Stellingen behorend bij het proefschrift "Strucutral and mechanistic aspects of soluble quinoprotein glucose dehydrogenase from Acinetobacter calcoaceticus" door Arjen Olsthoorn.

1. De door Alberty et al. afgeleide en door Stewart en Klinman toegepaste vergelijking, welke poogt cooperatieve kinetiek uit te drukken in twee paar Michaelis-Menten-parameters die de twee fases in een Lineweaver-Burk of Eadie-Hofstee grafiek beschrijven, is niet correct.

2. Hoewel glucoseoxydase het meest toegepaste enzym is in het gebied van biosensor-onderzoek, is de titel van het overzichtsartikel van Wilson en Turner hierover onjuist en in tegenspraak met de inhoud ervan.

3. Opheffering van de ruimtelijke structuur via Röntgenkristallografie biedt meer zekerheid omtrent het aantal gecombineerde metaalionen in eiwitten dan bepalingen via analytisch chemische methoden.

4. Bij hun analyse van de substraat-specificiteit van membraanbonden glucose-dehydrogenase uit Escherichia coli zien Cozier et al. over het hoofd dat L-aldoses de spiegelbeelden zijn van D-aldoses.

5. De levensvatbaarheid van een kinetisch model voor de werking van een enzym is een kwestie van "survival of the fit-test".

6. Koolhydraten zijn geen koolhydraten.

7. In de wetenschap is het vinden van de juiste vraag minstens zo belangrijk als het vinden van het juiste antwoord.

8. Voor microbiologisch werk dient men goed bij de pinken te zijn.

9. Een werkelijke uitbanning van bloeddoping uit de wieler sport zal zich uiten in het weer op hoogtestage gaan van de renners.

10. De slechte verbale hanteerbaarheid van de ‘euro’ zal aanleiding geven tot een veelvuldig gebruik van onofficiële namen voor deze munt.

11. Dit is een stelling.
Structural and mechanistic aspects of soluble quinoprotein glucose dehydrogenase from *Acinetobacter calcoaceticus*

Proefschrift
ter verkrijging van de graad van doctor
aan de Technische Universiteit Delft,
on gezag van de Rector Magnificus prof.ir. K.F. Wakker,
in het openbaar te verdedigen ten overstaan van een commissie,
door het College voor Promoties aangewezen,
op dinsdag 14 september 1999 te 16.00 uur
door

Adriaan Joseph Jan OLSTHOORN
scheikundig ingenieur
geboren te Schiedam
This study was carried out at the Department of Microbiology and Enzymology in the Kluiver Institute for Biotechnology of the Delft University of Technology, The Netherlands, and was financially supported by Boehringer Mannheim GmbH, Mannheim, Germany.
Dubium sapientiae initium
René Descartes

Aan mijn ouders
Contents

Chapter 1. General introduction 1

Chapter 2. Production, characterization, and reconstitution of recombinant quinoprotein glucose dehydrogenase (soluble type; EC 1.1.99.17) apoenzyme of Acinetobacter calcoaceticus 29

Chapter 3. Ca2+ and its substitutes have two different binding sites and roles in soluble, quinoprotein (pyrroloquinoline-quinone-containing) glucose dehydrogenase 37

Chapter 4. Negative cooperativity in the steady-state kinetics of sugar oxidation by soluble quinoprotein glucose dehydrogenase from Acinetobacter calcoaceticus 45

Chapter 5. On the mechanism and specificity of soluble, quinoprotein glucose dehydrogenase in the oxidation of aldose sugars 53

Summary 63

Samenvatting 65

Curriculum vitae 67

Nawoord 69
Chapter 1

General introduction
Contents

1. Biological oxidation of glucose
   1.1 Metabolism of glucose
   1.2 Direct oxidation of glucose
      \[\text{NAD(P) dependent glucose 1-oxidoreductase}\]
      \[\text{Flavoprotein glucose 1-oxidoreductases}\]
      \[\text{Quinoprotein glucose 1-oxidoreductase}\]

2. Quinoproteins
   2.1 Introduction
   2.2 The cofactor \textit{PQQ}
   2.3 \textit{PQQ}-dependent quinoproteins

3. Soluble quinoprotein glucose dehydrogenase (sGDH)
   3.1 Primary structure
   3.2 Quaternary structure
   3.3 Catalytic properties of sGDH

4. Application of sGDH in glucose determination

5. Scope and outline of this thesis
1. Biological oxidation of glucose

1.1 Metabolism of glucose

Glucose metabolism plays a prominent role in the chemistry of life. Glucose is the most abundant building block of bio-organic material and acts as the most common source of organic carbon and energy for growth and maintenance of living organisms.

The metabolic conversion of glucose itself is usually initiated by its phosphorylation to glucose-6-phosphate (Fig. 1), which occurs either intracellularly via hexokinase or during the transmembrane uptake process of glucose into the cytoplasm (1, 2). Glucose-6-phosphate is further metabolized or used in the biosynthesis of polysaccharides, glycosides or other sugars. In most organisms further metabolism proceeds (mainly) via the well known fructose-1,6-bisphosphate (FBP) pathway, through subsequent isomerization to fructose-6-phosphate. Other important routes, especially in bacteria, are the pentose phosphate (PP) pathway and the 2-keto-3-deoxy-6-phosphogluconate (KDPG) pathway (more commonly known as the Entner-Doudoroff pathway), which proceed via the formation of 6-phosphogluconic acid (Fig. 1). Although these pathways differ in many aspects, e.g. occurrence, efficiency in conservation of chemical energy and of organic carbon, or the ability to generate various biosynthetic precursors, they all provide in the intracellular conversion of glucose into glyceraldehyde-3-phosphate and pyruvate to feed the central intermediary metabolism.

![Diagram of glucose metabolism](image)

Figure 1. Phosphorylative initiation of glucose metabolism.
Enzymes: 1) hexokinase or phosphotransferase transport system, 2) phosphoglucone isomerase, 3) phosphofructokinase, 4) glucose-6-P dehydrogenase, 5) 6-P-gluconolactonase, 6) 6-P-gluconate dehydrogenase, 7) 6-P-gluconate dehydratase.
1.2 Direct oxidation of glucose

Distinguishable from the phosphorylative approach to glucose conversion described above, various microorganisms exhibit the ability for a direct oxidation of glucose at the C1-position. This initially yields the compound glucono-δ-lactone, which is readily hydrolysed, either spontaneously or via catalysis by lactonase, to gluconic acid (Fig. 2). As this oxidative process often occurs outside the cytoplasm of the organism, it results in the accumulation of gluconic acid in the external environment. Conversions such as this, which lead to the extracellular accumulation of oxidation products other than CO₂, are commonly referred to as incomplete oxidations. Other types of direct glucose oxidation also occur. Oxidation at the C2-position to yield 2-keto-glucose, has been observed in lignin-degrading fungi (3), and oxidation at the C3-position, to 3-keto-glucose is catalyzed by some bacterial enzymes (4). These conversions however occur much less abundantly than C1-oxidation and are not further discussed here.

![Chemical structures](image)

Figure 2. Direct oxidation of glucose, followed by hydrolysis, yielding gluconic acid.

Bacterial gluconic acid production was first described by Boutroux in 1880 (5) in cultures of *Mycoderma aceti* (presently known as *Acetobacter aceti* var. *xylinum*), and was subsequently observed in a variety of Gram-negative bacteria as well as filamentous fungi. Both species of *Gluconobacter* and the fungus *Aspergillus niger* are presently used for the industrial production of gluconic acid and gluconates, which amounts up to 40-50000 tons annually (6). Applications of these compounds are based mainly on the chelating properties of gluconic acid; sodium gluconate and gluconic acid are used as a calcium sequestering agent in technical applications such as calcium deposit removal and retardation of concrete hardening, while other gluconates (e.g. calcium, potassium, iron, magnesium, manganese, zinc, cobalt or copper gluconate) are applied medically or in health food and animal feeds, to treat or prevent mineral deficiency (6).

The actual oxidation of glucose to glucono-d-lactone is catalysed by a variety of quite different enzymes (Table 1). Apart from their protein structures, these enzymes differ with respect to their mechanisms in the following aspects. The (initial) redox reaction with glucose occurs either with a coenzyme (NAD(P)+), which in fact acts as a co-substrate as it is released after becoming reduced, or with a cofactor (PQQ, FAD, etc.), being firmly bound to the enzyme in all steps of the catalytic cycle, including those involved in its reoxidation by an electron acceptor. When the electron acceptor is oxygen, the oxidoreductase is called an oxidase, otherwise a dehydrogenase.
Table 1. Glucose 1-oxidoreductases

<table>
<thead>
<tr>
<th>EC name</th>
<th>EC number</th>
<th>acceptor</th>
<th>source (location)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>NAD(P)^+ -dependent enzymes</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>glucose 1-dehydrogenase (NAD(P)^+)</td>
<td>1.1.1.47</td>
<td>NAD^+ / NADP^+</td>
<td>pig, bovine, trout, salmon (liver) Bacillus spp., Sulfolobus solfataricus, Nostoc sp., Thermoplasma acidophilum, Corynebacterium sp. (cytoplasm)</td>
</tr>
<tr>
<td>glucose 1-dehydrogenase (NAD^+)</td>
<td>1.1.1.118</td>
<td>NAD^+</td>
<td>Pseudomonas sp. (cytoplasm)</td>
</tr>
<tr>
<td>glucose 1-dehydrogenase (NADP^+)</td>
<td>1.1.1.119</td>
<td>NADP^+</td>
<td>Gluconobacter spp. (cytoplasm)</td>
</tr>
<tr>
<td>aldose 1-dehydrogenase (NAD^+)</td>
<td>1.1.1.121</td>
<td>NAD^+</td>
<td>Pseudomonas sp., Bacterium strain 58 (cytoplasm)</td>
</tr>
<tr>
<td>glucose-fructose oxidoreductase (tight bound NADP^+)</td>
<td>1.1.99.28</td>
<td>fructose</td>
<td>Zymomonas mobilis (periplasm)</td>
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<td><strong>Flavoproteins</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>glucose oxidase (FAD)</td>
<td>1.1.3.4</td>
<td>O_2</td>
<td>Penicillium spp. (excreted) Aspergillus spp. (peroxisomes)</td>
</tr>
<tr>
<td>hexose oxidase (flavin)</td>
<td>1.1.3.5</td>
<td>O_2</td>
<td>red seaweeds; e.g. Chondrus crispus, Citrus spp. (in fruit)</td>
</tr>
<tr>
<td>glucose dehydrogenase (FAD)</td>
<td>1.1.99.10</td>
<td>?, dyes</td>
<td>Aspergillus oryzae (membrane)</td>
</tr>
<tr>
<td><strong>Quinoproteins (PQQ)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>membrane-bound glucose dehydrogenase (PQQ)</td>
<td>1.1.99.17</td>
<td>coenzyme Q</td>
<td>Gram-negative bacteria; e.g. Pseudomonas, Gluconobacter, Acetobacter, Acinetobacter, Klebsiella (cytoplasmic membrane)</td>
</tr>
<tr>
<td>soluble glucose dehydrogenase (PQQ)</td>
<td>1.1.99.17</td>
<td>?, dyes</td>
<td>Acinetobacter calcoaceticus (periplasm)</td>
</tr>
</tbody>
</table>

**NAD(P)^+-dependent glucose 1-oxidoreductases**

Glucose-dehydrogenases requiring NAD^+ or NADP^+ (Fig. 3) as a coenzyme have been isolated from prokaryotes, eukaryotes and archaeae. In to the EC-numbering system these have been further subdivided on the basis of (un)specificity towards the coenzyme and the substrate (Table 1). As the redox potentials of the couples glucose-gluconolactone and NAD(P)^+-NAD(P)H are not much different, the oxidation of glucose catalysed by this group of enzymes is reversible.

In archaebacteria (Sulfolobus, Thermoplasma) NAD(P)^+-dependent glucose dehydrogenase performs the first step in glucose metabolism, proceeding via a modified Entner-Doudoroff pathway in the absence of any phosphorylation, leading to glyceraldehyde and pyruvate (7, 8). In Gram-positive bacteria (Bacillaceae) the enzyme is produced in the early stages of sporogenesis (9). In animals, it is found in the endoplasmatic reticulum of
liver cells; its physiological role is not clearly established (10). The significance of the cytoplasmic NAD(P)+-dependent glucose dehydrogenases found in Gram-negative bacteria (Pseudomonas, Gluconobacter), is also unclear, since gluconate production by these organisms has been found to proceed mainly via the membrane bound quinoprotein glucose dehydrogenase (11, 12). In the fission yeast Schizosaccharomyces pombe a NADP+-dependent glucose dehydrogenase provides, together with a gluconate kinase, catalysing phosphorylation of gluconate to 6-phosphogluconate, an alternative entrance route for glucose metabolism via the pentose phosphate pathway (13).

The bacterium Zymomonas mobilis possesses a glucose-fructose oxidoreductase that contains firmly bound NAD+, acting as a cofactor rather than as a coenzyme. After oxidation of glucose, the resulting NADH remains enzyme bound and is reoxidized via reaction with fructose, thus yielding the overall reaction products gluconolactone and sorbitol (14). The sorbitol accumulates inside the cell and acts to provide tolerance of the organism towards the high osmotic pressure, due to very high sugar concentrations, in its natural environment. Glucose metabolism in Z. mobilis proceeds via the Entner-Doudoroff pathway (8), so that further metabolism of gluconic acid may possibly occur via phosphorylation to 6-phosphogluconate.

**Flavoprotein glucose 1-oxidoreductases**

The key enzyme in gluconate formation by fungi is a FAD (flavin adenine dinucleotide, Fig. 3) containing glucose oxidase (15), which catalyses the oxidation of glucose with molecular oxygen, thus also yielding hydrogen peroxide. It is a homodimeric glycoprotein containing one non-covalently bound FAD cofactor per subunit. Penicillium species excrete glucose oxidase (16), whereas in Aspergillus spp. the enzyme is located in special organelles, the peroxisomes (17). These peroxisomes also contain catalase, catalysing the decomposition the H₂O₂, which is toxic for the cell, into H₂O and O₂, and a lactonase, accelerating the hydrolysis of gluconolactone to gluconic acid. Glucose oxidase from A. niger is commercially available and is much used in systems for glucose determination and in the preservation of processed food.

While the fungal glucose oxidases display a high specificity for glucose, the hexose-oxidases found in red algae or Citrus fruits (Table 1) are active with a much broader range of aldoses. A recent study of the enzyme from Chondrus crispus (18) indicates that it contains a covalently-bound substrate-reducible flavin.

The in vivo function of gluconic acid production through oxidases is not directly obvious; as the reducing equivalents derived from glucose are passed directly to O₂ and not via the electron transport chain, the oxidation does not provide useful biochemical energy for the organism. Its function may be related to resulting changes in the environment of the organism; solubilisation of mineral salts, increased competitiveness towards other organisms due to pH-decrease, glucose removal and/or production of the biocidal H₂O₂ (6) (due to the latter effect the excreted glucose oxidase from Penicillium notatum was originally characterized as an antibiotic agent and named penicillin A or notatin (15)).
A FAD-dependent glucose-dehydrogenase has been found in *Aspergillus oryzae* (19) and
other fungi (B.W. Groen and J.A. Duine, unpublished results) but has not been further
caracterized to date.

![Molecule structures](image)

**Figure 3. Coenzymes / cofactors of various glucose 1-oxidoreductases.**

A: Nicotinamide adenine dinucleotide (NAD\(^+\)), B: Nicotinamide adenine dinucleotide phosphate
(NADP\(^+\)), C: Reduced form (NAD(P)H), D: Flavin adenine dinucleotide (FAD), E: reduced form
(FADH\(_2\)), F: pyrroloquinoline quinone (PQQ), G: reduced form (PQQH\(_2\) = quinol).

**Quinoprotein glucose 1-oxidoreductases**

This class concerns the topic of this thesis and is discussed below.

2. Quinoproteins

2.1 Introduction

In the early 1960s, Hauge isolated and partially characterized a (soluble) glucose
dehydrogenase from *Bacterium anirratum* (now *Acinetobacter calcoaceticus*) and established
that this enzyme contained a dissociable organic cofactor of hitherto unknown nature (20, 21). Independently, a similar finding was made in 1967 by Anthony and Zatman for the enzyme methanol dehydrogenase from *Pseudomonas* sp. (22). The structure of the methanol
dehydrogenase cofactor was elucidated in 1979 independently by the groups of Forrest (23) and of Duine (24). Duine proposed to name the new compound pyrroloquinoline quinone
(PQQ), and, upon demonstrating that the glucose dehydrogenase described by Hauge
contained the same cofactor as methanol dehydrogenase, he introduced the term quinoprotein
to define the class of PQQ-dependent enzymes (analogous to the term flavoprotein for enzymes containing flavin cofactors and haemoprotein for enzymes that contain haem groups) (25).

In the two decades following its structural elucidation, various redox enzymes have been shown to contain or require PQQ as a cofactor (some microorganisms have been found to produce enzymes that require PQQ for activity but not to synthesize PQQ itself so that the enzymes are present as the inactive apo-form). Furthermore, other redox enzymes were found to contain quinonoid groups which are covalently linked to the protein, and which seemed to be derived from PQQ. Further research has shown, however, that these covalently linked quinonoid groups are no PQQ-derivatives, but are derived directly from aromatic amino acid residues in the protein primary chain, yielding the structures tryptophyl tryptophanquinone (26) (TTQ, occurring in amine dehydrogenases), topaquinone (27) (TPQ, the quinone form of 2,4,5-trihydroxy phenylalanine, in copper containing amine oxidases) and lysyl tyrosinquinone (28) (LTQ, in lysyl oxidase). The redox enzymes which contain these groups are also termed quinoproteins, but have no structural similarity or relationship to those that contain or require PQQ (for a general review of quinoproteins, see (29, 30)).

2.2 The cofactor PQQ

PQQ is an orthoquinone (see Fig. 3) with the systematic name 2,7,9-tricarboxy-1H-pyrrrolo[2,3-β] quinoline-4,5-dione. In the process of enzyme-catalyzed oxidation of substrate it is reduced to the quinol form (PQQH₂). Free PQQ can be easily reduced to PQQH₂ by a variety of reducing agents, and the free quinone/quinol couple has a two-electron midpoint potential of +90 mV at pH 7 (31). At neutral pH, free PQQH₂ is readily reoxidized to PQQ by dissolved oxygen, but at pH 2 it is fairly stable (32). In methanol dehydrogenase PQQ has been observed to occur in a stable semiquinone form (PQQH⁻), indicating that reoxidation of PQQH₂ in this enzyme may take place in two steps, each involving a single electron transfer (33).

PQQ is soluble in water, in aqueous buffers and in other highly polar solvents such as DMSO. In accordance with its aromatic heteropolycyclic nature it has characteristic spectral properties. At neutral pH it forms a brick-red aqueous solution with an absorbance spectrum characterized by maxima at 249 nm, 273 nm (shoulder), 331 nm and 475 nm (very broad) with molar extinction coefficients of 20800, 16350, 10050 and 680 M⁻¹cm⁻¹, respectively (Fig. 4). The absorbance spectrum of the quinol form is quite different, showing a distinct peak at 302 nm with a molar extinction coefficient of 28300 M⁻¹cm⁻¹ (31). The absorbance spectrum of aqueous PQQ-solutions is strongly influenced by pH and temperature. The effect of pH is related to the acid-base equilibria of the three carboxyl groups, the pyridine nitrogen and the pyrrole proton (PQQ has at least 5 pKₐ's) (34). The effect of temperature is due to the occurrence of hydration of the C5-carbonyl group; lowering the temperature shifts the equilibrium in favour of the hydrate (35). The hydrated species is also the causative agent of the green fluorescence that is observed with aqueous solutions of PQQ. When enzyme-bound, the spectral absorbance of PQQ is readily distinguishable from the protein
Figure 4. Absorbance spectra of PQQ and PQQH₂.
Spectra of PQQ (—) and PQQH₂ (---) in 50 mM potassium phosphate, pH 7 (31). PQQ was reduced to PQQH₂ with H₂ in presence of PtO₂.

absorbance, (although it is less clearly visible in the case of quinohaemoproteins, due to the overshadowing absorption bands of the haem-groups), and, not surprisingly, differs from that of the free form. These features are suitable in the study of these enzymes, yielding e.g. information regarding enzyme-cofactor-interaction or the redox-state of PQQ.

The C₅-carbonyl group of PQQ is susceptible to nucleophilic addition. PQQ reacts easily with acetone and aldehydes to yield blue fluorescent C₅-aldol adducts that are fairly stable in aqueous solution (32, 35). The X-ray crystallographic analysis to solve the structure of PQQ was actually performed on the acetone adduct (23). Reversible adduct formation takes place with alcohols, ammonia, cyanide and water (to form the hydrate already mentioned above) (35, 36). Adduct-formation at the C₅ position is also thought to be a key step in PQQ-catalyzed biomimetic oxidation of amines, thiols and amino acids with oxygen (34) and in the formation of PQQ-oxazoles with amino acids (37, 38). Reduction of PQQ with alkaline NaBH₄, in the presence of oxygen, yields the 4,5-dihydrodiol PQQH₄ (39), presumably via a

Figure 5. Reduction of PQQ with NaBH₄ to PQQH₄.
Intermediary formed PQQH₂ is either further reduced or reoxidized to PQQ by oxygen.
mechanism involving the intermediate C₅-reduced hydroxydienone (40) (see Fig. 5). Of course, the observed chemical reactivity of PQQ also has implications regarding the mechanism of action of PQQ-dependent oxidoreductases (see § 3.3).

