Hoofdstukken II tot en met X.

De isolatie van PQQ uit de methylotrofe bacterie *Hyphomicrobium X* vond aanvankelijk plaats uitgaande van het enzym methanoldehydrogenase (MDH). De zuivering en eigenschappen van dit enzym zijn het onderwerp van Hoofdstuk II. Heel opmerkelijk was dat in het enzym een organisch vrij radicaal waargenomen kon worden met behulp van ESR spectroscopie. Het ESR signaal werd gekarakteriseerd door een giiso = 2,0045 en een top-top lijnbreedte van 0,7 mT. Een dergelijk signaal kon niet afkomstig zijn van een flavine semichinin of pteridine radicaal, maar wel van een chinon radicaal. Snelle oxidatie van gereduceerd methyleenblauw door de geïsoleerde cofactor (heetge wijst op een hoge redox potentiaal) was met dit laatste in overeenstemming.

De zuivering van PQQ, uitgaande van MDH of hele cellen, en enkele van zijn eigenschappen worden beschreven in Hoofdstuk III. Aangezien PQQ sterk gebonden werd aan een anionwisselaar en geen fosfaatgroepen bleek te bevatten, werd geconcludeerd dat het molecuul een of meerdere sterkzure carboxyl groepen bezit. Spectrale veranderingen rond pH 2,5 waren hiermee in overeenstemming. Reactie van PQQ met 3,4-dimethoxyaniline wees op een orthochinon structuurelement in een midden ring, in overeenstemming met de ESR gegevens. Van groot praktisch belang was de ontwikkeling van een HPLC analyse methode, zoals beschreven in dit Hoofdstuk. De geschiktheid van deze methode werd nog vergroot door gebruik te maken van een gemakkelijke derivatisering van PQQ met aceton tot een karakteristiek adduct.

Toen het mogelijk bleek om in een oplossing van het geïsoleerde PQQ een radicaal op te wekken waarvan het ESR signaal dezelfde eigenschappen vertoonde als dat van MDH kon geconcludeerd worden dat de chromofore groep van MDH betrokken was bij de door het enzym gekatalyseerde redox reacties. Aangezien het PQQ niet langer aan eiwit gebonden gebonden was vertoonde het ESR spectrum zogenaamde hyperfijn-structuur. Simulatie bleek mogelijk en de interpretatie van dit spectrum, zoals beschreven in Hoofdstuk IV, bracht aan het licht dat het vrije electron gekoppeld was met twee stikstofatomen en drie waterstofatomen, waarvan er één uitwisselbaar bleek te zijn. Dit resultaat klopte goed met de brutoformule verkregen met hoge resolutie massaspectrometrie. ¹H-NMR en massaspectrometrie van PQQ en van de tetrahydroverbinding worden behandeld in Hoofdstuk V. De verkregen gegevens bleken de structuur, afgeleid van die van een door middel van röntgen diffraactie opgeheelder structuur van een derivaat van PQQ, te ondersteunen. Minstens even betekenisvol was de waarneming dat het uit MDH geïsoleerde PQQ in staat was het apoenzym van glucosedehydrogenase te activeren, zodat duidelijk was dat de aldus geïsoleerde cofactor biologisch actief was. Na bepaling van de molaire absorptie coëfficiënt van PQQ, kon vastgesteld worden dat MDH één PQQ molecuul per enzym molecuul bevatte. Echter, al spoedig werd duidelijk dat bij de chromatografische analyse van de gexeexeerde cofactor een even grote hoeveelheid van de gereduceerde vorm van PQQ niet waargenomen was. De nadere karakterisering van PQQH₂ is het onderwerp van Hoofdstuk VI. Zowel de spectrale (λmax = 302 nm bij pH 7,0), als de chromatografische en redox eigenschappen (E½ = + 90 mV) werden bepaald. Onder bepaalde condities kon bij hoge pH de vorming waargenomen worden van het semichinin PQQ⁺, gekenmerkt door een absorptie maximum bij 475 nm. De redoxegenschappen van het radicaal
(E_m = -218 respectievelijk -242 mV voor de redox koppels PQQ/PQQH^- en PQQH^-/PQQH_2 bij pH 13.0) konden uit potentiometrische titraties bij pH 13.0 worden berekend.