The biosynthesis of PQQ has been studied in several bacterial species. Cultivation of bacteria in the presence of specific $^{13}$C and $^{15}$N labeled compounds has revealed that PQQ is built up from the amino acids glutamate and tyrosine (41-43) (see Fig. 6). Genetic studies have shown that PQQ biosynthesis is encoded by an operon containing 5 to 7 genes. One of these genes codes for a hypothetical polypeptide of 24 amino acids (pqqIV, Acinetobacter calcoaceticus (44)), 23 amino acids (pqqA, Klebsiella pneumoniae (45)) or 29 amino acids (pqqD, Methylobacterium extorquens (46)) containing a conserved glutamate and tyrosine residue. This small polypeptide is therefore presumed to form a precursor compound from which PQQ is derived, via a biosynthetic pathway involving oxidation, ring closure and peptide cleavage steps, catalysed by the proteins encoded by the other genes on the PQQ biosynthesis operon (47). However, strains of Methylobacterium extorquens containing a deletion or insertion mutation in the gene encoding the alleged precursor peptide (renamed pqqA) are still able to produce PQQ, suggesting that either this organism produces a different peptide which can substitute for PqqA, or that PqqA-like peptides may not be obligatory precursors of PQQ (48).

2.3 PQQ-dependent quinoproteins

The quinoprotein enzymes that are at present recognized to require PQQ as cofactor are all found in Gram-negative bacteria, and are situated either in the periplasm or bound to the cytoplasmic membrane with the substrate binding site directed towards the periplasm. Some of these PQQ-dependent enzymes also contain haem c and are therefore known as quinohaemoproteins. The most thoroughly characterized to date are those that catalyse the oxidation of methanol, ethanol (and other primary alcohols) or glucose (and other aldoses), presented in Table 2. Other PQQ-dependent enzymes are involved in the oxidation of quinate (49), polyvinyl alcohol (a secondary alcohol) (50), lupanine (51), glycerol (52) or fructose (53) (the nature of the cofactor of fructose dehydrogenase has however not yet been unambiguously established as PQQ).

Methanol dehydrogenase (MDH) has been isolated from many methylotrophic bacteria (54), organisms distinct from others by the ability to grow on organic compounds containing
<table>
<thead>
<tr>
<th>Substrate</th>
<th>Enzyme</th>
<th>Source</th>
<th>Structure 1</th>
<th>Location</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>methanol</td>
<td>MDH</td>
<td>Methylophilic bacteria</td>
<td>αβ</td>
<td>p</td>
<td>(56, 127)</td>
</tr>
<tr>
<td>ethanol (alcohol)</td>
<td>ADH</td>
<td><em>Pseudomonas</em> spp.</td>
<td>αβ</td>
<td>p</td>
<td>(58, 59)</td>
</tr>
<tr>
<td>QH-ADH&lt;sub&gt;1&lt;/sub&gt;</td>
<td>e.g. <em>Comamonas testosteroni</em></td>
<td>α</td>
<td>p</td>
<td>(110, 128)</td>
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</tr>
<tr>
<td>QH-ADH&lt;sub&gt;2&lt;/sub&gt;</td>
<td>Acetic acid bacteria, e.g.</td>
<td>αβ</td>
<td>m</td>
<td>(61, 129)</td>
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<td>Acetoener aeri,</td>
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<td><em>Gluconobacter suboxidans</em></td>
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<tr>
<td>glucose (aldose)</td>
<td>mGDH</td>
<td>Gram-negative bacteria</td>
<td>α</td>
<td>m</td>
<td>(69, 82)</td>
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<td></td>
<td>sGDH</td>
<td><em>Acinetobacter calcoaceticus</em></td>
<td>α</td>
<td>p</td>
<td>(83, 91)</td>
</tr>
</tbody>
</table>

1) α, β, γ represent different subunits, not necessarily identical for each enzyme; however, all enzymes contain 1 PQQ per α-subunit. An underline denotes a haem c containing (sub)unit.

2) p: periplasm (soluble enzyme), m: bound to cytoplasmic membrane.

one carbon atom. It catalyses the oxidation of methanol to formaldehyde, but is also active with ethanol and other primary alcohols. The 3-dimensional structures of MDH from *Methylophilus W3A1* (55, 56) and from *Methylobacterium extorquens* (57) have been determined via X-ray crystallography, revealing that these enzymes display a so-called superbarrel or propeller structure, exhibiting 8 propeller blades consisting of W-shaped beta-sheets.

The other alcohol dehydrogenases show little or no activity with methanol. They catalyse the oxidation of a wide variety of alcohols and in most cases also of the resulting aldehydes, leading to the formation of acids. Based on their structural properties, these enzymes have been classified in three groups (see Table 2). Quinoprotein alcohol dehydrogenase (Q-ADH) is, apart from substrate specificity, very similar to MDH (58). Besides a similar quaternary structure, both enzymes have a cytochrome c as natural electron acceptor, and require a high pH and activation by ammonia or a primary amine in the reaction with artificial electron acceptors. Some controversy exists concerning the presence of the smaller subunit in Q-ADH (59).

Quinohaemoprotein alcohol dehydrogenase type I (QH-ADH<sub>1</sub>) is isolated as an apoenzyme from *Comamonas testosteroni* (60), but as a holoenzyme from *Pseudomonas putida* (61) or *Rhodopseudomonas acidiphila* (62). It contains one haem c group. Besides with simple alcohols the enzyme is also active with larger substrates such as vanillyl alcohol (62) or polyethylene glycol (63) and with some secondary alcohols (63).

The type II quinohaemoprotein alcohol dehydrogenase (QH-ADH<sub>2</sub>) is responsible for the rapid oxidation of ethanol by acetic acid bacteria (64, 65), utilized in the production of vinegar. They consist of three subunits, the largest one probably containing PQQ and a haem c, and the middle one being a cytochrome c553 containing two haems. All three haem groups appear to be involved in the transfer of electrons from the cofactor PQQ to the natural electron acceptor, coenzyme Q (66). *Acetobacter polyoxoxogenes* contains an alcohol
dehydrogenase which is very similar to QH-ADH₂, but consists of only two subunits of 74 and 44 kDa, respectively (67).

**Membrane-bound quinoprotein glucose dehydrogenase**

The ability to oxidize glucose to glucono-δ-lactone is widespread among Gram-negative bacteria. It is due to the constitutive or inducible presence in these organisms of a PQQ-dependent membrane-bound glucose dehydrogenase (mGDH). Surprisingly, various organisms, e.g. *Rhodopseudomonas sphaeroides* (68), *Escherichia coli* (69) and *Agrobacterium tumefaciens* (70), contain mGDH in the apoenzyme-form as they do not synthesize PQQ at the growth conditions normally applied. Thus, their ability to produce gluconic acid depends on the external presence of PQQ. The oxidation of glucose by mGDH is linked to the electron transport chain via coenzyme Q (ubiquinone) (71) and has been established to serve as a source of energy for the organism (72). As the enzyme has a fairly high Kₘ for glucose (around 1 mM) and its active site is facing the periplasm, it will function only in environments that contain relatively high glucose concentrations (73).

The physiological significance of mGDH-catalysed glucose oxidation differs for various organisms. In pseudomonads it functions as an alternative, oxidative, initiation of glucose metabolism (74); the resulting gluconate is phosphorylated to 6-phosphogluconate which is further metabolized via the Entner-Doudoroff pathway, which is the common route of glucose metabolism in these organisms. *Escherichia coli* normally metabolizes glucose via the FBP-pathway. When it is grown in the presence of PQQ, it can oxidize glucose to gluconic acid via mGDH, and induction of the ED-pathway takes place, enabling further metabolism of the gluconate (75).

*Gluconobacter* species, utilized in industrial gluconate production, are able to convert glucose at high concentrations, with a high rate and in a nearly stoichiometrical manner (76). Under these conditions glucose is mainly used as an energy source. Only small amounts of glucose or gluconate are used as carbon source and this metabolism proceeds exclusively through the PP-pathway (77), again via conversion of gluconate to 6-phosphogluconate. However, glucose cannot serve as the sole carbon source for *Gluconobacter*, as it lacks a complete tricarboxylic acid cycle (78). *Gluconobacter* and the closely related *Acetobacter* also contain a PQQ-dependent alcohol dehydrogenase and are the main biocatalysts in vinegar (acetic acid) production.

**Soluble quinoprotein glucose dehydrogenase**

The bacterium *Acinetobacter calcoaceticus* contains both a soluble and a membrane-bound PQQ-dependent glucose dehydrogenase. The membrane bound enzyme is homologous to the mGDHs from other bacteria, described above, the soluble enzyme (sGDH), which occurs in the periplasm, is structurally quite different. While sGDH so far has only been found in A. *calcoaceticus*, genes coding for proteins with a similar structure have recently been observed in several other organisms (79).
The occurrence of glucose dehydrogenase activities with different substrate specificities in soluble and membrane fractions of *A. calcoaceticus* was already noted in early studies aimed at purification of glucose dehydrogenase from this organism. This was initially assumed to be due to different forms of one and the same enzyme (80, 81). The presence of two completely different glucose dehydrogenases was first established by genetic studies that resulted in the cloning of two different GDH-genes, one coding for a protein of 83 kDa (82), similar to membrane-bound GDHs from other sources, and the other coding for a protein of 50 kDa (83), in agreement with the subunit molecular weight of purified soluble GDH. At about the same time, other indications for the occurrence of two different glucose dehydrogenases were obtained, e.g. via antibodies raised against mGDH of *Pseudomonas fluorescens*, which showed cross-reactivity with the membrane fraction of *A. calcoaceticus* but not with partially purified sGDH (84, 85).

It is noteworthy that, despite their evident difference, sGDH and mGDH are (still) designated by the same EC number. Possibly this has to do with the fact that EC numbering is based on catalytic rather than on structural aspects. After the difference between sGDH and mGDH became established, it has been suggested however (86) to reserve the number E.C. 1.1.99.17 for sGDH, being in fact the first enzyme to which this number was attributed, and to give mGDH a new number.

*Acinetobacter* species are not able to metabolize glucose (or gluconic acid), but the oxidation of glucose to gluconic acid does provide some useful energy to the organism (72). When grown on the usual media, an *A. calcoaceticus* sGDH-mutant shows no defects and oxidizes glucose like the wild-type organism, while on the contrary a mGDH-mutant does not perform any glucose oxidation (83). Furthermore, the substrate specificity displayed by the organism is similar to that of the membrane bound enzyme, but not to that of the soluble enzyme (87). Thus, under the conditions that have been tested, sGDH is not functional in the oxidation of aldose sugars *in vivo*. In view of the periplasmic localization of sGDH, this suggests that there is no functional coupling of sGDH to the respiratory chain (under these conditions). Related to this, the physiological electron acceptor for sGDH is not clearly known, although a periplasmic haemoprotein, cytochrome *b*₅₆₂, has been suggested to play this role (88). The physiological role of sGDH is thus unclear and the present knowledge does not allow to make a unequivocal choice between the following hypotheses:
1) sGDH is a rudiment from evolution and is not functional (anymore) in *A. calcoaceticus*.
2) sGDH becomes only functional in aldose sugar oxidation under specific conditions, that result in its coupling to the respiratory chain.
3) sGDH has a function in the reduction of an external electron acceptor, present in its environment under specific conditions, coupled to aldose sugar oxidation
4) sGDH has an as yet unknown function, other than oxidation of aldose sugars.

However, *in vitro* (with artificial electron acceptors), sGDH has a much higher turnover number for glucose than mGDH. Since the structure and mechanism of action of sGDH are the subject of this dissertation, these topics are separately discussed in § 3, in comparison, where relevant, with other PQQ-dependent enzymes.
3 Soluble quinoprotein glucose dehydrogenase (sGDH)

3.1 Primary structure

The gene for sGDH (gdhB) encodes a protein of 478 amino acids. In agreement with the observed location of sGDH in the periplasm (89), the first 24 amino acids comprise a signal peptide, that is involved in - and removed during - transport of the protein across the cytoplasmic membrane to the periplasm, thus yielding a mature protein of 50.231 Da. The gene has been cloned in Escherichia coli (83), and proper removal of the signal peptide also takes place in this recombinant strain (Chapter 2).

Comparison of the aminoacid sequences of (the PQQ-binding subunits of) all types of PQQ-dependent dehydrogenases listed in Table 2, shows that these enzymes are all strongly related, with the exception of sGDH, which shows hardly any similarity to these or any other known protein sequence. Weak similarity of sGDH (4 identical residues), observed in a region (47 residues) of high similarity between five other PQQ-enzymes (two mGDHs, two MDHs and a QH-ADH II, 14 residues identical in all five), led to the proposal that this region is involved in PQQ-binding (90). However, the recently obtained 3-D structures of methanol dehydrogenase from Methylophilus W3A1 (55) and from Methylobacterium extorquens (57) have shown that the residues involved in the binding of PQQ are scattered all over the protein chain. The stretch bearing similarity with sGDH is not in the vicinity of the active site and no other suggestion for a possible specific function has emerged so far.

3.2 Quaternary structure

As isolated from Ac. calcoaceticus, or as obtained after reconstitution of the apoenzyme, the active sGDH holoenzyme is a dimer of identical subunits and contains one PQQ per subunit (91, 92). X-ray fluorescence spectrometry (93), reconstitution experiments and atomic absorption spectrometry (Chapter 3) have indicated that the active holoenzyme furthermore contains four Ca^{2+}-ions. Recent X-ray crystallographical data of sGDH however show the presence of three Ca^{2+}-ions per subunit, i. e. six per holoenzyme molecule (79). One of the Ca^{2+}-ions is coordinated to the C5-oxygen, N6 and C7-carboxyl group of PQQ, in the same way as has been observed for the Ca^{2+} present in methanol dehydrogenase (MDH) (55) (see Fig. 7), while the others are located near the dimer interface. As noted in Chapter 5, the values reported in Chapter 3 may have been underestimated due to inaccuracy of the specific absorption coefficient used to establish the enzyme concentration. Alternatively, it might be that one of the two non-PQQ-coordinated ions found in the X-ray structure is relatively loosely bound and not essential for activity, and is absent from the enzyme under the conditions used in the other metal ion determinations.

Requirement of Ca^{2+} (or of Mg^{2+}, which is the preferred metal ion in mGDH (94-96)) for the formation of active holoenzyme is a common trait of PQQ-dependent enzymes. Treatment with chelating agents such as EDTA or CDTA has been found to result in the liberation of PQQ from alcohol dehydrogenase (ADH) (97) or mGDH (39) of Pseudomonas aeruginosa. On the other hand, a similar treatment does not lead to inactivation of sGDH (21,
93), quinohaemoprotein alcohol dehydrogenase (QH-ADH\textsubscript{II}) (98) or mGDH (94) from \textit{Glucobacter suboxydans}, mGDH of \textit{A. calcoaceticus} (96) or MDH from \textit{Methyllobacterium extorquens} (99). Whereas the loss of PQQ upon Ca\textsuperscript{2+}-removal observed for some quinoproteins suggests an essential role of Ca\textsuperscript{2+} in PQQ binding, this is apparently not the case with QH-ADH\textsubscript{I} from \textit{Comamonas testosteroni}, where treatment with EDTA or CDTA does lead to calcium removal and enzyme inactivation, but not to detachment of the PQQ (100). Reconstitution experiments with sGDH indicate that Ca\textsuperscript{2+}-ions have two functions in this enzyme and are required for subunit dimerization as well as for activation of an inactive PQQ-containing enzyme-form (Chapters 2 and 3). The observed involvement of Ca\textsuperscript{2+} in enzyme activation suggests that the metal ion plays a specific role in the catalytic process (see also § 3.3), although its role could also be limited to proper positioning of the PQQ.

In the case of sGDH, Ca\textsuperscript{2+} may be substituted by Cd\textsuperscript{2+}, Mn\textsuperscript{2+} or Sr\textsuperscript{2+} (93, 101), yielding active enzymes that have fairly similar catalytic properties to the Ca\textsuperscript{2+}-containing enzyme (see Chapter 3 and 4). Other, differing metal ion preferences have been observed for ADH (97), mGDH (95), QH-ADH\textsubscript{I} (100) and QH-ADH\textsubscript{II} (98), suggesting differences in the properties of the active sites of these enzymes. Furthermore, for MDH, replacing Ca\textsuperscript{2+} by Sr\textsuperscript{2+} (102) or Ba\textsuperscript{2+} (103) results in enzymes with an increased maximal turnover rate.

Upon reconstitution in the presence of Ca\textsuperscript{2+}-ions, apo-sGDH displays high affinity for PQQ (see Chapter 2). This, in combination with the high turnover number of sGDH, makes the apoenzyme very suitable for the detection and assay of PQQ (38, 104). The protein-cofactor interaction is strongly affected by temperature. Reversible inactivation, accompanied by the release of PQQ, occurs at temperatures above 35°C; in the presence of Ca\textsuperscript{2+}-ions (1 mM CaCl\textsubscript{2}) this is shifted to temperatures above 50°C (93). Dissociation of the dimeric apoenzyme, in the absence of free Ca\textsuperscript{2+}-ions, takes place at temperatures above 30°C (Chapter 3), which suggests that in the observed thermal inactivation and PQQ release, subunit dissociation is involved.

The ultraviolet/visible absorbance spectrum of apo-sGDH is that of a protein (93), but due to the presence of PQQ(H\textsubscript{2}) the spectra of oxidized and reduced holoenzyme display additional absorbance maxima at higher wavelengths (see Chapter 2, Fig. 1). These maxima are significantly red-shifted compared to the maxima of free PQQ and PQQH\textsubscript{2}, which suggests that the PQQ-binding site of sGDH is fairly hydrophobic, as the spectrum of triesterified PQQ in organic solvent also shows a substantial red-shift, and similar red shifts are observed with MDH (33), which has PQQ present in a hydrophobic cavity (56). Interestingly, in mGDH from \textit{Acinetobacter calcoaceticus}, the optical properties of the cofactor are quite different from those of sGDH and MDH and much more resembling those of free PQQ (96). Another notable difference is, that while with sGDH reconstitution of the apoenzyme with PQQ directly leads to active holoenzyme, in the case of mGDH initially an anomalous enzyme form is obtained, which converts to the normal holoenzyme form upon treatment with sulfite or under assay conditions (96).
As reduction of sGDH by addition of substrate is accompanied by marked changes in the absorbance spectrum of the enzyme, absorbance spectroscopy has been useful in determination of the PQQ-content of the enzyme (21, 92), anomer specificity ((21), Chapter 5), the investigation of the reconstitution process and the role of Ca\textsuperscript{2+} in this (Chapters 2 and 3) and of the catalytic mechanism (Chapter 5).

3.3 Catalytic properties of sGDH

Although it appears not functional in vivo, sGDH displays a high glucose oxidation activity in in vitro assays with various artificial electron acceptors (dyes), such as dichlorophenol indophenol, phenazine methosulphate or Wurster's blue (20, 91, 92). In addition to glucose, many other aldoses, both hexoses and pentoses as well as disaccharides, act as substrates for sGDH (84, 87, 105). Its substrate specificity is further investigated in Chapter 5.

As is usual with dye-linked dehydrogenases, sGDH exhibits a ping-pong kinetic behaviour, indicating that the catalytic cycle consists of two successive redox reactions: one between enzyme and substrate in which the enzyme (actually the enzyme-bound cofactor) becomes reduced and oxidized product is released; and the other involving reduction of an electron acceptor, resulting in reoxidation of the enzyme(-cofactor). This kinetic behaviour is attended by mutual substrate inhibition by substrate and electron acceptor (91, 92, 105). The occurrence of negative cooperativity has been observed (84), and is analyzed in detail in Chapter 4. The observed kinetic behaviour can be well described by an equation accounting for biphasic cooperativity, and may be due to interaction between both enzyme subunits of the dimeric enzyme.

Experimental as well as modeling studies regarding the mechanism of action of PQQ-dependent dehydrogenases have so far mainly been focussed on methanol dehydrogenase (MDH), the latter studies being stimulated by the availability of the 3-D structure of MDH. Various mechanisms have been put forward for MDH, which have relevance for all other PQQ-dependent dehydrogenases as well, as the reactions catalysed by these enzymes in all cases involve the dehydrogenation of a \( >\text{CHOH} \) group to a \( >\text{C}=\text{O} \) group.

The formation of a C5-propanal adduct upon suicide inhibition of MDH with cyclopropanol and the observation of a fluorescent intermediate in the catalytic cycle of MDH with deuterated methanol led to the proposal of a mechanism involving base-catalysed formation of a C5-hemiketal intermediate (106, 107) (see Fig. 7A). Such a mechanism, thus proceeding via the formation of a covalent PQQ-substrate complex, finds further support in the tendency of PQQ to form fluorescent C5-adducts with various nucleophiles, including alcohols (see § 2.2). According to this mechanism base-catalysed proton abstraction takes place concerted with attack of the oxyanion on C5 of PQQ. The calcium ion that is coordinated to PQQ in the active site may act as a Lewis acid by way of its coordination to the C5-carbonyl oxygen, thus rendering the C5-atom more susceptible to nucleophilic attack; it may also coordinate to the substrate oxygen, thus assisting in substrate binding and/or activation (108). An aspartate residue present in the active site of MDH, and conserved in the
Figure 7. Proposed reaction mechanisms of PQO-dependent dehydrogenases.
A) formation of a hemiketal intermediate followed by oxidative elimination.
B) possible involvement of the pyrrole nitrogen.
C) formation of a hemiketal intermediate followed by hydride transfer to the C4-oxygen.
D) hydride transfer to C5 followed by enolization.
E) direct hydride transfer to the C4-oxygen.
sequences of PQQ-dependent dehydrogenases that show homology to MDH, has been suggested to provide the catalytic base proposed above (108). Following adduct formation the reaction is envisaged to proceed via proton abstraction by the C4-oxygen concerted with oxidative elimination (see Fig. 7A). Model studies with PQQ-analogs in anhydrous organic media have recently shown that Ca\(^{2+}\) enhances the rate of C5-hemiketal formation with primary alcohols and that the addition of a strong base results in the formation of reduced PQQ-derivatives and aldehydes. The first result supports a catalytic role of the PQQ-coordinated Ca\(^{2+}\) whereas the second one suggests the possible involvement of a basic residue in the oxidative elimination process, either via direct proton abstraction from the adduct or by facilitating the intramolecular proton abstraction via ionization of C4-oxygen through deprotonation at N1 (109) (Fig. 7B). Occurrence of deprotonation of N1 appears however not essential for activity of quinohaemoprotein alcohol dehydrogenase type I, since this enzyme shows significant activity (> 5%, compared to the PQQ containing enzyme) when reconstituted with N1-methyl-PQQ, which cannot undergo N1-deprotonation (110).

Zheng and Bruice (111) have argued that oxidative elimination, as presented in Fig. 7A, is unlikely as this is a retro-ene reaction, which is normally extremely slow. On the basis of ab initio molecular orbital calculations they propose that the reaction proceeds via proton ionisation of the intermediate, followed by elimination of substrate in the oxide-form concerted with hydride transfer to the oxygen of C4 (Fig. 7C).

Alternatively, mechanisms have been proposed that do not involve the formation of a covalent adduct. Anthony et al. (108) put forward a mechanism that involves base catalysed hydride transfer from the substrate carbon atom to the electrophilic C5 followed by enolisation of C5-reduced-PQQ to the quinol form (Fig. 7D). In this mechanism the Ca\(^{2+}\)-ion functions in a similar way as suggested for the covalent adduct mechanism. Zheng and Bruice (111) present an alternative which involves a general base proton removal of a (PQQ)Ca\(^{2+}\)O(H)CH<-complex concerted with hydride transfer to the oxygen of C4 (Fig. 7E). In this alternative, the role of Ca\(^{2+}\) is threefold; it contributes to the formation of the ES-complex, provides a modest decrease in the pKa of the substrate, and polarizes the C5 oxygen. Calculations show that such a mechanism leads to much the same transition state as the mechanism involving adduct formation.

Transient kinetic studies of the enzyme-reducing (half-)reaction of sGDH with substrate are described in Chapter 5. Although the high catalytic activity of sGDH limits transient kinetic studies with normal aldoses, the occurrence of a substantial deuterium isotope effect, on the observed overall turnover rate with C1-deuterated glucose, enabled a more detailed observation of the transient kinetics of sGDH reduction with this substrate. The resulting data indicate that enzyme reduction proceeds via one or more step leading to the formation of a fluorescent intermediate, having an absorbance spectrum similar to that of PQQ-adducts, followed by an irreversible step, yielding the reduced enzyme form, that involves hydrogen transfer, as judged from the size of the deuterium isotope effect, and determines the overall rate of the complete reaction under non-cooperative conditions. This scheme appears compatible with the mechanisms involving the formation of a C5-hemiketal adduct (Fig. 7A-
C), but also with the direct hydride transfer mechanism presented in Fig. 7D, since, in view of its structural similarity, the C5-reduced PQQ intermediate of this mechanism, may be expected to exhibit similar spectral and fluorescent behaviour as PQQ-adducts (see Discussion in Chapter 5). The mechanism of Fig. 7E can be rejected as it is not compatible with the experimentally observed fluorescent intermediate.

Although the data presented in Chapter 5, do not yet allow a definitive choice between either a covalent intermediate or a direct hydride transfer type of mechanism, subsequent transient kinetic (A. Dewanti et al., in preparation) and structural (A. Oubrie et al., in preparation) studies have at present yielded evidence in favour of the hydride transfer mechanism as presented in Fig. 7D.

4. Application of sGDH in glucose determination

In view of the prominent role of glucose as carbon and energy source in metabolism (see § 1) it is evident that its concentration forms a key parameter in various biological processes. Specific interest in the determination of glucose exists in the fields of industrial fermentation control, food processing, and medicine, in the latter especially regarding the diagnosis and management of diabetes. As diabetes is characterized by improper regulation of blood glucose levels, its therapy consists largely of frequent determination of blood glucose levels by the patient self, allowing subsequent regulation towards desirable levels by self-administration of insulin or glucose-providing food.

The methods currently in use for the determination of glucose concentrations in samples such as body or culture fluids are all based on glucose converting enzyme systems that couple the catalytic conversion of glucose to the formation of a detectable signal. Assays, based on glucose oxidase/peroxidase or hexokinase/glucosephosphate dehydrogenase, providing a detectable absorbance change upon oxidative conversion of glucose are widely used in (clinical) laboratory analysis. Furthermore the use of teststrips (containing glucose oxidase) that display a concentration-dependent colour change, is well established especially in general medical practice and self-management of diabetes. Teststrip readout is performed either visually or by means of a portable reflectance photometer. Another, more advanced detection principle is applied in the so-called amperometric biosensors. In such a device electrons are transferred from the substrate via the enzyme to an electrode, which allows the measurement of a substrate concentration dependent current. In principle, amperometric biosensors are suitable for continuous in situ concentration monitoring, or high throughput screening of samples. However, in practice several technical problems, mainly concerning electron transfer and system stability, still have to be solved. Thus, the construction of amperometric biosensors is a topic of much research, with most attention focussed on sensors for glucose (112).

In comparison to other glucose oxidizing enzymes, sGDH displays various attractive features for application in glucose determination: a high catalytic activity, the ability to react
with many artificial electron acceptors, insensitivity towards oxygen, independence of external factors or coenzymes, tight cofactor binding and favourable handling properties. In fact, its application in a teststrip system (Glucotrend®) has recently been commercialized by Boehringer Mannheim GmbH (113). In this system an aromatic nitroso-compound (N,N-bis-(2-hydroxyethyl)-4-nitrosoaniline) acts as primary electron acceptor; its reduced form reduces the indicator 2,18-phosphomolybdic acid to heteropoly blue.

The above-mentioned properties also make sGDH an attractive enzyme for the construction of amperometric glucose sensors, which until now has mainly been carried out with glucose oxidase. The first reports on glucose sensors based on sGDH, dating from the mid-eighties (114, 115), involve the use of mediators such as dichlorophenol indophenol, phenazine methosulphate, or dimethylferrocene to achieve electron transfer from enzyme to electrode. Although the use of soluble mediators is not suitable for the construction of biosensors for continuous or in situ applications, this principle is applied in the construction of single use, disposable enzyme electrodes, to be used as an alternative to test strips in systems for self monitoring or point-of-care testing of blood glucose (116). The application of sGDH in this type of electrodes has also reached commercialization (117).

One possible approach to achieve electron transfer between enzyme and electrode without soluble mediators, involves the use of conducting polymers. Enzyme electrodes with a high activity (current density of 1.8 mA/cm²) have been constructed by incorporation of sGDH into an osmium-complex-containing redox-conducting epoxy network on glassy carbon (118). As a consequence of their high activity, it has been shown that this type of sGDH containing electrodes has a potential applicability for the bioelectrochemical production of aldobionic acids from the corresponding aldose sugars (119).

Another approach has focused on the possibility of achieving direct electron transfer from enzyme to electrode by reconstitution of apo-sGDH on an electrode surface modified with long spacered PQQ (120). Although reconstitution to active enzyme took place, unmediated electron transfer from enzyme to electrode was not achieved.

Alternatively, the ability of sGDH to react with quinonoid electron acceptors has been exploited in the construction of biosensors for the detection of redox active phenolic compounds such as p-aminophenol or epinephrine (121-124). In this set up glucose is present at a fixed concentration, and the analyte to be detected acts as the mediator for electron transfer between enzyme and electrode.

5. Scope and outline of this thesis

The work described in this thesis was initiated on the basis of previous research projects carried out in our laboratory (125) and in Leiden (126). These studies provided an initial characterization of sGDH and opened the way for a convenient method for preparing the apoenzyme via a recombinant E. coli strain. The results further indicated that sGDH would be a suitable model enzyme for studying the mechanism of PQQ-binding and the catalytic
mechanism of PQQ-dependent dehydrogenases, a topic strongly embedded within the research interest and know-how of our research group concerning the enzymology of quinoproteins.

As the abovementioned results also pointed at a potential suitability of sGDH for application in glucose determination, Boehringer Mannheim GmbH showed a strong interest in this enzyme, and was prepared to finance fundamental research aimed at its structural and mechanistic characterization.

The first objective of the project, then, was the preparation of pure apo-sGDH in good quantities by means of the recombinant *E. coli*. The purification and reconstitution of the apoenzyme yielding a high-quality holoenzyme preparation are described in Chapter 2.

Second the reconstitution process, and especially the involvement of Ca$^{2+}$ in this, was explored using chromatographic and spectroscopic methods, as described in Chapter 3.

Then, the catalytic properties of the enzyme were studied. Initial rate measurements were performed in an assay giving high turnover rates with sGDH, and yield an insight in the kinetic behaviour of sGDH as described in Chapter 4.

Finally, as described in Chapter 5, the changes in the absorbance spectrum of sGDH occurring upon its reduction by substrate were exploited in stopped-flow measurements of the enzyme reducing half-reaction aimed at the elucidation of the catalytic mechanism and substrate specificity of sGDH.
References


Chapter 2

Production, characterization, and reconstitution of recombinant quinoprotein glucose dehydrogenase (soluble type; EC 1.1.99.17) apoenzyme of *Acinetobacter calcoaceticus*

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Production, Characterization, and Reconstitution of Recombinant Quinoprotein Glucose Dehydrogenase (Soluble Type; EC 1.1.99.17) Apoenzyme of *Acinetobacter calcoaceticus*

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Soluble, periplasmic quinoprotein glucose dehydrogenase of *Acinetobacter calcoaceticus* (sGDH; EC 1.1.99.17) was produced in good yield in the apoenzyme form (without the cofactor pyrroloquinoline quinone, PQQ) by an *Escherichia coli* recombinant strain provided with a plasmid containing the gene under control of a lac promoter. Structural analysis of the purified apoenzyme revealed that the *E. coli* strain used produces the correct mature protein. Titration of the apoenzyme with PQQ in the presence of Ca²⁺ showed that a linear relation exists between the amount of added PQQ and activity observed, and that the subunit and PQQ associate in a molar ratio of 1:1. Based on spectral and enzymatic criteria, it is concluded that the present holoenzyme preparation has a better quality than the previously described preparations of authentic holoenzyme. As isolated here, the recombinant apoenzyme was in the dimeric form. Partial monomerization occurred upon gel filtration in a buffer with chelator and the process could be reversed with Ca²⁺. PQQ binds to the dimer in the presence of chelator, not to the monomer. However, the PQQ-containing dimer was not active and showed an unusual absorption spectrum which was slowly converted into a PQQR-like spectrum when glucose was added. Full restoration of activity was achieved upon addition of Ca²⁺ and the spectra were immediately converted into those of normal holoenzyme in the oxidized and reduced form, respectively. Addition of chelator to holoenzyme did not lead to inactivation or monomerization. It is concluded, therefore, that Ca²⁺ has a dual role in this enzyme, being required for dimerization of the subunits as well as for functionalization of the bound PQQ, and that it is more firmly attached to the holoenzyme than to the apoenzyme.

**Key Words**: Quinoprotein; pyrroloquinoline quinone; glucose dehydrogenase; reconstitution; *Acinetobacter calcoaceticus*.

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Direct oxidation of glucose to glucono-δ-lactone can be catalyzed with quite a few different enzymes, the most striking property to distinguish them being the variation in cofactor or coenzyme identity. Recent examples are the quinoprotein glucose dehydrogenases, enzymes which have pyrroloquinoline quinone (PQQ)² as cofactor. One of these, the membrane-bound glucose dehydrogenase, occurs in many gram-negative bacteria, either in the apo- or holo-form. The other one, the soluble glucose dehydrogenase (sGDH; EC 1.1.99.17), is structurally quite different and so far has only been found in the periplasm of the bacterium *Acinetobacter calcoaceticus* (the physiological significance, the structural properties, and the confusion about the identity of these enzymes in the past have been described elsewhere (1, 2)).

² Abbreviations used: PQQ, pyrroloquinoline quinone (2,7,9-tricarbonyl-1H-pyrrolo[2,3-f]quinoline-4,5-dione); PQQR, quinol form of PQQ; sGDH, soluble quinoprotein glucose dehydrogenase; DCPIP, 2,6-dichlorophenol-indophenol; PMS, phenazine methosulfate; CDTA, trans-1,2-diaminocyclohexane-N,N',N"-tetraacetic acid; SDS, sodium dodecyl sulfate; FAGE, polyacrylamide gel electrophoresis; Hol, the enzyme form containing PQQ and the full complement of Ca²⁺; Hol-Δ, the enzyme form in which the Ca²⁺ required to functionalize the bound PQQ is lacking.

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sGDH is a homodimeric enzyme (subunits of 50 kDa) containing PQQ and Ca^{2+} (3, 4). It catalyzes the oxidation of glucose and other aldoses to their corresponding aldonol-5-lactones with concomitant reduction of its co-factor, PQQ. Reoxidation occurs easily with several artificial dyes. In view of its simple behavior and composition compared to other PQQ-containing dehydrogenases (only one type of subunit and cofactor, no activator is required for activity), sGDH seems an ideal model enzyme for investigating the structure and mechanism of this type of quinoproteins (1, 2). Moreover, the enzyme has a high turnover number and electrochemical cofactor regeneration, not affected by the O_2 tension in the sample, has been achieved (5), making it an attractive candidate for application in amperometric biosensors and test strips. Unfortunately, the level of sGDH induction in A. calcoaceticus and the purification yield are rather low, most probably forming the reason why only a tentative characterization of the enzyme has been carried out so far. In principle these problems can be circumvented, however, since the structural gene for sGDH has been cloned and preliminary investigations (6, 7) have shown that the gene can be brought to expression in an Escherichia coli strain. The work presented here shows that good production and correct processing to apoenzyme was achieved with the recombinant strain used. In view of the high quality of the reconstituted enzyme, the characteristics determined here may serve as reference points for investigations on this enzyme in the future.

MATERIALS AND METHODS

Organism and culture conditions. The E. coli recombinant strain pPG492, harboring the gene for sGDH, was kindly provided by Dr. N. Goosen (6). The organism was grown on adapted Luria broth, as described previously (7), at 28°C in a 150-liter pilot fermenter, aerated at 30 liters/min and stirred at 260 rpm. Cells were harvested at the end of the exponential growth phase.

Enzyme purification. Cell paste (500 g) was suspended in 500 ml 20 mM Tris-HCl buffer, pH 7.5, containing 3 mM CaCl_2 (buffer A) and disrupted in a French pressure cell at 110 MPa. To the extract (930 ml), 10 mg DNase I and 40 ml 0.1 M MgCl_2 were added. After incubation overnight at 4°C, the suspension was centrifuged at 40,000 g for 20 min. To the supernatant (750 ml), 2 liters buffer A was added and 300 ml S-Sepharose Fast Flow (Pharmacia). After incubation overnight at 4°C, the S-Sepharose was collected by filtration, mixed with some buffer A, and poured into a column. The column was washed with 1 liter buffer A, and the enzyme was eluted with buffer A containing 1 M NaCl. Active fractions were collected (39 ml) and diluted to 500 ml with buffer A, and the solution was applied to a S-Sepharose Fast Flow column (4.5 x 18.5 cm). The enzyme was eluted using a linear gradient of 0 to 1 M NaCl in buffer A (500 ml). To the pooled active fractions (45 ml), solid (NH_4)_2SO_4 was added to a concentration of 1.5 M. The resulting solution was applied to a TSK Fractogel Butyl 650 column (1.6 x 15 cm, Merck) and eluted using a linear gradient of 1.6 to 0 M (NH_4)_2SO_4 in 20 mM Mes buffer, pH 6.5 (80 ml). The active fractions (20 ml) were dialyzed against 1 liter buffer A for 24 h at 4°C and stored at -40°C.

Enzyme reconstitution. Halo-sGDH was obtained by addition of PQQ to apoenzyme in buffer A, followed by an incubation for 15 min at room temperature. If required, excess PQQ was removed from the holoenzyme by gel filtration on a PD10 column (Pharmacia) using 50 mM potassium phosphate buffer, pH 7.0, for equilibration and elution. PQQ was prepared by chemical synthesis and a molar absorption coefficient at 249 nm of 25,400 M^-1 cm^-1 (at pH 4) was used to determine PQQ concentrations (7).

Assay procedure. Enzyme activity was determined spectrophotometrically at 25°C by following the reduction of 2,6-dichlorophenol-indophenol (DCPIP) at 600 nm, using phenazine methosulphate (PMS) as a primary electron acceptor. The standard assay was prepared by mixing: 840 μl 50 mM potassium phosphate buffer, pH 7.0, 50 μl 1 M glucose; 50 μl 1 mM DCPIP (620 x 21.9 M^-1 cm^-1); 50 μl 20 mM PMS; 10 μl of enzyme sample, reconstituted as indicated above and, if necessary, diluted with buffer A containing 0.1% Triton X-100.

Gel filtration chromatography. Gel filtration chromatography was carried out on a Superdex 200 column (Pharmacia) in 20 mM Mops buffer, pH 7.0, containing 150 mM NaCl, at a flow rate of 0.5 ml/min. Monitoring occurred with a Hewlett-Packard 1040A diode array detector. Specific absorption coefficients at 280 nm for the purified apo and holoenzyme were calculated from absorbance measurements of peaks eluting from the gel filtration column at 265 and 280 nm, in 20 mM potassium phosphate buffer, pH 7.0, as described by Van Iersel et al. (8).

Polyacrylamide gel electrophoresis. Gel electrophoresis was performed on Phast System gels and equipment (Pharmacia). Gels were stained for protein with Coomassie brilliant blue R250. Homogeneity and subunit molecular mass of the purified sGDH apoenzyme were checked under denaturing conditions (9) with SDS–PAGE on a 10–15% gradient gel using a low-molecular-mass calibration kit for molecular mass estimations. Native PAGE was used to determine the quaternary structure of various apo- and halo-sGDH samples. Since sGDH has an isoelectric point of 3.5 (3), native PAGE was performed under reversed polarity conditions, using buffer strips containing 2% agarose, 0.50 M β-alanine, and 0.70 M acetic acid, giving a pH of 4.2 behind the buffer front (10). Quinohemoprotein alcohol dehydrogenase from Comamonas testosteroni, having an M_ of 74 kDa and an isoelectric point of 9.0 (11) was used as a marker protein.

N-terminal amino acid sequence analysis. The N-terminal amino acid sequence of sGDH apoenzyme was determined by automatic Edman degradation in a Model 477A pulsed liquid protein sequenator coupled to an ABI HPLC system (Applied Biosystems).