Bereiding van het apoenzym van glucose dehydrogenase uit Acinetobacter calcoaceticus bleek niet altijd reproduceerbaar. Hetzelfde enzym uit Pseudomonas aeruginosa was beter geschikt voor dat doel. Naast deze biologische bepalingsmethode ontstond er behoefte aan een gevoelige chromatografische analyse methode, teneinde ook de aanwezigheid van biologisch niet actieve derivaten van PQP te kunnen detecteren. De ontwikkeling van een dergelijke methode, gebaseerd op de afbraak tot een sterk fluorescerende verbinding, wordt beschreven in Hoofdstuk VII en bleek veertig maal minder gevoelig te zijn dan de biologische test.

Tussen het fluorescentie-excitatie spectrum en het absorptie spectrum van PQQ bleek een groot verschil te bestaan. In Hoofdstuk VIII wordt aannemelijk gemaakt dat dit samenhangt met het feit dat de C_5-carbonyl groep van PQP in water gedeeltelijk gehydrateerd is en dat alleen de gehydrateerde moleculen fluoresceren. De verschuiving van de ligging van het hydratatie-evenwicht, veroorzaakt door verandering van de temperatuur, komt zowel in het ¹H-NMR-spectrum als in het absorptie spectrum en de intensiteit van de fluorescentie tot uiting. Met behulp van deze gegevens konden de spectra van de zuivere componenten berekend worden. Naast water bleken ook amines, alcoholen, ammonia en HCN te adderen aan de C_5-carbonyl groep. Deze verbindingen zijn nauw betrokken bij de enzymologie van quinoproteinen (PQQ bevattende enzymen), zodat de eigenschappen van deze adducten inzicht zouden kunnen verschaffen in het gedrag van de enzymen. De amine adducten bleken na verloop van tijd over te gaan in PQQH_2, de overige adducten waren stabiel.

De vondst van de groep van Abeles in Boston dat MDH irreversibel geremd wordt door cyclopropanol leidde bij nadere bestudering tot meer inzicht in het functioneren van dit enzym. De veronderstelling van Abeles c.s. dat cyclopropanol uitsluitend reageert met de PQP semichinin bevattende MDH moleculen bleek niet juist te zijn. Stoechiometrische hoeveelheden cyclopropanol ten opzichte van PQQ waren nodig om het enzym volledig te inactiveren, waarbij alle PQQ omgezet werd tot een sterk fluorescerend en spectraal op het aceton adduct van PQQ gelijkend product (Hoofdstuk IX). Het absorptie spectrum van het geïnactiveerde MDH leek sterk op dat van de geoxideerde vorm van MDH, gecombineerd met cyanide (MDHox). PQQ bleek niet spontaan met cyclopropanol te reageren, echter wel in aanwezigheid van koper- of zilveroxide. Het hoofdproduct dat hierbij gevormd werd was identiek met het gemodificeerde PQQ dat uit het geïnactiveerde MDH geëxtraheerd kon worden. Met behulp van NMR kon worden vastgesteld dat het hier gaat om het C_5-3-propanal adduct van PQQ. Additie aan de C_5-carbonyl groep werd verder ondersteund door de waarneming dat na reductie van het adduct, gevolgd door dehydratatie, geen 8-hydroxychinoline-achtige structuur ontstond (Hoofdstuk X). Aangezien tijdens de redox cyclus van MDH een intermediaire vorm waargenomen kon worden met hetzelfde absorptie spectrum als dat van het met cyclopropanol geïnactiveerde enzym, krijgt het opheffen van de structuur van het gemodificeerde PQQ meer betekenis. Het is nu immers zeer aannemelijk dat het enzym-intermediair een enzym-substraat complex is, waarbij methanol geadaardeerd is aan de C_5-carbonyl groep van PQQ.