RESULTS AND DISCUSSION

Production and characteristics of the recombinant apo-sGDH. From a 100-liter batch fermentation, 2.0 kg of wet cell paste was obtained. Cultivation was routinely carried out in the absence of inducer of the lac operon (isopropyl-β-d-thiogalactoside) since no higher enzyme yields were obtained in its presence. Results of a typical purification are presented in Table I. The first step, overnight incubation of the extract at room temperature in the presence of DNase and MgCl_2, proved to be necessary to increase the yield of enzyme able to bind to the cation exchanger. Moreover, without applying this step, the major part of enzyme activity, about 80%, was found in the pellet fraction after centrifugation, whereas this was only 15% by including the first step (Table I). The total activity of the cell-
TABLE I
Purification* of Recombinant αGDH Apoenzyme from E. coli pQP492

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Total protein (mg)</th>
<th>Total activity (kU)</th>
<th>Specific activity† (U/mg)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Extract</td>
<td>n.d.</td>
<td>1950</td>
<td>n.d.</td>
<td>100</td>
</tr>
<tr>
<td>Extract after incubation</td>
<td>n.d.</td>
<td>1670</td>
<td>n.d.</td>
<td>84</td>
</tr>
<tr>
<td>and centrifugation</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S-Sepharose (1 M NaCl)</td>
<td>924</td>
<td>1750</td>
<td>1900</td>
<td>87</td>
</tr>
<tr>
<td>S-Sepharose (gradient)</td>
<td>415</td>
<td>1200</td>
<td>2900</td>
<td>61</td>
</tr>
<tr>
<td>Butyl Pratogel and dialysis</td>
<td>108</td>
<td>800</td>
<td>7400</td>
<td>41</td>
</tr>
</tbody>
</table>

*The typical purification indicated here was carried out with 500 g wet cell paste. Protein determinations were based on the specific absorption coefficient of apo-αGDH, assuming that contaminating proteins in the last stages of the purification have a similar value. For that reason, no determinations (n.d.) were carried out for the first steps.
†1 U = 1 μmol glucose oxidized/min.

free extract remained unaltered by the overnight incubation. This suggests that the incubation is not required for processing of a proenzyme form but for detachment of the apoenzyme from cellular debris.

The purified apo-αGDH gave one single band of about 50 kDa on SDS–PAGE, in accordance with the calculated value from the amino acid sequence (50,231 Da (6)). Since the inserted piece of DNA in the recombinant strain did contain not only the structural gene for αGDH but also the leader sequence for this periplasmic protein, whether correct processing occurred was checked. Sequencing of the N-terminal amino acids of the purified apo-αGDH for a stretch of 10 amino acids revealed that the protein was homogeneous and that it had a sequence identical to that deduced from the structural gene and that determined for authentic αGDH (6).

The absorption spectrum of the purified protein (Fig. 1A), having a maximum at 278 nm, exhibits the characteristics expected for a cofactorless enzyme. The absence of absorbance above 300 nm is in agreement with the absence of activity, observed in the assay when the reconstitution step with PQQ was omitted. Apparently, this E. coli strain does not produce PQQ or any other chromophore having affinity for the protein, in agreement with earlier findings on the absence of PQQ in other E. coli strains (12). The specific absorption coefficient of the apoenzyme at 280 nm appeared to be 1.28 liters g⁻¹ cm⁻¹.

Reconstitution to activity. Apo-αGDH in buffer A reconstituted rapidly upon addition of PQQ. Since the holoenzyme has a high turnover number, dilution to a concentration in the order of 1 μg protein/ml was required for a reliable estimate of the specific activity with the assasy. However, on using buffer A for this, a more than proportional decrease in activity was observed, probably due to instability or adsorption effects becoming a disturbing factor at low enzyme concentrations. This phenomenon was not observed when 0.1% Triton X-100 was added to the dilution buffer. It was found that the presence of Ca²⁺ in the reconstitution buffer was essential for adequate reconstitution.

The purified apoenzyme, reconstituted with a slight excess of PQQ, and diluted as described above, has a specific activity of 7400 U/mg in the assay described under Materials and Methods. In the assay described by Hauge (13), which is based on DCPIP as sole artificial electron acceptor at pH 6.0, the specific activity of the recombinant enzyme was 810 U/mg. From the published data by Hauge (14) and Dokter et al. (3) it was calculated that their preparations of authentic enzyme had a much lower specific activity, estimated to be 574 and 490 U/mg protein, respectively, under this condition.

Incubation of diluted holoenzyme samples at room temperature for more than 1 day resulted in a partial loss of activity, which could be completely restored upon addition of extra PQQ. This suggests that PQQ
became partly inactivated, perhaps by reacting with Tris base present in the buffer used (which is in fact a primary amine, in principle able to react with PQQ (15)). In agreement with this, such phenomena were not observed with holoenzyme stored in phosphate buffers.

From the plot of activity versus amount of PQQ added to apoenzyme (Fig. 2), it is calculated that full activity is attained at a ratio of 0.96 mol of PQQ per mole of subunit. Thus, within experimental error, all subunit molecules appear to be able to bind one PQQ. This stoichiometry and the linearity observed for the curve at subsaturating amounts of PQQ, indicate that all molecules are active. The linearity also indicates that the affinity of the apoenzyme for the cofactor is very high. Consequently, fitting of these data provides only a rough estimate of the dissociation constant of PQQ, being in the order of 10 pm. From time-dependent measurements it was found that under the conditions used here reconstitution was completed within 15 min.

**Spectral properties of holoenzyme.** On reconstitution with PQQ, holoenzyme is obtained in the oxidized form. Its absorption spectrum (Fig. 1B) shows a peak with a maximum at 278 nm, a broad band around 352 nm and a low broad band from 420 to 600 nm, centered around 520 nm. Reduction of the holoenzyme by addition of glucose results in the occurrence of a sharp peak with a maximum at 338 nm while the peak in the UV region shifts from 278 to 280 nm with a concomitant decrease in absorbance (Fig. 1B). The reduced holoenzyme has a specific absorption coefficient at 280 nm of 1.48 liters g⁻¹ cm⁻¹.

It is proposed to take the A₃₈₅/₈₂₅₀ absorbance ratio of reduced holo-sGDH as a convenient indicator for purity, cofactor content, and catalytic competence of a preparation. Our samples showed a constant ratio of 0.47, whereas from the published spectra of authentic reduced holo-sGDH, values of about 0.30 (14) and 0.40 (3, 4) were derived. This indicates that in the latter cases the preparations were not homogeneous, subsaturating amounts of cofactor were present, or the preparations partly consisted of catalytically inactive enzyme. This view is supported by the lower specific activities of these preparations, as indicated above.

**Quaternary structure of apo- and holo-sGDH.** Holo-sGDH, as isolated from *A. calcoaceticus*, is a dimeric enzyme (3). To assess the quaternary structure of the recombinant apo- and holo-sGDH, prepared as indicated above, gel filtration chromatography was performed (Fig. 3). Under the conditions applied, both apo- and holoenzyme eluted as one peak at a position corresponding with a molecular mass of 74 kDa (since the molecular mass calculated from the amino acid sequence is 100.5 kDa (6), the lower value must be due to anomalous behavior). On supplementing the elution buffer with 1 mM CDTA and incubating the apoenzyme with 5 mM CDTA before applying it to the column, two partly overlapping peaks were obtained corresponding with molecular masses of 74 and 51 kDa. These condi-
tions did not affect the elution behavior of holo-sGDH. Since these observations could be ascribed either to dissociation of dimeric apoenzyme or to an artifact of the chromatography system, similar experiments with a different technique seemed necessary to discriminate between the two possibilities.

PAGE of native holo-sGDH (Fig. 4) showed one band, even when incubation of the sample occurred with 10 mM CDTA, and its position on the gel in comparison with that of the marker protein suggested that it was in the dimeric form. On pretreatment of apoenzyme with 10 mM CDTA and removal of the chelator by gel filtration, the electrophoretogram showed a faint band at the position of the dimer and a strong band at a position expected for the monomer (Fig. 4). Addition of Ca\textsuperscript{2+} reversed the situation nearly completely. All these observations indicate that apo-sGDH as isolated occurs in the dimeric form (Apo-dimer, see Scheme 1) but that removal of the bound Ca\textsuperscript{2+} with chelator induces monomerization (Apo-monomer), a process which can be reversed with Ca\textsuperscript{2+}. Apparently, the Ca\textsuperscript{2+} ions in holo-sGDH (those already present and those extra required for PQQ functionalization, see below) are more strongly bound since (a short) treatment with the chelator did not lead to inactivation or monomerization. Although the presence of PQQ does not affect the monomerization or dimerization of apoenzyme (see below), as present in holoenzyme containing the full complement of Ca\textsuperscript{2+} (Holo, see Scheme 1), it prevents monomerization by chelator.

**Binding of PQQ to apoenzyme.** When samples of Apo-monomer and Apo-dimer, prepared by gel filtration using CDTA-containing buffer (Fig. 3), were incubated with PQQ, no activity was observed in the assay, indicating that reconstitution to active holoenzyme did not take place. Upon removal of excess PQQ by gel filtration (Fig. 5), the chromatograms taken at 280 nm were similar to those of fractions not incubated with PQQ (not shown), indicating that the presence of PQQ did not lead to dimerization or monomerization. However, the chromatograms taken at 338 nm indicated that binding of PQQ occurred to Apo-dimer (Fig. 5B) but not to Apo-monomer (Fig. 5A). The absorption spectrum (Fig. 6) of the resulting dimeric PQQ-containing but inactive enzyme form (Holo-X, see Scheme 1) shows a maximum at 340 nm, which is 12 nm lower than that of Holo (Fig. 1B) but 12 nm higher than that of free PQQ (16). Since the spectrum of triesterified PQQ in an organic solvent also shows a substantial red shift and bivalent metal ions also induce such a shift when added to PQQ in water (4, 17), this suggests that the active site of sGDH is hydrophobic and that the Ca\textsuperscript{2+} ions are in the vicinity of PQQ so that they can compensate the negative charges of the carboxylate groups. The former suggestion is supported by the fact that PQQ in the holoenzyme is nonfluorescing, indicating

**FIG. 4.** Gel electrophoresis of holo- and apo-sGDH. Native PAGE was performed under reversed polarity conditions as described under Materials and Methods. Lanes: (1) quinohemoprotein alcohol dehydrogenase; (2) holo-sGDH incubated with 10 mM CDTA; (3) holo-sGDH; (4) apo-sGDH, pretreated with CDTA; (5) as in lane 4, after subsequent addition of 1 mM CaCl\textsubscript{2}. The arrow indicates the direction of electrophoresis.

**FIG. 5.** Binding of PQQ to apo-sGDH in the presence of chelator. Samples of Apo-monomer and Apo-dimer, obtained via gel filtration chromatography on Superdex 200 using 20 mM Mops buffer, pH 7, containing 0.15 M NaCl and 1 mM CDTA, were incubated with excess PQQ for 30 min and resubjected to gel filtration chromatography under the same conditions. A. Apo-monomer; B. Apo-dimer. Absorbance was monitored at 280 nm (---) and 338 nm (-----).

**SCHEME 1.** The various enzyme forms of sGDH and their interconversion. Holo-X is the inactive enzyme form in which the Ca\textsuperscript{2+} required to functionalize PQQ is lacking.
that its o-quinone moiety is intact (reaction of PQQ at its C5 position with water or other nucleophiles gives a red shift in its absorption maximum due to the formation of fluorescent adducts (16)). The latter suggestion is supported by the position of Ca2+ and PQQ as found in the three-dimensional structure of methanol dehydrogenase (18), although it should be noted that the amino acid sequences of this enzyme and sGDH in the stretches concerned are completely different (19).

When glucose was added to Holo-X, slow reduction of the cofactor took place, as suggested by the PQQH2-like spectrum eventually obtained (Fig. 6, PQQH2 has a maximum at 302 nm (20)). Since reduced Holo (Holo-red) has a maximum at 338 nm (Fig. 1B), the suggestions made above with respect to the environment of PQQ in oxidized holoenzyme may also apply to the reduced form. Addition of Ca2+ ions to Holo-X and Holo-red rapidly converted both forms into fully active enzyme with spectra belonging to Holo and Holo-red, respectively (Fig. 6). Apparently binding of Ca2+ converts PQQ into an orientation which affects its absorption spectrum and gives it catalytic competence. Similar phenomena have been observed for methanol dehydrogenase (20) and quinohaemoprotein ethanol dehydrogenase (17). However, differences exist among these enzymes with respect to binding of PQQ to apoenzyme in the absence of Ca2+, release of PQQ from holoenzyme by addition of chelators, and the possibility of replacing Ca2+ by other bivalent metal ions in reconstitution (22).

**CONCLUSIONS**

1. Processing of the gene for sGDH from A. calcoaceticus in the E. coli strain used here led to adequate production (a yield of 10 mg pure protein per liter of cultivation fluid was achieved) and correct processing to mature apoenzyme.

2. Upon titration with PQQ in the presence of Ca2+, complete saturation of the subunits was achieved at a molar ratio of 1. The fully reconstituted enzyme had a specific activity which was substantially higher than that of all authentic enzyme preparations described so far.

3. In view of the high quality of the preparation, the characteristics determined here (specific activity, specific absorption coefficients, absorbance ratios) may function as reference data for future research on this enzyme.

4. Binding of PQQ occurs to dimeric enzyme, not to enzyme in its monomeric form.

5. Ca2+ seems to play a dual role in this enzyme as it is required for dimerization as well as for PQQ functionalization. Binding of Ca2+ is much stronger in the holoenzyme than in the apoenzyme.

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Chapter 3

Ca$^{2+}$ and its substitutes have two different binding sites and roles in soluble, quinoprotein (pyrroloquinoline-quinone-containing) glucose dehydrogenase

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Ca\(^{2+}\) and its substitutes have two different binding sites and roles in soluble, quinoprotein (pyrroloquinoline-quinone-containing) glucose dehydrogenase

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To investigate the mode of binding and the role of Ca\(^{2+}\) in soluble, pyrroloquinoline-quinone (PQQ)-containing glucose dehydrogenase of the bacterium *Acinetobacter calcoaceticus* (sGDH), the following enzyme species were prepared and their interconversions studied: monomeric apoenzyme (M); monomer with one firmly bound Ca\(^{2+}\) ion (M*); dimer consisting of 2 M* (D); dimer consisting of 2 M and 2 PQQ (Holo-Y); dimer consisting of D with 2 PQQ (Holo-X); fully reconstituted enzyme consisting of Holo-X with two extra Ca\(^{2+}\) ions (Holo) or substitutes for Ca\(^{2+}\) (hybrid Holo-enzymes). D and Holo are very stable enzyme species regarding monomerization and inactivation by chelator, respectively, the bound Ca\(^{2+}\) being locked up in such a way that it is not accessible to chelator. D can be converted into M* by heat treatment and the tightly bound Ca\(^{2+}\) can be removed from M* with chelator, transforming it into M. Reassociation of M* to D occurs spontaneously at 20\(^\circ\)C; reassociation of M to D occurs by adding a stoichiometric amount of Ca\(^{2+}\). Synergistic effects were exerted by bound Ca\(^{2+}\) and PQQ, each increasing the affinity of the protein for the other component. Dimerization of M to D occurred with Ca\(^{2+}\), Cd\(^{2+}\), Mn\(^{2+}\), and Sr\(^{2+}\) (in decreasing order of effectiveness), but not with Mg\(^{2+}\), Ba\(^{2+}\), Co\(^{2+}\), Ni\(^{2+}\), Zn\(^{2+}\), or monovalent cations. Conversion of inactive Holo-X into active Holo, was achieved with Ca\(^{2+}\) or metal ions effective in dimerization. Although it is likely that activation of Holo-X involves binding of metal ion to PQQ, the spectral and enzymatic activity differences between normal Holo- and hybrid Holo-enzymes are relatively small. Titrations experiments revealed that the two Ca\(^{2+}\) ions required for activation of Holo-X are even more firmly bound than the two required for dimerization of M and anchoring of PQQ. Although the two binding sites related with the dual function of Ca\(^{2+}\) show similar metal ion specificity, they are not identical. The presence of two different sites in sGDH appears to be unique because in other PQQ-containing dehydrogenases, the PQQ-containing subunit has only one site. Given the broad spectrum of bivalent metal ions effective in reconstituting quinoprotein dehydrogenase apoenzymes to active holoenzymes, but the limited spectrum for an individual enzyme, the specificity is not so much determined by PQQ but by the variable metal-ion-binding sites.

**Keywords:** quinoprotein; pyrroloquinoline quinone; glucose dehydrogenase; reconstitution; calcium.

The bacterium *Acinetobacter calcoaceticus* produces two quite different PQQ-containing (quinoprotein) glucose dehydrogenases, the membrane-bound enzyme (mGDH) and the soluble enzyme (sGDH). mGDH occurs in many gram-negative bacteria [1] and glucose oxidation via this enzyme provides the organism with useful energy [2]. The deduced amino acid sequence of this

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**Abbreviations.** PQQ, pyrroloquinoline quinone (2,7,9-tricarboxy-1H-pyrrolo[2,3-f]quinoline-4,5-dione); PQQH\(_2\), semiquinone form of PQQ; PQQH\(_{\text{red}}\), quinol form of PQQ; sGDH, soluble quinoprotein glucose dehydrogenase; mGDH, membrane-bound glucose dehydrogenase; CD1A, trans-1,2-diminocholeoxazine-4,11'-N,N'-tetraacetic acid; M, monomeric apo-GDH; M*, M with one firmly bound Ca\(^{2+}\) ion; D, dimeric apo-sGDH consisting of 2 M*; Holo-Y, dimer consisting of 2 M and 2 PQQ; Holo-Y\(_{\text{red}}\), reduced Holo-Y; Holo-X, dimer consisting of D with 2 PQQ; Holo-X\(_{\text{red}}\), reduced Holo-X; fully reconstituted sGDH; Holo\(_{\text{red}}\), reduced holoenzyme.

**Enzymes.** Glucose dehydrogenase (pyrroloquinoline-quinone) (EC 1.1.99.17); alcohol dehydrogenase (acceptor) (EC 1.1.99.8).

enzyme [3] has similarity with those of quinoprotein alcohol dehydrogenases. However, in contrast with the latter enzymes, mGDH prefers Mg\(^{2+}\) instead of Ca\(^{2+}\) in the reconstitution of apoenzyme with PQQ to active holoenzyme [4]. sGDH has so far been found only in *A. calcoaceticus* strains [5]. Although the enzyme is an extremely efficient catalyst for *in vitro* glucose oxidation, making it an attractive candidate for analytical applications [6], such a role is absent in whole cells, implying that the physiological function of sGDH is unknown [7]. Except for a small region, the amino acid sequence of sGDH [8] is completely different from mGDH and from those of the quinoprotein alcohol dehydrogenases [9]. However, like alcohol dehydrogenases, sGDH requires Ca\(^{2+}\) in the reconstitution of apoenzyme with PQQ [10]. sGDH holoenzyme (Holo; see Scheme 1 for definitions of the various enzyme forms) has been purified from *A. calcoaceticus* strain LMD 79.41 [11, 12]. The aforpom of this enzyme (D), i.e., the homodimer containing Ca\(^{2+}\) but not PQQ, has been isolated from an *Escherichia coli* recombinant strain [13] (a non-PQQ-producing bacterium). Previous studies [13] on reconstitution suggested that Ca\(^{2+}\) has a dual role in this process and that these can be separately studied. To provide evidence for this, efforts were made to prepare a good-quality monomer (M) preparation with respect to reconstitution ability and ab-
sence of chelator), to detect species occurring in the pathway of reconstitution, and to study their interconversion.

It is already known that various bivalent metal ions can replace the natural one (Ca$^{2+}$ or Mg$^{2+}$ in the case of mGDH) in the reconstitution of apoenzymes with PQQ to holoenzymes [5, 10]. However, except for methanol dehydrogenase, the results of these studies only revealed the ability of the metal ions to replace the natural ion in the activity assays. Replacement of Ca$^{2+}$ by Sr$^{2+}$ (by growing the methylobacterium in the presence of Sr$^{2+}$ [14]) gave a methanol dehydrogenase form with a spectrum of higher absorbance as well as an enzyme with higher specific activity [15]. This could be explained by assuming that the metal ion is in close interaction with the cofactor (not PQQ but either PQQH$_2$ or PQQH$_3$), depending on the enzyme isolation procedure used [16], the differences (as compared to the natural enzyme) reflecting the specific properties of the metal ion involved in the interaction. The three-dimensional structure of methanol dehydrogenase shows that Ca$^{2+}$ is close to the 7-carboxyl group, and the N6 and the C5 carbonyl group in the quinoline ring of PQQH$_2$ [17]. Since the Ca$^{2+}$ in sGDH can be replaced by several other bivalent cations [5], the putative dual role of Ca$^{2+}$ and the nature of the binding sites connected with these functions were investigated by probing several metal ions for their capability to replace Ca$^{2+}$. The effects exerted by these ions were studied in a qualitative way.

**EXPERIMENTAL PROCEDURES**

**Dissociation of D.** D was prepared as described [13] in 20 mM Mops/NaOH, pH 7.0. To study the effect of heat on dissociation, samples of D (20–30 μM subunit) were heated in a water bath at a certain temperature for 5 min and immediately analysed by HPLC gel filtration for their dimer/monomer content (see below). The reversibility of this process was determined by keeping the heated samples for 1 h at 20°C before applying HPLC. The ability of trans-1,2-diaminocyclohexane-N,N,N',N'-tetraacetic acid (CDTA) to block the reassociation was tested in the same way and CDTA was added either before heating or just after cooling down the heated sample to 20°C.

To determine the effect of pH on dissociation, concentrated D (250 μM subunit) was diluted ten times with 50 mM potassium phosphate of the appropriate pH. The samples were incubated at 35°C for 5 min and immediately analysed by HPLC.

**Preparation of M from D.** D was transferred to 20 mM Caps/NaOH, pH 10.0, via a PD 10 gel-filtration column (Pharmacia), equilibrated with the same buffer. Enzyme containing eluate (1 ml, 7.1 mg protein) was applied to a centrifuge column containing 3 ml Chelex 100 chelating resin in the Na$^+$ form (Bio-Rad), equilibrated with the same buffer, and for which the interstitial liquid between the particles was removed by centrifugation. After incubating for 5 min at room temperature, the enzyme solution was removed by centrifugation. Subsequently, the enzyme was transferred to bivalent-metal-ion-depleted 20 mM Mops/NaOH, pH 7.0 (buffer A), via a PD 10 gel-filtration column equilibrated with this buffer. The preparation was stored at 4°C. Buffer A was prepared by incubating 20 mM Mops for one week with the chelating resin Serdolit CHE (Serva) in the Na$^+$ form (this automatically brought the pH to 7.0).

**Dimerization of M with various metal ions or PQQ.** M (7.9 μM subunit) in buffer A was incubated with 1 mM metal salt (mostly chloride salts, the anion moiety being irrelevant for the effect) or 32 μM PQQ for 1 h. Subsequently, the extent of dimerization was determined with HPLC gel filtration.

**Preparation of an inactive, PQQ-containing dimer (Holo-X) from D.** D was transferred to buffer A via a PD 10 gel-filtration column. To remove adventitious Ca$^{2+}$ as much as possible, the procedure was repeated once. The eluted enzyme (1.8 ml, 7.2 mg protein) was incubated with an approximately threefold excess of PQQ (with respect to subunit concentration) for 30 min, after which excess PQQ was removed via a PD 10 column equilibrated with buffer A. The preparation was stored at 4°C.

**Reconstitution of M and Holo-X with various metal ions to (hybrid) Holo-enzyme.** Apo-enzyme (40 μM subunit) in buffer A was incubated at room temperature for 1 h with PQQ (80 μM) and various metal salts (1 mM). Holo-X (16 μM subunit concentration) in buffer A was incubated at room temperature for 1 h with various metal salts (1 mM).

**HPLC gel filtration.** HPLC gel-filtration chromatography was carried out with a Waters HPLC system equipped with a Superdex 200 gel-filtration column (Pharmacia) in 20 mM Mops/NaOH, pH 7.0, containing 150 mM NaCl, at a flow rate of 0.5 ml/min. The eluate was monitored with a Hewlett-Packard 1040A diode array detector operating under HP ChemStation software.

**Spectrophotometry.** Ultraviolet/visible absorption spectra were measured in buffer A with a Hewlett-Packard 8524A photodiode array spectrophotometer at 20°C. To remove any adhering Ca$^{2+}$, the cuvettes were extensively rinsed with 10 mM CDTA and subsequently with buffer A.

**Determination of protein and PQQ concentrations.** Enzyme forms and PQQ concentrations were determined spectrophotometrically. For M and D, a specific absorption coefficient at 280 nm of 1.281 g$^{-1}$ cm$^{-1}$ was used. Specific absorption coefficients at 280 nm of 1.671 g$^{-1}$ cm$^{-1}$ for Holo-X and 1.551 g$^{-1}$ cm$^{-1}$ for Holo-X$_{apo}$ were calculated from the value for Holo$_{apo}$ of 1.481 g$^{-1}$ cm$^{-1}$ [13] by converting Holo-X into Holo$_{apo}$ with glucose and Ca$^{2+}$. A molar absorption coefficient at 249 nm of 25400 M$^{-1}$ cm$^{-1}$ (at pH 4.0) was used for PQQ [18].

**Enzyme assay.** Assays for glucose dehydrogenase activity were carried out as described [13]. Dilution of the samples to 10–40 mM subunit was performed with buffer A containing 0.05% Triton X-100 (depleted of bivalent metal ions with chelating resin). As indicated, in certain experiments the metal ion used for reconstitution was also included in the dilution buffer (at a concentration of 1 mM). Enzyme activity is given as nmol dichloroindophenol reduced min$^{-1}$ mg enzyme$^{-1}$ under the specified assay conditions.

**Determination of Ca$^{2+}$ in Holo.** Holo-enzyme, reconstituted from D as described [13], was transferred to buffer A via a PD 10
RESULTS

Dissociation of D and preparation of M. As reported previously [13], the isolated apo-sGDH from the E. coli recombinant strain is in the dimeric form, contains Ca\(^{2+}\), but not PQ (indicated in the present nomenclature with D, see Scheme 1). The preparations remained completely dimeric upon storage in the bivalent-metal-ion-free buffer A (4 weeks at 4°C), and even in the presence of a large excess of CDTA. However, heating a solution of D at certain temperatures for a constant time, followed by immediate HPLC gel filtration, showed that monomerization occurred in a narrow temperature range, as indicated in Fig. 1 since the separation of dimer and monomer is not perfect on the HPLC gel-filtration column (Fig. 3A), the curves in Figs 1 and 2 represent the dissociation process in a qualitative way]. The dissociation to monomer appeared to be reversible since the heat-dissociated sample completely reassociated to dimer after standing at 20°C for 1 h. However, the reassociation was blocked under the indicated conditions by adding CDTA, with a stoichiometric amount when added before the heating step, with a tenfold excess when added just after cooling to 20°C. These phenomena are explained as follows: dissociation of D by heat produces a monomer species (M*) containing firmly bound Ca\(^{2+}\), which is removable at elevated temperature, but which is much less easily removed at 20°C (the accessibility of Ca\(^{2+}\) for CDTA would be higher if some melting of the structure of M* occurred at elevated temperature); removal of Ca\(^{2+}\) from M* gives a monomer species (M) that is unable to dimerize. Although in principle heating D in the presence of CDTA could provide M preparations for further studies, such a procedure was considered unsuitable for two reasons: whether CDTA removes Ca\(^{2+}\) or just binds to Ca\(^{2+}\) still liganded to the protein is uncertain (the latter possibility would lead to unreliable results with respect to experiments in which Ca\(^{2+}\) is replaced by other metal ions); steps applied to remove excess CDTA from the M preparations could introduce contaminating Ca\(^{2+}\), provoking (partial) dimerization.

Since addition of 0.15 M NaCl to the buffer led to a higher melting temperature for dissociation of D (data not shown), charged amino acid residues could keep the subunits together. Fig. 2 shows that the phenomenon is pH dependent, the dissociation occurring more easily at elevated pH. Thus, the preparation of M was attempted by treatment of D with chelator resin at low ionic strength and high pH (since sGDH has a high isoelectric point (9.5 [11]), the high pH will also avoid adsorption of the enzyme to the cation-binding chelator resin). On applying the indicated procedure for fresh preparations, M was obtained in high yield without contamination with D, and was fully convertible into D with stoichiometric amounts of Ca\(^{2+}\) (old preparations required an excess of Ca\(^{2+}\) for dimerization to take place, probably because conversion of M to a less ordered structure occurs upon storage).

Dimerization of M. Upon incubating M in buffer A supplemented with 1 mM metal ion salts, subsequent HPLC gel filtration and measurement of the peak areas in the chromatogram (Fig. 3A) enabled us to estimate the extent of dimerization (Table 1). This showed that addition of Cr\(^{3+}\), Cd\(^{2+}\), Mn\(^{2+}\), and Sr\(^{2+}\) induced dimerization (in decreasing order of effectiveness,
Table 1. Enzyme species obtained after incubating M with various metal ions, with or without PQQ. M (7.9 μM) in buffer A was incubated for 1 h with various metal ions (1 mM) in the absence or presence of PQQ (32 μM). Subsequently, the monomer/dimer content was determined by HPLC gel filtration as described in the Experimental Procedures section. In the cases where both a monomer and a dimer peak were observed, monomer/dimer ratios (%) are indicated (calculated from the peak areas). n.d., not determined.

<table>
<thead>
<tr>
<th>Metal ion</th>
<th>Enzyme species without PQQ</th>
<th>with PQQ</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>monomer</td>
<td>50/50</td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>dimer</td>
<td>dimer</td>
</tr>
<tr>
<td>Cd²⁺</td>
<td>dimer</td>
<td>dimer</td>
</tr>
<tr>
<td>Mn²⁺</td>
<td>60/40</td>
<td>dimer</td>
</tr>
<tr>
<td>Sr²⁺</td>
<td>dimer</td>
<td></td>
</tr>
<tr>
<td>Mg²⁺</td>
<td>monomer</td>
<td>n.d.</td>
</tr>
<tr>
<td>Ba²⁺</td>
<td>monomer</td>
<td>30/70</td>
</tr>
<tr>
<td>Co²⁺</td>
<td>monomer</td>
<td>monomer</td>
</tr>
<tr>
<td>Ni²⁺</td>
<td>monomer</td>
<td></td>
</tr>
<tr>
<td>Zn²⁺</td>
<td>monomer</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

the dimerization by Sr²⁺ being scarcely visible in the chromato-
gram), but that Mg²⁺, Ba²⁺, Co²⁺, Ni²⁺, or Zn²⁺ or monovalent
cations did not. In interpreting this result, it should be realized that
incubation occurred for 1 h and gel filtration was carried out
in buffer A so that negative results could be due to a low
rate of association or a high rate of dissociation for the metal
ion concerned. The last possibility seems most likely since
incubation overnight did not increase the extent of dimerization
(except for a slight increase with Mn²⁺).

Upon incubating M in buffer A with PQQ (32 μM, fourfold excess
over the subunit concentration), partial dimerization (50%) was
observed and PQQ was bound only to the dimeric form and not to the
monomeric form (Fig. 3 B). After an incubation period of 16 h, the
same ratio was observed, indicating that incomplete dimerization
was not due to a low rate of association but to a relatively high rate
of dissociation. This was confirmed with experiments in which higher
concentrations of PQQ were applied, the chromatogram showing an
increase in the ratio of dimer to monomer. Subsequent incubation with
1 mM EDTA for 1 h resulted in an extent of dimerization of only 10% of
that observed in the absence of EDTA. Since HolO or the inactive,
PQQ-containing dimeric species obtained upon incubation of D
with PQQ in the absence of Ca²⁺ (HolO-X, [13] and below) is
not dissociated by EDTA, it is concluded that the dimerization
is not caused by the introduction of traces of bivalent metal ions,
but is really due to PQQ. A mixture of M and PQQ prepared as
described above, which contains substantial amounts of dimer
with bound PQQ, was not active in the enzyme assay. Neverthe-
less, since reduction took place when glucose was added to this
mixture (see below), the dimer can be regarded as an enzyme
form. In view of the difference with HolO-X (containing Ca²⁺),
it is proposed that the new enzyme form is indicated as HolO-Y
(Scheme 1).

Incubation of M with both PQQ (equimolar concentration)
and a dimerization-effective metal ion (1 mM) under the same
conditions as above resulted in complete dimerization with
each of them, even with Sr²⁺ (Table 1). The synergistic effect
of PQQ on dimerization is apparently so strong that even inef-
fектив Ba²⁺ showed some dimerization: In this case, PQQ was
bound only to the dimer. Co²⁺ and Ni²⁺ apparently counteracted
the effect of PQQ since no dimer was found and PQQ was at-
tached to the monomer.

Fig. 4. Absorption spectra obtained during the conversion of M into
Holo-Y+enzyme with PQQ, glucose, and increasing amounts of Ca²⁺.
To M (9.7 μM) in buffer A (spectrum a), PQQ (11.5 μM) was added
(spectrum b), and subsequently glucose (2.5 mM). After incubation
for 5 min (spectrum c), nine aliquots of CaCl₂ solution were added
and spectra were taken between the steps after incubating for 7 min.
Finally, an excess of CaCl₂ (257 μM final concentration) was added (spectrum
d). The spectra were corrected for the dilutions (up to 5%) caused by
the additions. The inset shows the relative changes in the absorbance at
340 nm as a function of the amount of added CaCl₂, the absorbance
values taken from spectra c and d used as reference points.

Fig. 5. Enzymatic activities determined during the conversion of M
into HolO-Y+enzyme with PQQ and increasing amounts of Ca²⁺. M
(40 μM) in buffer A was incubated for 1 h with PQQ (80 μM) and
varying amounts of CaCl₂. Subsequently, the mixture was diluted
and assayed as described in the Experimental Procedures section. Results
are means ± SE of three measurements.

Reconstitution of M. On addition of glucose to a HolO-Y-
containing preparation (a mixture of M and PQQ), the resulting
spectrum (Fig. 4) had a shape resembling that of reduced HolO-
X (HolO-X, Fig. 7) or that of PQQH₂ (having a maximum at
302 nm [19]). Although free PQQ does not react with glucose,
bound PQQ is apparently able to do so, leading to reduction of
HolO-Y and oxidation of glucose. This was confirmed by the
observation that addition of Ca²⁺ immediately changed the
spectrum of HolO-Ymax into that of HolOmax with a maximum at
338 nm [11]. From the spectral changes observed upon stepwise
addition of CaCl₂ solution (Fig. 4), it was calculated that com-
plete conversion of M into HolOmax is achieved with 2.2 mol
Ca²⁺/mol M. On following the reconstitution in a separate ex-
Fig. 6. Absorption spectra of Holo-X and Holo-enzyme. Holo-X was prepared as described in the Experimental Procedures section and its spectrum taken in buffer A (-----). Subsequently, conversion into Holo-enzyme was carried out by adding CaCl₂ (1 mM) and the spectrum (-----) was taken after 2 min incubation.

Experiment via activity measurements (Fig. 5), it was estimated that complete reconstitution was achieved with 2.4 mol Ca²⁺/mol M. It is concluded, therefore, that formation of one molecule of Holo₄ from 2 M requires 4 Ca²⁺ ions. This value is in agreement with that found here for Holo with atomic absorption spectrometry (4.5 ± 0.4) and by others [10] with X-ray fluorescence spectrometry (3.90 ± 0.16).

On screening all metal ions tested for dimerization for their ability to reconstitute M to Holo, as judged from the normal enzyme assay, the same specificity and order of activity was found as for dimerization [Ca²⁺ (100%), Cd²⁺ (90%), Mn²⁺ (75%), and Sr²⁺ (45%)]. To prevent dissociation of the metal ion other than Ca²⁺ from the enzyme upon dilution required to perform the assay, the corresponding salts (1 mM) were included in the dilution buffer, which appeared to be especially effective in the case of Mn²⁺.

Reconstitution of Holo-X. Using the procedure described in the Experimental Procedures section, stable preparations of Holo-X were obtained. On addition of Ca²⁺ to Holo-X, the spectrum changed into that of Holo (Fig. 6) and concomitant induction of enzymatic activity was observed, indicating that Holo-X is a Ca²⁺-deficient form of the latter and that the Ca²⁺ required for the conversion interacts with PQQ. Enzymatically active hybrid Holo-enzymes were only obtained with metal ions active in dimerization and in converting M into active Holo-enzymes, except for Co²⁺, the order being Ca²⁺ (100%), Cd²⁺ (97%), Mn²⁺ (92%), Sr²⁺ (57%), and Co²⁺ (23%). Co²⁺ is apparently able to activate Holo-X, but not to dimerize the subunits.

It has already been reported [13] that addition of glucose to Holo-X gives rise to slow reduction of this enzyme form, as was also found for the present preparations. Addition of Ca²⁺ to a Holo-X₄ preparation led to Holo₄ formation, as judged from the absorption spectra (Fig. 7). Since this conversion is accompanied by a large red shift of the maxima (much larger than in the case of the oxidized enzyme forms), reliable titrations could be performed. The straight line and the isobestic points in Fig. 7 indicate that Ca²⁺ has a high affinity for the enzyme and that no spectrophotometrically detectable intermediates are formed during the conversion. From the titration curve, it was calculated that 0.81 mol Ca²⁺/mol subunit was required for complete conversion. From this value and that for the conversion of M into

Fig. 7. Absorption spectra taken during the conversion of Holo-X into Holo₄ with glucose and increasing amounts of Ca²⁺. To Holo-X (7.2 µM) in buffer A (spectrum a), 1 mM glucose was added. After incubation for 7 min (spectrum b), four aliquots of CaCl₂ solution were added and spectra were taken between the steps after incubation for 11 min. Finally, an excess of CaCl₂ (383 µM final concentration) was added (spectrum c). The spectra were corrected for dilution (up to 2%) caused by the additions. The inset shows the relative changes in absorbance at 340 nm as function of increasing concentration of CaCl₂. The absorbance values taken from spectra b and c used as reference points.

Holo-enzyme, it is concluded that dimerization of subunits and activation of Holo-X each requires addition of 1 Ca²⁺ ion/subunit molecule. The straight line in the latter case (Fig. 5) also indicates that the affinity for Ca²⁺ of the binding site connected with activation of Holo-X must be higher than that involved in dimerization of the subunits.

Conversion of Holo-X into hybrid Holo-enzyme with several metal ions was also studied by obtaining absorption spectra. Incubation of Holo-X in the presence of glucose and 1 mM Cd²⁺, Mn²⁺, Sr²⁺, or Mg²⁺ salts led to the following observations: both the shape and absorbancies of the spectra of the hybrid Holo₄-enzymes prepared with Cd²⁺ and Sr²⁺ were very similar to those of normal Holo₄ except that they showed red and blue shifts of the maxima of 2 nm, respectively; the spectrum of the hybrid Holo₄-enzyme prepared with Mn²⁺ showed similar shape but lower absorbancies than that of normal Holo₄ and a red shift of 2 nm, the lower absorbance probably indicating that association of the metal ion was not so strong, which was confirmed by the observation that dilution buffer containing Mn²⁺ gave higher activities than dilution buffer lacking this ion; Mg²⁺ addition did not change the spectrum of Holo-X₄, indicating that this metal ion does not bind, in agreement with the fact that subsequent Ca²⁺ addition led to the complete conversion into normal Holo-enzyme, as judged from the absorption spectra and specific activities, and that Mg²⁺ was inactive to induce enzymatic activity; Ba²⁺ addition resulted in an absorption spectrum intermediate between that of Holo-X₄ and Holo₄, suggesting that binding of this metal ion is weak, which is supported by the observation that subsequent Ca²⁺ addition led to a complete conversion, as judged from the absorption spectrum, and that Ba²⁺ was inactive with respect to induction of enzymatic activity; addition of Co²⁺ or Ni²⁺ induced unfamiliar spectral changes, suggesting that they react with Holo-X in an abnormal way.

DISCUSSION

The results show that Ca²⁺ in D is locked up in such a way that it is not accessible to chelator. Although binding of Ca²⁺ to
M* is strong, it can be removed by chelator, especially at elevated temperature. This strong affinity is in agreement with the observation that M can be converted into D (possibly via M*) with stoichiometric amounts of Ca*++. Since the presence of chelator does not affect the enzymatic activity of Holo enzyme, the Ca*++ ions required for activation of Holo-X are also locked up. Such a conclusion seems to contradict the fact that isolated D contains two but not four Ca*++ ions per molecule. To explain this, we assume that PQQ exerts a synergistic action, that is, its binding induces affinity for Ca*++ of the sites involved in activation of Holo-X and increases the affinity of the sites involved in dimerization. This view is supported by the following observations: titration of M or Holo-X to Holo with Ca*++, as monitored by enzymatic activity, gives a straight line and stoichiometric amounts of the metal ion give complete conversion, which indicates that the induced affinity of the binding sites for activation must be higher than that for dimerization; metal ions inducing only partial dimerization of M do this completely in the presence of PQQ (Table 1). Metal ions active in dimerization also exert a synergistic action, as follows from the observations that PQQ is loosely bound in Holo-Y but strongly bound in Holo-X.

Since the absorption spectra of Holo-Y and Holo-X are roughly similar and they can be converted into Holo-enzyme without forming spectrophotometrically detectable transients, it seems that the two PQQ molecules in Holo-Y (the amount estimated from the absorbance ratios) are bound at the appropriate site already. The synergistic action of PQQ and metal ion in Holo-X might be the result of conformational changes taking place in the conversion of D into Holo-X. However, although the roughly similar spectral characteristics of Holo-Y and Holo-X suggest that no direct interaction occurs between metal ion and PQQ in Holo-X, anchoring of PQQ and stabilization of the complex as a whole can also be explained by assuming that neutralization of the negatively charged carboxylate groups of PQQ by the positively charged metal ions, without causing a large spectral change, is responsible for this. The fact that Ca*++ addition to free PQQ or PQQH2 gives only a small red shift of the absorption maximum [20] could be interpreted in favor of the second hypothesis.

As judged from the gel-filtration chromatograms, PQQ is bound to the dimer and not to the monomer. This could indicate that the ligands for binding of a PQQ molecule derive from both subunits. However, it cannot be excluded that M can spontaneously dimerize (with rapid dissociation, so that no dimer is observed with gel filtration), the dimer having a conformation enabling PQQ to bind only to one subunit. Since quinohemoprotein alcohol dehydrogenase is a monomeric enzyme [21], it is clear that in principle PQQ binding to only one subunit is possible. Methanol dehydrogenase shows that this is even possible in a multimeric enzyme. PQQ in this tetrameric enzyme (αβ2γ2 configuration) is only bound to the α subunits and subunit association and PQQ binding do not require the participation of Ca*++ [17]. The two Ca*++ ions per enzyme molecule that are required for inducing activity, do this by binding to and probably also by additional anchoring of the two PQQ molecules to this enzyme. The examples also illustrate that sGDH is unique among the PQQ-containing dehydrogenases, having two different binding sites related to the different functions of Ca*++ and its substitutes in this enzyme.

Since the absorption maximum of Holoαα and the fully reduced form of methanol dehydrogenase [16] are similar, it is tempting to speculate that the Ca*++ ions are involved in activation of the cofactor in sGDH and are bound to PQQ in the same way as in methanol dehydrogenase. A large red shift occurs in the absorption maximum of Holo-X when those metal ions are added that convert inactive Holo-X into active Holo. The shift is much larger (in energetic terms) for the reduced than for the oxidized enzyme forms. This could mean that interaction of the metal ion with PQQH2 is more intense than with PQQ in the enzyme. Since the three-dimensional structure elucidated for methanol dehydrogenase [17] most probably concerns the semiquinone form of the enzyme, corroboration of the hypothesis must await structure elucidation of the other redox forms.

In accepting the view that Ca*++ involved in activation of PQQ affects the spectroscopic properties of PQQ in sGDH, it is somewhat surprising that the absorption spectra of the hybrid Holoαααα-enzymes are so similar to that of the normal Holoαααα-enzyme, only showing small red or blue shifts of the maxima. The differences in properties of the metal ions are apparently neither translated into significant differences between the maxima or absorbancies, nor between the enzymatic activities. Since the activities of all hybrid Holo-enzymes were lower than the normal one, it seems that abnormality, either expressed by a small red or a blue shift, just lowers the catalytic performance.

The small spectral differences observed here for the hybrid Holoαααα-enzymes are not in agreement with the large difference in absorbance reported for Sr*++-containing and Ca*++-containing methanol dehydrogenase enzymes [15]. However, on close inspection of the absorption spectra of the enzymes, it seems likely that the Sr*++-enzyme was isolated in the fully reduced form and the Ca*++-enzyme in the usual semiquinone form [16], which could explain the large difference in absorbance (and the small difference in the maxima). This interpretation is supported by a recent report [22] in which it was mentioned that reconstruction of a Holo-Y-like methanol dehydrogenase (produced by a mutant strain being unable to insert the Ca*++ ions in the enzyme) with Ba*++ gave holoenzyme with a normal spectrum. Although in this interpretation the normal and substituted enzyme forms are spectrally similar, for methanol dehydrogenase as well as for sGDH, this is not the case with respect to enzymatic activity. For instance, it has been found that the Vmax values of the substituted methanol dehydrogenases were much higher than the Vmax of the normal enzyme [15, 22]. Since our assay for sGDH is carried out at practically saturating substrate and electron acceptor concentrations, but the hybrid Holo-enzymes showed lower activities than the normal enzyme, it seems that the effects of the metal ions on the catalytic mechanism of the enzymes via activation of the cofactor cannot unequivocally be translated into a specific effect on the kinetic parameters of the quinoprotein dehydrogenase. To establish the relationship for sGDH, an extensive kinetic analysis of normal enzyme, substituted enzyme forms, and hybrid Holo-enzymes with various substrates is in progress.

The similarity with respect to metal ions active in dimerization of monomer and in activation of PQQ suggests that the dimensions and the affinities of the binding sites for the metal ion connected with the two functions are also similar. However, in accepting the view that the Ca*++ for activation binds to PQQ (and in the hypothesis that Ca*++ for dimerization binds to PQQ, other moieties of the molecule are involved), it is clear that the ligands in both cases are different.

On comparing the metal ion specificity of sGDH with the known properties of the metal ions, no obvious correlation seems to exist between them. On comparing the specificity with that for other PQQ-containing dehydrogenases, it appears that variations exist (e.g. Mg*++ is active for mGDH [4], Ba*++ for methanol dehydrogenase [22], but both are inactive for sGDH reconstitution). Thus the suitability of a bivalent metal ion to function in these enzymes seems to be governed not so much by PQQ but by the specific nature of the binding site in each enzyme.
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Amsterdam and Boehringer Mannheim GmbH for financial support.

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Chapter 4

Negative cooperativity in the steady-state kinetics of sugar oxidation by soluble quinoprotein glucose dehydrogenase from *Acinetobacter calcoaceticus*

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Negative cooperativity in the steady-state kinetics of sugar oxidation by soluble quinoprotein glucose dehydrogenase from Acinetobacter calcoaceticus

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Steady-state-kinetics investigations were carried out for the oxidation of aldose sugars by soluble quinoprotein glucose dehydrogenase (GDH) from Acinetobacter calcoaceticus using N-methylphenazinum methyl sulfate (PMS) as artificial electron acceptor. As is not uncommon for a dye-linked dehydrogenase, the enzyme showed ping-pong behaviour and double-substrate inhibition. However, under conditions that avoided its masking by sugar-substrate inhibition as much as possible, negative kinetic cooperativity with respect to sugar substrate oxidation by this enzyme was demonstrated. Arguments are presented that exclude trivial factors as a cause for the phenomenon observed. Experimental data could be fitted with an equation accounting for biphasic cooperativity containing two sets of apparent kinetic parameters, \( V_1 \) and \( K_1 \), and \( V_2 \) and \( K_2 \), representing the enzyme’s Michaelis-Menten behaviour at low and high substrate concentrations, respectively. Assuming that subunit interaction causes the cooperativity effect, the sets express the performance of soluble GDH’s two subunits in two states of mutual interaction. From fitting the experimental data for several sugars with this equation, it appeared that their \( V_2 \) values were similar, although their \( K_1 \) values varied considerably. This showed that the cooperativity effect dramatically changes the performance of soluble GDH, as reflected by the \( V_2 \) and \( K_2 \) values for glucose (in phosphate buffer) being about 10-fold and 100-fold higher than the \( V_1 \) and \( K_1 \) values, respectively. Substituting the Ca\(^{2+}\) involved in activation of pyrroloquinoline quinone (PQQ) in soluble GDH by Sr\(^{2+}\) affected the cooperativity effect (an increase of the \( K_1 \) value) but not the two turnover rates of the hybrid enzyme for glucose. The data suggest that the two catalytic cycles of soluble GDH have different rate-limiting steps compared with that of PQQ-containing methanol dehydrogenase.

Keywords: quinoprotein; glucose dehydrogenase; enzyme kinetics; cooperativity.

The bacterium Acinetobacter calcoaceticus produces two pyrroloquinoline quinone (PQQ)-containing glucose dehydrogenases (GDH), a soluble one and a membrane-bound one. The homodimeric soluble GDH has been purified from this bacterium in the holocomplex [1-5] and in the apoenzyme from an overproducing Escherichia coli recombinant strain [6]. The enzyme has a broad specificity with respect to aldose sugars (hexoses, pentoses, monosaccharides, and disaccharides) and artificial electron acceptors (two-electron [e.g. N-methylphenazinum methyl sulfate (PMS) and 2,6-dichloroindophenol (Cl\(_2\)Ind)] and one-electron acceptors [e.g. Wurster’s Blue]). Previous studies [7] have shown that the Ca\(^{2+}\) present in this enzyme are required for dimerization of the subunits and for activation of PQQ. Starting with apoenzyme (dimer containing Ca\(^{2+}\)), reconstitution to active hybrid holoenzyme can be achieved by adding PQQ and Cd\(^{2+}\), Mn\(^{2+}\), or Sr\(^{2+}\). Hybrid enzymes prepared with Cd\(^{2+}\) or Mn\(^{2+}\) are nearly as active as normal holo-enzyme (in the standard assay with 50 mM glucose) but enzyme prepared with Sr\(^{2+}\) showed only half of the activity. The reason for this suboptimal performance is unknown.

As isolated or as obtained after reconstitution of the apoenzyme with PQQ holoGDH contains PQQ in the oxidized form only [3, 6]. On addition of glucose, reduction of the cofactor takes place, accompanied by production of gluconic acid, the amount being equivalent to 1 turnover per subunit (Dewanti, A. R. & Duine, J. A., unpublished results), indicating that the enzyme operates essentially via a ping-pong type of mechanism, the cofactor becoming reduced upon oxidation of the aldose sugar and subsequently reoxidized by the electron acceptor Lineweaver-Burk plots of initial rates determined at varying concentrations of Cl\(_2\)Ind [1] or Wurster’s blue [3, 4], gave parallel straight lines, indicative of straightforward ping-pong kinetics, at least in the narrow range of substrate and electron-acceptor concentrations tested (the narrowness being required because aldose sugar and electron acceptor cause substrate inhibition). However, using PMS as electron acceptor coupled to Cl\(_2\)Ind, Matsuhashita et al. [5] observed that Lineweaver-Burk plots for soluble GDH deviated from linearity in regions where substrate inhibition was still absent. Since this phenomenon was not fur-
ther explored and the kinetic behaviour of soluble GDH under a variety of conditions is not known, this prompted us to perform steady-state-kinetics studies on this enzyme and its Sr\(^{2+}\)-containing hybrid form.

**EXPERIMENTAL PROCEDURES**

**ApoGDH and holoGDH preparations.** Apoenzyme was isolated from an *E. coli* recombinant strain containing the gene for soluble GDH from *A. calcoaceticus* [8], as described [6]. HoloGDH or Sr\(^{2+}\)-containing hybrid holoenzyme were prepared by adding excess PQQ to the apoenzyme, in the presence of Ca\(^{2+}\) or Sr\(^{2+}\), respectively, followed by gel filtration to remove unbound PQQ, as described [6, 7].

**Steady-state-kinetics measurements.** Initial reaction rates were determined by following the reduction of Cl\(_2\)Ind at 600 nm on a Perkin-Elmer 555 spectrophotometer at 20 °C, using PMS as a primary electron acceptor. The assay mixture (1 ml) contained 50 mM potassium phosphate, pH 7.0, 50 μM Cl\(_2\)Ind (e\(_{600} = 20.6\) mM\(^{-1}\) cm\(^{-1}\), [9]), substrate (50 mM glucose unless indicated otherwise) and PMS (1 mM unless indicated otherwise). The reaction was started by addition of 10 μl enzyme at a suitable concentration (enzyme samples were diluted with 20 mM Tris/HCl, pH 7.0, 3 mM CaCl\(_2\) and 0.1% Triton X-100). Solutions of PMS in water were kept shielded from light to ensure low background activities of the assay (rates of absorbance changes due to chemical degradation were lower than 0.001 min\(^{-1}\)). To be sure that the sugar substrate had the correct and reproducible anomer ratio in the assay, sugar solutions were prepared in advance to allow equilibration to take place. 1 U enzyme activity corresponds to the conversion of 1 μmol aldose/min under the conditions specified above. To determine the amounts of activity, enzyme activities were measured out with the standard assay using a specific activity value of 7400 U/mg protein [6].

**Kinetic equations.** Although PMS is a far better electron acceptor for soluble GDH than Cl\(_2\)Ind (at least at pH 7.0) [6], as encountered in these investigations, especially at low PMS concentrations, the direct contribution of Cl\(_2\)Ind to the reoxidation rate of reduced enzyme cannot be neglected (Fig. 1). Assuming that both electron acceptors compete for the same site in the reduced enzyme and that a ping-pong kinetic mechanism applies, the relationship between the initial rate, \(v\), and the varying concentrations of electron acceptors at a fixed sugar substrate concentration is given by

\[
\nu = \frac{[P]V_0K_0 + [D]V_0K_D}{[P]K_0 + [D]K_D + K_{0D}}
\]

in which \(V_0\) and \(K_D\), and \(V_0\) and \(K_0\) are the sets of apparent kinetic parameters of soluble GDH for PMS (P) and Cl\(_2\)Ind (D), respectively. Transformation of Eqn (1) to give the dependence of the initial rate of the PMS concentration at a fixed concentration of Cl\(_2\)Ind yields

\[
\nu = \frac{[P][V_0 - \nu_0]}{[P] + C} + \nu_0[C = K_D[D] + K_0/K_{0D}]
\]

in which \(\nu_0\) is the initial rate at \([P] = 0\).

**Kinetic cooperativity in the enzymatic conversion of a substrate, apparent from biphasic behaviour in a Lineweaver-Burk or Eadie-Hofstee plot, can be mathematically described [10] by the following second-order rate equation:**

\[
\nu = \frac{[A]^2a + [A]b}{[A]^2 + [A]c + d}
\]

in which \([A]\) is the substrate concentration and \(a, b, c\) and \(d\) are coefficients representing terms consisting of one or more parameters. When the mechanistic cause is unknown, cooperative behaviour is most simply described with two sets of apparent kinetic parameters, \(V_1\) and \(K_1\), and \(V_2\) and \(K_2\), representing the enzyme's Michaelis-Menten performance at low and high substrate concentrations, respectively, [10, 11] according to

\[
\nu = \frac{[A]^2V_2 + [A]V_2K_2}{[A]^2 + [A]K_2 + K_1K_2}
\]

When the cooperativity is due to subunit interaction, displaying itself as indicated in Scheme 1A, the aforementioned kinetic parameters can be related directly to the different rate constants of Scheme 1A [10, 11]: \(V_1 = [E][k_2]; K_1 = (k_{-1} + k_2)/k_2; V_2 = [E][k_5]; K_2 = (k_{-3} + k_5)/k_5\), where \([E]\) denotes the total enzyme concentration.

To take experimentally observed substrate inhibition into account (competition of the sugar with PMS for the reduced enzyme form), a third-order term with one apparent inhibition constant, \(K_{a}\), was incorporated in the denominator of Eqn (4), this being the minimal requirement necessary to fit the experimental results:

\[
\nu = \frac{[A]^2V_2 + [A]V_2K_2}{[A]^2K_a + [A]^2 + [A]K_2 + K_1K_2}
\]

Assuming that ping-pong kinetics apply to the simple mechanism proposed in Scheme 1A, and that no cooperativity effect is exhibited towards the electron acceptor, the kinetic mechanism of soluble GDH is as depicted in Scheme 1B. To derive an equation for this, symbolic software was used [12].

**Fitting of experimental data.** Direct non-linear least-squares computer fitting of the experimental data to the equa-
Fig. 1. Activity of soluble GDH at varying PMS concentration. Activity was measured with the standard assay with 10 mM glucose and varying PMS concentration. The line represents the fit of the experimental data with Eqn (2), yielding $V_0 = 3953 \pm 46$ U/mg, $C = 51.9 \pm 3.4$ mM, and $\varepsilon_0 = 529 \pm 45$ U/mg.

Fig. 2. Activity/pH profiles for soluble GDH. Enzyme activities were measured with 1 mM PMS, with 50 mM (A) or 1 mM glucose (B), in 50 mM potassium phosphate, pH 6.0–7.0 (○), or 50 mM Tris/HCL, pH 7.0–9.0 (△).

Fig. 3. Lineweaver-Burk plots for soluble GDH at varying concentrations of glucose and PMS. (A) Glucose as the variable substrate. PMS concentrations were 1 (○), 0.3 (●), 0.1 (△), 0.04 (■) and 0.02 (▲) mM. (B) PMS as the variable substrate. Glucose concentrations were 50 (○), 25 (●), 10 (△), 5 (■), 3 (▲), 2 (▲) and 1 (★) mM.

Fig. 4. Activity/[PMS] plots for soluble GDH at varying concentrations of glucose and PMS. (A) Glucose as the variable substrate. PMS concentrations were 1 (○), 0.3 (●), 0.1 (△), 0.04 (■) and 0.02 (▲) mM. (B) PMS as the variable substrate. Glucose concentrations were 50 (○), 25 (●), 10 (△), 5 (■), 3 (▲), 2 (▲) and 1 (★) mM.

RESULTS

Assay-system performance. Starting the assay either by addition of enzyme or of PMS and Cl$_2$Ind, both conditions gave linear absorbance decreases over time without the occurrence of bursts or lag phases, indicating that the measurements reflected true steady-state kinetic behaviour. In the absence of enzyme, a control absorbance decrease of less than 0.001 min$^{-1}$ was observed. Enzyme concentrations were chosen that resulted in rate values between 0.05 and 1.0 min$^{-1}$. Within this range, activities were linear with respect to enzyme concentration. At the Cl$_2$Ind concentration and pH used, interference due to reoxidation of PMSH$_2$ by dissolved O$_2$ did not occur, as judged from the absence of O$_2$ uptake by a Clark electrode under the standard assay conditions and from the similar values obtained with the assay carried out under anaerobic (the solutions flushed with N$_2$) compared with those under aerobie conditions. The latter observation indicates that reaction of O$_2$ with reduced enzyme, as has been proposed to take place for quinoprotein methanol dehydrogenase [13], can be excluded. It is concluded that Cl$_2$Ind reduction was proportional to sugar-substrate oxidation.

Using twice or half as much Cl$_2$Ind as in the standard assay, no effect on the initial rate was observed, indicating that oxidation of PMSH$_2$ by Cl$_2$Ind is not a rate-limiting step under the assay conditions. Substantial enzymatic activity was observed at [PMS] = 0, the contribution of direct Cl$_2$Ind reduction to the overall rate explaining why deviations seem to occur from the kinetic model in this region. Fig. 1 also shows that Eqn (2) was perfectly fit to the experimental data, indicating that the underlying assumptions for Eqn (2) are valid and that substrate inhibition by PMS does not occur up to 1 mM PMS in the presence of 10 mM glucose.

Using a glucose concentration of 50 mM, the pH optimum for soluble GDH was about 7.0, and activities appeared to be slightly higher in potassium phosphate than in Tris/HCL of the same pH (Fig. 2A). When the pH profile was measured at 1 mM glucose, again a pH optimum of about 7.0 was found, but the activities obtained with potassium phosphate were markedly higher than those found with Tris/HCL especially at pH 7.0 (Fig. 2B). Since activities were highest with potassium phosphate, pH 7.0, this buffer was normally used in the experiments.

Steady-state kinetics with glucose and PMS. Reciprocal plots of initial reaction rates versus varying concentrations of glucose at fixed concentrations of PMS gave parallel lines with concave downward curvature (Fig. 3A), suggesting negative cooperative behaviour to be present. On the other hand, lines representing data sets for a low PMS concentration showed upward curvature, suggesting the occurrence of substrate inhibition by glucose, competitive with respect to PMS for the reduced enzyme form. When the data were replotted with PMS as the variable substrate (Fig. 3B), lines were obtained that are straight from 0.04 mM to 1 mM PMS but curve weakly downward at lower PMS concentrations. The latter effect is due to that Cl$_2$Ind can act as a direct electron acceptor for soluble GDH, as discussed above. Since Eqn (2) fitted the experimental data presented in Fig. 1 very well and the curves (also those for a low glucose concentration) in Fig. 3B did show anomalies, there are no indications for cooperative behaviour of the enzyme towards PMS or for substrate inhibition by PMS. However, the latter occurs because the onset of substrate inhibition by PMS (competing with glucose for the oxidized enzyme form) was observed at concentrations above 2 mM (data not shown).

To establish the negative-cooperativity effect more firmly, steady-state-kinetics studies were performed over a broad range of glucose concentrations. A fixed PMS concentration of 1 mM was used to avoid masking of the cooperativity effect by substrate inhibition (competition of glucose with PMS for the re-
Table 1. Apparent kinetic-parameter values determined from steady-state kinetics of normal or hybrid (Sr²⁺-containing) soluble GDH. Substrate concentrations were varied as indicated in the legends of the figures. Glucose and lactose data were fitted with Eqn (5), galactose and xylene data with Eqn (4).

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Buffer</th>
<th>Vₐ</th>
<th>Kₐ</th>
<th>Vᵦ</th>
<th>Kᵦ</th>
<th>Kᵦ</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>kU/mg</td>
<td>mM</td>
<td>kU/mg</td>
<td>mM</td>
<td>mM</td>
</tr>
<tr>
<td>Glucose</td>
<td>phosphate</td>
<td>1.82 ± 0.22</td>
<td>0.74 ± 0.18</td>
<td>20.2 ± 1.9</td>
<td>69 ± 12</td>
<td>92 ± 13</td>
</tr>
<tr>
<td></td>
<td>Mops/NaOH</td>
<td>1.96 ± 0.26</td>
<td>0.87 ± 0.37</td>
<td>22.8 ± 9.1</td>
<td>363 ± 216</td>
<td>156 ± 79</td>
</tr>
<tr>
<td>Lactose</td>
<td>Tris/HCl</td>
<td>0.51 ± 0.22</td>
<td>0.20 ± 0.31</td>
<td>16.4 ± 2.0</td>
<td>52 ± 12</td>
<td>150 ± 27</td>
</tr>
<tr>
<td>Galactose</td>
<td>phosphate</td>
<td>1.90 ± 0.21</td>
<td>1.68 ± 0.35</td>
<td>18.2 ± 3.2</td>
<td>154 ± 45</td>
<td>61 ± 14</td>
</tr>
<tr>
<td>Xylene</td>
<td></td>
<td>1.98 ± 0.06</td>
<td>6.97 ± 0.38</td>
<td>4.2 ± 1.1</td>
<td>1080 ± 740</td>
<td>521 ± 44</td>
</tr>
</tbody>
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* Hybrid (Sr²⁺-containing) soluble GDH.

Fig. 4. Eadie-Hofstee plots for soluble GDH at varying glucose concentrations and in various buffers. Activities were measured in 50 mM potassium phosphate (●) or 50 mM Tris/HCl (○) at 1 mM PMS. Glucose concentrations were in the range 0.1–450 mM. The lines represent fits of the experimental data with Eqn (5).

Fig. 5. Eadie-Hofstee plot for hybrid (Sr²⁺-containing) soluble GDH with varying glucose concentrations. Experiments were carried out with the standard assay. Glucose concentrations were in the range 0.5–1000 mM. The line represents the fit of the experimental data with Eqn (5).

duced enzyme form) and substrate inhibition by PMS (competition with glucose for the oxidized enzyme form) as much as possible. Data obtained with 50 mM potassium phosphate or 50 mM Tris/HCl are presented in Eadie-Hofstee plots in Fig. 4. Biphase behaviour with downward concavity, especially pronounced in Tris/HCl, indicative of negative kinetic cooperativity, was observed with both buffers, as well as substrate inhibition by glucose, becoming already evident at a concentration around 70 mM. The experimental data could be adequately fitted with Eqn (5), and values for the apparent kinetic parameters calculated from these fits are given in Table 1. It appears that, in potassium phosphate, the cooperativity effect leads to a 100-fold increase in the apparent Michaelis constant (Kᵥ versus Kᵥ') and to a 10-fold increase in apparent maximal activity (Vᵥ versus Vᵥ'). In addition, it can be concluded that the enzyme behaves differently in potassium phosphate than in Tris/HCl and Mops/NaOH with respect to the values found for Vᵥ and Kᵥ representing the enzyme's kinetic behaviour at low glucose concentrations, but not with respect to the values found for Vᵥ’ and Kᵥ’ representing the enzyme's behaviour at high glucose concentrations (however, the values for the specificity constant, represented by Vᵥ'/Kᵥ', are the same). The difference is not due to an ionic-strength or salting-out effect of the buffers since the activity found in Tris/HCl at 1 mM glucose was not affected by addition of KCl (up to 0.2 M) or Na₂SO₄ (up to 60 mM). This can be explained by assuming that the phosphate ions change the enzyme, the behaviour in the Tris/HCl or Mops/NaOH representing the normal situation at low glucose concentrations. The results of experiments in which the phosphate concentration was varied (data not shown) are consistent with this view since they revealed that an increase of the activity occurs up to 0.2 M potassium phosphate for assays with 1 mM glucose. Therefore, phosphate ions exert a specific effect on the enzyme, increasing the enzyme's catalytic turnover and its saturation value for glucose in the low region of substrate concentrations to the same extent.

The Eadie-Hofstee plot (Fig. 5) and the apparent kinetic parameter values (Table 1) for the oxidation of glucose by Sr²⁺-containing hybrid holoGDH indicate that negative cooperativity is affected by the type of metal ion present (the Kᵥ value for the hybrid enzyme is higher than for the normal enzyme). The metal ion must be involved in a non-rate limiting step in the catalytic mechanism exhibited at high glucose concentrations since the Vᵥ, Vᵥ' and Kᵥ values of the normal and hybrid enzyme are similar.

Steady-state kinetics with other aldose sugars. To determine how the substrate identity affects the kinetic behaviour of soluble GDH, a disaccharide (lactose), a hexose sugar (galactose) and a pentose sugar (xylene) were investigated. Negative cooperativity applies to soluble GDH in the oxidation of aldose sugars other than glucose (Fig. 6). The values for the apparent kinetic parameter values for lactose (Table 1) were similar for Vᵥ and Vᵥ' but were twofold higher for Kᵥ and Kᵥ' compared with those for glucose. Since soluble GDH shows another specificity, e.g. having a preference for the β-anomer of glucose ([1, 2] un-
published results), this difference could be due to different anomer ratios for the two sugars. However, this is not the case since the anomer ratios for glucose and lactose are similar [14] and soluble GDH shows the same anomer preference as for glucose (unpublished results). Since it is the glucose moiety in lactose (4-O-β-galactosylglucose) that becomes oxidized, the galactose moiety apparently hinders binding of lactose to the enzyme but it does not affect the rate-limiting steps in the catalytic cycles. Visual inspection of their Edel-Hofstee plots (Fig. 6) indicated that galactose and xylose did not exhibit substrate inhibition; therefore the experimental data were fitted with Eqn (4). However, it cannot be excluded that the inhibition occurs at higher substrate concentrations than those tested here and that its effects are already present in the experimental data obtained. This might explain why large standard deviations were found for $V_I$ and $K_I$, but much smaller ones for $V_L$ and $K_L$. Comparing $V_I$ and $V_L$ for all the aldose sugars investigated, it appears that the $V_I$ values are more or less the same but the $V_L$ values are different. Thus, the catalytic performances of soluble GDH for xyllose and galactose are the same as for glucose at low substrate concentrations but suboptimal at high concentrations. Perhaps the ability of soluble GDH to bind these two substrates is much lower than for glucose, consistent with the higher $K_I$ and $K_L$ values and the visual absence of substrate inhibition in the plots for them.

An overall kinetic equation for soluble GDH. Eqns (2) and (5) described the half reactions of the catalytic cycle very well, as judged from the fits of the experimental data. Based on the underlying assumptions for these equations, we attempted to derive a general equation for soluble GDH, taking the cooperative effect for the sugar substrate into account. Although an equation could be derived for the mechanism depicted in Scheme 1B, it was not practicable since it contained too many terms in the numerator and denominator, the substrate or electron-acceptor concentration being up to the fourth order (incorporation of the observed substrate inhibition would make the equation even more complicated). Attempts to simplify the equation by introducing assumptions with respect to the cause of the negative cooperativity kinetics disappointing results, as the equations obtained were so complicated that they could not be used in practice.

DISCUSSION

The performance of the assay was verified, and it appears that appropriate conditions were used during the measurement of the initial rates in the steady-state kinetics of soluble GDH. The observation that the poor electron acceptor Cl₂Ind contributed directly to the overall rate at low PMS concentrations and that it could be quantitatively described by Eqn (2), enabled us to establish the kinetic mechanism at low PMS concentrations. Other causes for non-linearity or non-parallelity of the lines in the Lineweaver-Burk plots, i.e. negative cooperativity and substrate inhibition, could be quantitatively described by Eqn (5). Taking all three phenomena into account, the results demonstrated that soluble GDH acts via a ping-pong kinetic mechanism. This is consistent with our previous observations [7] that the reduced form of soluble GDH can be isolated after adding sugar substrate to the oxidized form.

The reason that negative cooperativity was not observed in previous publications [1, 3, 4] is probably due to the use of Wurster’s blue or Cl₂Ind as the sole electron acceptor. These compounds have a much lower affinity for the enzyme than PMS, so that substrate inhibition by glucose occurs at a lower concentration, which may have masked the cooperativity effect, as it did here at low PMS concentrations. The concentration of 1 mM PMS used here appeared to be the most adequate one with glucose for the following reasons: substrate inhibition by PMS does not occur at this concentration; and substrate inhibition by glucose occurs at such a high concentration that the observation of negative cooperativity in the plots is not masked.

When plots indicate that negative cooperativity occurs, the cause may be a trivial or a mechanistic one. Trivial causes include heterogeneity of the enzyme preparation with respect to catalytic performance [14], due to the presence of different enzyme species or to the occurrence of a protomer-oligomer equilibrium under the conditions used in the assay, and a non-constant heterogeneity with respect to the substrate (e.g. a shifting anomic ratio of a sugar under the conditions of the assay). All
data available for soluble GDH indicate, however, that heterogeneity did not occur in the preparations used. As has been reported [6], the recombinant apo-enzyme used to prepare the holoenzyme is homogeneous, as judged from its behaviour on electrophoresis, N-terminal amino acid sequencing, the linear increase of activity on titration of the apoenzyme with PQO or Ca^{2+}, the homogeneity of holoGDH on chromatography (only dimeric enzyme is observed), and the proportionality of the assay (with respect to activity and enzyme concentration, in the range 0.9–14 ng enzyme/ml). Heterogeneity of the substrates can be excluded as a cause of the negative cooperativity because, although d-glucose is present in solution in two anomic forms (α- and β-D-pyranose), the ratio of the two forms is not dependent on the glucose concentration [15] so that the substrate can be regarded as homogeneous. Therefore, the cause of negative cooperativity of soluble GDH in sugar oxidation must be a mechanistic one.

Several mechanistic causes for cooperative effects in enzyme kinetics have been proposed [10, 14]: the presence of a regulatory non-active binding site (an allosteric site) in the enzyme, which alters the catalytic mechanism upon occupation of the site by substrate; the occurrence of a monomolecular enzyme transition [16], i.e. the presence of an equilibrium between two enzyme conformations that can both bind the substrate to form an active complex, one of which is reformed after one productive cycle; random-order binding of substrates in an enzymatic conversion requiring a ternary complex; and interaction between subunits, i.e. binding of a substrate to a subunit or transformation of it into the reduced state, altering the properties of the other subunit (with respect to binding and/or conversion of the substrate) in the dimeric enzyme. The first two possibilities cannot be excluded, the third can be excluded because of the ping-pong kinetics observed, and the fourth is the most likely one for soluble GDH because it can be envisaged that when the active site of a subunit is occupied by substrate, or when the PQO in the subunit becomes reduced by reaction with the substrate, an effect will be exerted on the other subunit in the homodimeric enzyme molecule, leading to a change in its properties. Assuming that binding and catalytic properties are affected, the catalytic cycle can be represented by Scheme 1A, from which Eqn (4) was derived. Although this equation (after transforming it into Eqn (5) to take the experimentally observed substrate inhibition by some sugars into account) fitted the experimental data very well, mechanisms other than that proposed here (described by equations similar to Eqn (4) but with other lumped parameter sets) cannot be excluded with the results obtained.

According to knowledge of the catalytic mechanism of soluble GDH, one of the steps in this is adduct formation between glucose at the C1 position and PQO at the C5 carbonyl group (unpublished results). Therefore, it is understandable that the catalytic performances of soluble GDH for glucose and lactose are the same but that the $K_v$ values for the two substrates differ. However, although at low substrate concentration the catalytic performances for xylose and galactose were the same as for glucose, those at high concentrations of these substrates were far below that for glucose. Two explanations can be given for this: (a) The negative cooperativity is due to subunit interaction between the reduced and oxidized subunits in the enzyme, but the resulting enzyme form has different catalytic performances for the various substrates (the same $V_v$ values for glucose and lactose but lower ones for xylose and galactose). (b) Negative cooperativity is induced by substrate binding, but the extent to which the catalytic performance is affected depends on the identity of the inducing substrate (glucose and lactose being better inducers than galactose and xylose). Unfortunately, the limitations of the experimental and mathematical accessibility of enzymatic cooperativity effects prohibit the discrimination between the two possibilities from the present results. The turnover rate of soluble GDH for sugars is so high (unpublished results) that stopped-flow experiments are feasible in the low region but not in the high region of glucose concentrations, implying that the previously-stated kinetics of the cooperativity effect cannot be explored. Moreover, titration of apoenzyme with PQO or Ca^{2+} showed that a linear relationship exists between activity and the amount of titrant [6, 7], suggesting that there are no active monomeric species or dimeric species with one active and one inactive subunit. On the other hand, the observation that independent steps are involved in the induction process, as suggested by the finding that substitution of Ca^{2+} by Sr^{2+} changes the $K_v$ value but not the $K_s$, $V_0$ or $V_i$ values, and the different $V_i$ values for the substrates tested means that further investigations on different substrates and/or metal-ion substitution could give a clue in this respect.

Substitution of Ca^{2+} by Sr^{2+} has been carried out for several other quinoprotein dehydrogenases [17–21], leading to a lower specific activity, except for methanol dehydrogenase where up to tenfold higher values were found. Only for methanol dehydrogenase have the steady-state kinetics been investigated, showing that substitution resulted in higher values of $V$ and $K_i$ [18, 21]. Since in soluble GDH the $V$ values are not affected by the substitution, assuming that the metal ion exerts its effect on the same step in the catalytic cycle for both enzymes, it seems that the rate-limiting steps in the enzymes are different. The observation that in Tris/HCl and Mops/NaOH a lower $V_i$ value for glucose was obtained but the $V_i$ values were not affected, compared with the values in phosphate buffer, could mean that the rate-limiting steps in the two catalytic states of soluble GDH are different.

Negative-cooperativity kinetics have not been observed for other PQO-containing dehydrogenases, including the membrane-bound glucose dehydrogenase [15], Dewanti, A. R. and Duine, J. A., unpublished results). Studies on reconstitution of soluble GDH [7] have revealed that strong synergism is exerted by Ca^{2+} and PQO in dimerization of the monomers, leading to a very stable complex in which the metal ions are locked up, making them unavailable for removal by chelators. This may be favorable for transfer of conformational changes inducing the cooperativity effect, which may occur either on substrate binding or on reduction of PQO in one of the subunits of the enzyme molecule. It is expected that elucidation of the three-dimensional structure of soluble GDH may provide information regarding possible conformational changes.

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REFERENCES


Chapter 5

On the mechanism and specificity of soluble, quinoprotein glucose dehydrogenase in the oxidation of aldose sugars

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On the Mechanism and Specificity of Soluble, Quinoprotein Glucose Dehydrogenase in the Oxidation of Aldose Sugars†

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ABSTRACT: Kinetic and optical studies were performed on the reductive half-reaction of soluble, quinoprotein glucose dehydrogenase (sGDH), i.e., on the conversion of sGDHred plus aldose sugar into sGDHex or plus corresponding aldonolactone. It appears that the nature and stereochemical configuration of the substituents at certain positions in the aldose molecule determine the substrate specificity pattern: absolute specificity exists with respect to the C1-position (only sugars being oxidized which have the same configuration of the H/OH substituents at this site as the β-anomer of glucose, not those with the opposite one) and with respect to the overall conformation of the sugar molecule (sugars with a C1 chair conformation are substrates, those with a C2 one are not); the nature and configuration of the substituents at the 3-position are hardly relevant for activity, and an equatorial pyranose group at the 4-position exhibits only a specific hindering of the binding of the aldose moiety of a disaccharide. The pH optimum determined for glucose oxidation appeared to be 7.0, implying that reoxidation of sGDHex is rate-limiting with those electron acceptors displaying a different value under steady-state conditions. The kinetic mechanism of sGDH consists of (a) step(s) in which a fluorescing intermediate is formed, and a subsequent, irreversible step, determining the overall rate of the reductive half-reaction. The consequences of this for the likelihood of chemical mechanisms where glucose is oxidized by covalent catalysis in which a C5-adduct of glucose and PGQ are involved, or by hydride transfer from glucose to PQQ, followed by tautomerization of C5-reduced PGQ to PGQH2, are discussed. The negative cooperative behavior of sGDH seems to be due to substrate-occupation-dependent subunit interaction in the dimeric enzyme molecule, leading to a large increase of the turnover rate under saturating conditions.

Soluble, quinoprotein glucose dehydrogenase (EC 1.1.99.17; sGDH)† is a homodimeric enzyme which occurs in the bacterium Acinetobacter calcoaceticus (1) [quinoprotein, PQQ-containing; PQQ, pyrroloquinoline quinone (Figure 1A)]. The enzyme oxidizes a broad range of aldose sugars, the spectrum comprising hexoses as well as pentoses and mono- as well as disaccharides. The sugars are converted into their corresponding aldonolactones, and a variety of unnatural compounds such as N-methylphenazonium methyl sulfate (PMS) (2), 2,6-dichlorophenindophenol (DCIP) (3), Wurster’s Blue (4), N-substituted nitrosoanilines (unpublished results), and electroconducting polymers (5) act as electron acceptor for the enzyme in this. Structural aspects regarding the dimerization of the subunits, the binding of PQQ, and the role of Ca2+ and substitutes of it in this and in enzyme activation have already been described (6).

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Abbreviations: sGDH, soluble quinoprotein glucose dehydrogenase; PQQ, pyrroloquinoline quinone (2,7,9-tricaproyl-1H-pyrrole-2,3,5-triquinoline-4,5-dione); PQQH2, quinol form of PQQ; PQQH2, 4,5-dihydro-PQQ; (2,7,9-tricaproyl-1H-pyrrole-2,3,5-triquinoline-4,5-dione); PMS, N-methylphenazonium methyl sulfate; DCIP, 2,6-dichlorophenindophenol; MBS, 2-(N-morpholino)ethanesulfonic acid; CHES, 2-(cyclohexylamino)-ethanesulfonic acid; CAPS, 3-(cyclohexylamino)-1-propanesulfonic acid.

Figure 1: Structure of PQQ, some of its derivatives, and PQQH2.
(A) PQQ; (B) PQQH2; (C) PQQ adducts (R=H, PQQ-H2O adduct); (D) C5-reduced PQQ; (E) PQQH2.

Just like for other types of NAD(P)-independent, dye-linked dehydrogenases, the catalytic cycle of PQQ-containing dehydrogenases proceeds according to a ping-pong mechanism. So far, the best studied quinoprotein enzyme was methanol dehydrogenase (EC 1.1.99.8). However, although additional details of its kinetic mechanism (7) and the 3-D structure of its reduced form (8, 9) are known, the chemical mechanism of alcohol oxidation by this enzyme is still obscure. Progress in this is hampered by the following difficulties: the instability of the oxidized form of methanol.
dehydrogenase (10); the presence of endogenous substrate in enzyme preparations and of alcohols/alddehydes in buffer solutions (11); the still not understood role of NH₃ as activator in the catalytic mechanism of the enzyme (1). Since these difficulties are not met with sGDH, it seems an ideal model enzyme to study the mechanism of POQ-containing dehydrogenases. In addition, since sGDH has large potentials for analytical applications (5, 12), insight into its substrate and anomer selectivity is highly desirable.

The oxidized and the reduced enzyme forms (containing POQH₂, pyrroloquinoline quinol, Figure 1B) of sGDH have been isolated (2), the properties and mechanism of formation being in line with a ping-pong mechanism for the enzyme. The results of steady-state kinetic investigations on sGDH supported this but also revealed some complicating factors: depending on the electron acceptor used, a different optimal pH was found (2, 4); substrate inhibition occurs (3, 4, 13, 14) (i.e., the sugar competes with electron acceptor for interaction with the reduced enzyme form); negative cooperative behavior is observed in the oxidation of sugars in the higher concentration ranges (14, 15). To obtain more insight into this and to increase our knowledge on the mechanism and selectivity of sGDH, the first part of the catalytic cycle, i.e., the reductive half-reaction, was investigated. This was carried out in two ways: the stepwise reaction in which oxidized enzyme is titrated with sugar (anomers); the fast reaction in which oxidized enzyme is rapidly mixed with an excess of sugar. To detect any intermediate and to determine the reaction rates, changes in the status of POQ were monitored in time with ultraviolet/visible and fluorescence spectroscopy.

**EXPERIMENTAL PROCEDURES**

**Apo- and Holo-sGDH Preparations.** Apo-enzyme was isolated from an Escherichia coli recombinant strain containing the gene for sGDH from Acinetobacter calcoaceticus (16) as described (2). Holo-sGDH was prepared by adding excess POQ to the apo-enzyme, in the presence of Ca²⁺ ions, followed by gel filtration to remove unbound POQ, as described (6).

**Spectrophotometry.** Ultraviolet/visible absorption spectra were measured with a Hewlett-Packard 8524A photodiode array spectrophotometer. When necessary, O₂ was removed from the samples by sparging with N₂. During the measurements and the addition of anaerobic glucose solution, the cuvettes were kept anaerobic by passing N₂ above the sample.

**Steady-State Kinetics.** Initial reaction rates were determined spectrophotometrically at 20 °C, as described previously (2), by monitoring the reduction of DCTP, using PMS as the primary electron acceptor. Based on the assumption that the observed negative cooperative behavior is due to subnet interaction (15), the dependence of the steady-state rate, ν, on substrate concentration, [A], is described by

$$v = \frac{[A]^2 k_{cat2} + [A] k_{cat1} K_2}{[A]^2 + [A] K_2 + K_1 K_2}$$

where $k_{cat1}$ and $K_1$, and $k_{cat2}$ and $K_2$ are sets of apparent kinetic parameters representing the enzyme's catalytic (Michaelis–Menten) performance at low and high sugar substrate concentration ranges, respectively (17, 18). The specificity constant, defined as $k_{cat}/K_M$, was determined here from the initial slope in a plot of rate versus substrate concentrations (low range), represented by $k_{cat}/K_1$.

**Stopped-Flow Kinetics of the Reductive Half-Reaction.** Stopped-flow experiments were performed by rapid mixing in a Hi-Tech Scientific SF53 apparatus and monitoring the absorbance at 337 nm (this wavelength giving the maximal difference in absorbance between oxidized and reduced sGDH). Experiments in which fluorescence or multiple wavelength absorbance changes were monitored were carried out with an Applied Photophysics SX.17MV stopped-flow reaction analyzer. All experiments were performed at 15°C.

**Mathematical Analysis of Stopped-Flow Trace.** The reductive half-reaction was assumed to proceed according to the following scheme:

**Scheme 1**

$$E + S \xrightleftharpoons{k_e}{k_i} ES \xrightarrow{k_i} E'P$$

where E and E' represent the oxidized and reduced enzyme form, respectively. The formation of E'P from ES is considered to proceed in an irreversible way. This assumption is based on the large difference in redox potential between the couples glucose/glucono-δ-lactone [$E^{\circ} = -364$ mV (19)] and POQ/POQH₂ [$E^{\circ} = +50$ mV, in sGDH (20)] and the observation that addition of glucono-δ-lactone did not affect reaction rates in the steady-state assay. Provided that [S] is considerably larger than [E], the formation of E'P can be described by

$$\frac{[E'P]}{[E]_0} = 1 - \frac{1}{k_a - k_b} \left( k_a e^{-k_P k_i} - k_b e^{-k_i} \right)$$

with $k_a$ and $k_b$ as the high and low observed rate constants, respectively, given by

$$k_a = \frac{1}{g} \left( p + \sqrt{p^2 - 4q} \right)$$

$$k_b = \frac{1}{g} \left( p - \sqrt{p^2 - 4q} \right)$$

with $p = k_i[S] + k_1 + k_2$ and $q = k_i[S] k_0 k_2$ (21). Note that the relative amplitudes of the exponential terms are directly related to the observed rate constants and that apparent single-exponential E'P formation, with $k_b$ as observed rate constant, will thus occur under conditions where $k_b \ll k_a$ (which depends both on the relative values of the kinetic rate constants (e.g., when $k_1[S] \ll k_2$ or when $k_i[S] \gg k_0$)). From the first derivative of eq 3b, it follows that the initial slope of $k_b$ vs [S] equals the steady-state specificity constant, $k_{cat}/K_M$, since

$$\frac{d[k_b]}{d[S]} (S = 0) = \frac{k_b k_i}{k_2 + k_3} = \frac{k_{cat}}{K_M}$$

**Fitting of Experimental Data.** Computer fitting of experimental data was carried out with the Igor Pro v. 2.02 software package (WaveMetrics Inc., Lake Oswego, OR).

**RESULTS**

**Anomer Preference of sGDH.** Titration of holo-sGDH (in the oxidized form) with glucose under anaerobic conditions
resulted in gradual reduction of the enzyme without any indication for the existence of intermediates spectroscopically distinct from oxidized or reduced enzyme (Figure 2). Since glucose dissolved in water anomerizes to an equilibrium of its two anomic forms and we wanted to establish the anomer specificity of sGDH, conversion experiments were carried out with fresh and equilibrated solutions of the two glucose anomers. Addition of a substoichiometric amount of a freshly (about 5 min earlier) prepared solution of either α-D-glucose or β-D-glucose resulted in immediate increases (i.e., within the time required to mix the sample) in absorbance at 338 nm of 8.7% and 149%, respectively, compared to that observed with the same amount of a solution of α-D-glucose which had been allowed to mutarotate to anomer equilibrium for 1 day. Furthermore, with fresh α-D-glucose, the immediate absorbance increase was followed by a slow gradual absorbance increase, which can be attributed to mutarotation of the α- to the β-anomer. Since the immediate increase in absorbance observed with the fresh solution of α-D-glucose can be well accounted for by the expected amount of β-D-glucose formed by mutarotation within 5 min (22), it is concluded that sGDH only acts on the β-anomer of D-glucose. This is in agreement with experiments carried out with an amperometric sGDH enzyme electrode (A. J. J. Olsthoorn, unpublished results), also showing that an absolute preference exists for β-D-galactose, β-D-xylose, and α-L-arabinose (aldoses all having the same configuration at the anomeric C-atom as β-D-glucose). Based on an anomic equilibrium composition for D-glucose of 37% α- and 63% β-D-pyranose (22), the following molar extinction coefficients for the catalytic center (=subunit) of sGDH were determined from the spectra shown in Figure 2: ε338 (difference of reduced minus oxidized enzyme) = 27.0 M^{-1} cm^{-1}; ε440 (reduced form) = 87.0 M^{-1} cm^{-1}; ε340 (oxidized form) = 95.9 M^{-1} cm^{-1}. Using a value of 50 kDa for the subunit molecular mass (16), the following specific absorption coefficients were calculated: 1.74 L g^{-1} cm^{-1} at 280 nm for reduced sGDH; 1.92 L g^{-1} cm^{-1} at 278 nm for oxidized sGDH.

Steady-State Kinetics with Glucose-1-d. Analogous to previous results with glucose and other substrates (15), pronounced negative cooperative behavior was observed in the steady-state kinetics of sGDH with glucose-1-d (Figure 3). The experimental data could be adequately fitted with eq 1. The apparent kinetic parameter values obtained with glucose-1-d (Table 1) differ significantly from those found with nondeuterated glucose, resulting in substantial deuterium isotope effects, k_d/k_o. Substrate inhibition, occurring with glucose at high concentrations (15), has not been taken into account in fitting the data of glucose-1-d, as it was not observed in the range of substrate concentrations used here. However, since it cannot be excluded that it occurs, some caution applies to the reliability of the values obtained for k_d and K_s.

Stopped-Flow Studies of the Reductive Half-Reaction. Upon mixing sGDH with glucose-1-d, the absorbance at 337 nm increased in a non-single-exponential manner (Figure 4), but the trace could be adequately fitted with eq 2. The latter implies that the molar absorption coefficients of E and ES must be very similar at this wavelength. To obtain the individual rate constants indicated in Scheme 1, values of k_d and k_s were determined for several concentrations of glucose-1-d (Figure 5), and the data were simultaneously fitted to eq 3a for k_d, and to eq 3b for k_s, the fits yielding the parameter values given in Table 2.

Based on the reaction sequence shown in Scheme 1, a calculated absorption spectrum (Figure 6) of the intermediate species in this was obtained by analyzing the absorbance changes of sGDH in the range from 260 to 410 nm, caused by rapid mixing of the enzyme with glucose-1-d (0.1 mM),

![Figure 2: Absorption spectra taken during titration of sGDH with glucose. To sGDH (0.5 mg/mL) in 20 mM potassium phosphate buffer, pH 7.0 (spectrum a), were added 5 aliquots of glucose solution (prepared 1 day in advance as to be in anomic equilibrium) with 30 s intervals, and spectra were taken between the steps. Finally excess glucose (90 µM final concentration) was added (spectrum b). The spectra were corrected for dilution (up to 3%) caused by the additions. The inset shows the difference spectrum obtained by subtraction of spectrum a from spectrum b.](image1)

![Figure 3: Eadie-Hofstee plot for sGDH with varying concentrations of glucose-1-d. Enzyme activities were measured as described under Experimental Procedures. The solid curve represents the fit of the experimental data with eq 1.](image2)

| Table 1: Apparent Kinetic Parameter Values of sGDH for Glucose-1-d and for Glucose As Determined with Steady-State Kinetics* |
|---|---|---|
| | glucose-1-d | glucoseb | k_d/k_o |
| k cat (s^{-1}) | 253 ± 4 | 1550 ± 190 | 6.1 ± 0.8 |
| K_s (µM) | 171 ± 6 | 740 ± 180 | 4.3 ± 1.1 |
| k_cat (s^{-1}) | 4490 ± 610 | 17200 ± 1600 | 3.9 ± 0.6 |
| K_s (µM) | 69 ± 12 | 69 ± 12 | 1.0 ± 0.3 |
| K (µM) | 92 ± 13 | — | — |

*a Measurements were carried out as described in the legend of Figure 3, and kinetic parameter values ± SE were obtained by fitting the data in Figure 3 with eq 1.* Data taken from ref 15.
with global analysis. As it is not possible to determine $k_1$, $k_2$, and $k_3$ independently at a single substrate concentration, in this case fitting was performed with a fixed value for $k_2$ of 10.2 s$^{-1}$. The values calculated for $k_1$ and $k_3$ with global analysis were close to those found for the single-wavelength experiments (Table 2). As illustrated by the spectra in Figure 6, this approach also shows that E and ES have similar absorbances at 337 nm, justifying the use of eq 2 at this wavelength.

![Figure 3:](image1)

**Figure 3:** Absorption spectrum of sGDH with glucose-1-d.

![Figure 4:](image2)

**Figure 4:** Calculated absorption spectrum of the reaction intermediate occurring in the reduction of sGDH with glucose-1-d. Experiments were performed as described in the legend of Figure 4, at a final glucose-1-d concentration of 0.10 mM. Absorbance changes were monitored at wavelengths of 260–410 nm in 10 nm steps and at 275 nm. The data were globally analyzed with ProKinetec software (version 4.10, AppliedPhotonics Ltd), using the reaction model of Scheme 1 and a fixed value for $k_2$ of 10.2 s$^{-1}$. Spectra originate from the oxidized (---), intermediate (--), and reduced (•••) forms of sGDH.

![Figure 5:](image3)

**Figure 5:** Plot of $k_1$ and $k_2$ versus glucose-1-d concentration. Values of $k_1$ (closed circles) and $k_2$ (open circles) were obtained by fitting the monitored absorbance change at 337 nm to eq 2. Results are means ± SE of three measurements. The solid curves represent the fitting of these values to eq 3a and eq 3b, respectively, yielding the rate constant values given in Table 2.

![Figure 6:](image4)

**Table 2:** Rate Constant Values of the Reductive Half-Reaction of sGDH with Glucose-1-d.

<table>
<thead>
<tr>
<th>rate constant</th>
<th>from varying concentration</th>
<th>from multiple wavelength</th>
</tr>
</thead>
<tbody>
<tr>
<td>$k_1$ (mM$^{-1}$ s$^{-1}$)</td>
<td>1129 ± 24</td>
<td>1136 ± 7</td>
</tr>
<tr>
<td>$k_2$ (s$^{-1}$)</td>
<td>10.2 ± 2.4</td>
<td>fixed as 10.2</td>
</tr>
<tr>
<td>$k_3$ (s$^{-1}$)</td>
<td>206.4 ± 4.4</td>
<td>218.4 ± 3.2</td>
</tr>
</tbody>
</table>

*Values ± SE were obtained as indicated in the legends of Figures 5 and 6.

On monitoring the fluorescence intensity when sGDH was mixed with glucose-1-d, a transient species was observed. The trace could be adequately fitted with a general double-exponential equation (Figure 7), which yielded values for the observed rate constants similar to those found from absorbance measurements (Figure 5). Therefore, this approach is also in accordance with the reaction sequence proposed in Scheme 1, with the fluorescing transient and the transient for which the absorption spectrum was calculated (Figure 6) being identical.

At higher concentrations of glucose-1-d, the observed rate constants (data not shown) became much higher than could be expected according to the mechanism and rate constant values deduced above for the low concentration range. This phenomenon is completely in line with the negative cooperative behavior observed in steady-state measurements, the
Table 3: Specificity Constant Values of sGDH for Various Aldoses

<table>
<thead>
<tr>
<th>Aldose</th>
<th>Side group configuration</th>
<th>k_{cat}/K_{M} (mM^{-1} s^{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td>D-glucose</td>
<td>1 (d)</td>
<td>1700</td>
</tr>
<tr>
<td>1-deutero</td>
<td>2</td>
<td>2100</td>
</tr>
<tr>
<td>2-deoxy</td>
<td>2</td>
<td>1090</td>
</tr>
<tr>
<td>3-deoxy</td>
<td>3</td>
<td>1500</td>
</tr>
<tr>
<td>6-deoxy</td>
<td>4</td>
<td>3.3</td>
</tr>
<tr>
<td>D-mannose</td>
<td>A</td>
<td>2500</td>
</tr>
<tr>
<td>D-allose</td>
<td>A</td>
<td>590</td>
</tr>
<tr>
<td>D-galactose</td>
<td>A</td>
<td>50</td>
</tr>
<tr>
<td>D-lactose</td>
<td>A</td>
<td>1470</td>
</tr>
<tr>
<td>D-fucose</td>
<td>A</td>
<td>440</td>
</tr>
<tr>
<td>D-xylene</td>
<td>A</td>
<td>0.24</td>
</tr>
<tr>
<td>D-ribose</td>
<td>A</td>
<td>11</td>
</tr>
<tr>
<td>L-arabinose</td>
<td>60°</td>
<td>1450</td>
</tr>
<tr>
<td>Maltose</td>
<td>α-gluc</td>
<td>800</td>
</tr>
<tr>
<td>Cellobiose</td>
<td>β-gluc</td>
<td>1080</td>
</tr>
<tr>
<td>Lactose</td>
<td>β-gal</td>
<td>790</td>
</tr>
<tr>
<td>Melibiose</td>
<td>α-gal</td>
<td>960</td>
</tr>
</tbody>
</table>

Values were calculated using eq 4 from observed rate constants for enzyme reduction obtained from stopped-flow measurements at three different substrate concentrations. Schematic presentation of substrate structure as compared to β-D-glucose (Figure 8): %, percentage of β-D-pyranose anomer present at amino acid equilibrium (22, 34); H, hydrogen instead of hydroxyl group (deoxyaldoses); A, configuration of hydroxyl group axial instead of equatorial; X, hydrogen instead of hydroxymethyl group (pentoses); α, gal, β-glucose- or β-D-galactopyranose instead of hydroxyl group (disaccharides). Apparent specificity constants based upon total substrate concentration, i.e., disregarding the existence of different (ratio of) anomeric forms. Data taken from ref 15. α-Pyranose anomer; pentose nomenclature is based on the configuration at C₅, causing the pentose analogue of β-D-galactopyranose to be named α-L-arabinopyranose.

Figure 8: Stereochemical structure of D-glucose. The hydrogen which is deuterated in D-glucose-1-d is represented in boldface type.

Figure 9: Dependence on the pH of k_{cat} for the reduction of sGDH with glucose. Experiments were performed as described in the legend of Figure 3, at a final glucose concentration of 0.20 mM, in 50 mM of the following buffers: citrate, pH 5.0; MES, pH 6.0; potassium phosphate, pH 7.0; CHES, pH 9.0; and CAPS, pH 10.0. The values of k_{cat} were calculated by fitting of the absorbance increase at 337 nm to a general single-exponential equation.

Discussion

Specificity. The difference molar absorption coefficient for glucose-reduced minus oxidized enzyme at 338 nm of 27.0 mM⁻¹ cm⁻¹ corresponds fairly well with the value of 24.4 mM⁻¹ cm⁻¹ found by Hauge (23). However, in our case, the calculation is based on an absolute preference of
the enzyme for $\beta$-D-glucose whereas that of Hauge is not because he observed that all added glucose reacts with the enzyme. This suggests that under his conditions $\alpha$-D-glucose mutarotated almost completely to the $\beta$-anomeric form in the time span between glucose addition and measurement of the resulting spectral change, although the cause of this rapid mutarotation is not clear and the phenomenon was not observed in our experiments.

Based on a previously obtained value for the specific absorption coefficient for reduced sGDH of 1.48 L g$^{-1}$ cm$^{-1}$ by ultraviolet-visible spectroscopy, it was calculated that holo-sGDH contains 2.3 Ca$^{2+}$ ions per subunit (6). Recalculation, making use of the value for the specific absorption coefficient derived here (1.74 L g$^{-1}$ cm$^{-1}$), yields 2.7 Ca$^{2+}$ ions per subunit. Therefore, further research is required to establish the correct number of Ca$^{2+}$ ions per subunit molecule in holo-sGDH.

In comparing the specificity constant data determined with steady-state and stopped-flow experiments (Table 3), it appears that both methods yield fairly similar values (note that the values obtained with the former method are nearly always somewhat higher than those obtained with the latter one, which is most probably due to the higher temperature at which the steady-state experiments were performed). Since the $k_{cat}$ (and thus $k_{j}$) values for the substrates tested were the same (15), differences in specificity constant values determined here must concern the performance of the substrate in the process leading to the fluorescent intermediates formation (related to $k_{j}$ and $k_{s}$). Thus, comparison of the specificity constants of the various substrates (Table 3) yields qualitative insight into the structural requirements posed by the active site of sGDH toward the substrate. The relatively low values observed with 2-deoxy-D-glucose, mannose, and lyxose and the high values found with 3-deoxy-D-glucose and allose indicate that the C$_4$-hydroxyl group of the substrate plays an important role in this process, whereas the C$_2$ substituents do not. The values for the disaccharides show that equatorial pyranosyl substituents at C$_4$ decrease the relative specificity by about a factor of 2, irrespective of the precise nature of the pyranose, suggesting that decreased relative specificities are due to specific hindering of the interaction between the enzyme and the glucose moiety of the substrate. Interpretation of the results with other substrates differing from glucose with respect to their C$_2$ and/or C$_4$ substituents is less straightforward. The data seem to indicate a subtle interplay between C$_4$-hydroxyl and C$_2$-(hydroxy)methyl groups: an axial C$_2$-(hydroxy) group can apparently compensate for a missing C$_2$-(hydroxymethyl) group (high value with arabinose compared to xylose) whereas the presence of both groups has an adverse effect (low value with galactose), and as suggested by the data for 6-deoxy-D-aldooses, the position of the C$_2$-(hydroxy) group is irrelevant for the process (similar values for 6-deoxy-D-glucose and fucose = 6-deoxy-D-galactose).

In assays for sGDH, the pH optimum appears to vary significantly with the artificial electron acceptor used, with reported values of pH 6 for DCIP (3), pH 7 for PMS/DCIP (2), and pH 9 for Wurster's Blue (4). This variation may be due to the cationic or anionic nature of the dye and to different amino acids residues involved in the reaction between dye and reduced enzyme, the residues exhibiting different pK$_a$ values. The results reported here show that the reductive half-reaction exhibits an optimum around pH 7.0, indicating that reoxidation of reduced sGDH with electron acceptor is rate-limiting for DCIP and Wurster's Blue but not for PMS (the assay with the latter has a pH optimum of 7. and $k_{cat}$ and $k_{j}$ are similar).

**Kinetic Mechanism.** The similar values of $k_{cat}$ (Table 1) and $k_j$ (Table 2) for glucose-1-4 indicate that the maximal attainable steady-state turnover rate (in the absence of the negative cooperativity effect) is mainly determined by the rate of the step in which reduced sGDH is formed (the small difference between the parameter values is probably due to the different temperatures at which they were determined). Thus, to explain the much higher value of $k_{cat}$, it must be this step which is primarily affected by the mechanism underlying the cooperative behavior of sGDH. Whether this means just a removal of the barriers causing the rate-limitation or a complete change in mechanism remains to be elucidated.

The steady-state kinetic parameters obtained with glucose and glucose-1-4 revealed a deuterium isotope effect of about 6 on $k_{cat}$. In view of the reasoning given above, in fact the effect is exerted on $k_j$. Since hydrogen transfer reactions (which may concern a proton, a hydrogen radical, or a hydride ion) generally exhibit a primary isotope effect in the range of 6-10 (24), such a transfer may occur in the $k_{j}$-related reaction leading to reduced sGDH.

The isotope effect on the specificity constant for glucose can be used to establish whether isotope effects occur only in the step associated with $k_j$ or also in the previous steps connected with $k_1$ and $k_2$. When only $k_j$ shows an intrinsic isotope effect, the observed isotope effect on the specificity constant can be given (25) by

$$
\left(\frac{k_{cat}}{k_{cat}^0}\right)_D = \frac{k_{jD}}{k_{jH}} \left(\frac{k_1 + k_2}{k_1k_2} = \frac{1 + k_{jD}k_{jH}}{k_{jD}k_{jH} + k_{jD}k_{jH}}\right)
$$

where the subscripts H and D denote parameters associated with normal and deuterated glucose, respectively. From the values of $k_j$ and $k_j$ found for glucose-1-4, it follows that the isotope effect on the specificity constant in this case could not exceed the value of 1.05 (obtained at infinite $k_{jD}$). The observed value is about 1.5 (1.4 from steady-state measurements, 1.6 from stopped-flow measurements (Table 3)), indicating the presence of an isotope effect on the step(s) associated with $k_j$ and $k_j$ as well. Whether it concerns a secondary isotope effect or a primary one on a step which is scarcely rate-limiting remains to be elucidated.

Regarding the mechanistic cause of the observed negative cooperativity, the results of the stopped-flow measurements indicate that it is not due to an enzyme preparation consisting of different sGDH species, as has also been concluded from steady-state measurements (15). Reduction of sGDH with glucose by titration did not reveal enzyme forms spectrally distinct from reduced or oxidized sGDH. This means that after one of the subunits becomes reduced, either it does not affect the absorption spectrum of the other, still oxidized subunit or the affinity of the latter for glucose is so increased that enzyme molecules in which only one of the two subunits is reduced do not occur. Both the latter explanation and the one proposing that the observed negative cooperativity is due to redox-state-dependent subunit interaction (i.e., reduction of a subunit affects the kinetic properties of the other...
subunits) are unlikely since this would mean that the enzyme molecules do not behave uniformly during the course of the reaction, which was not observed in the stopped-flow measurements (at a low glucose concentration). It is suggested therefore that reduction of one of the subunits does not affect the optical or kinetic properties of the other, still oxidized subunit. This lack of communication between POQ and POQH₂ in the enzyme molecule also applies to electron transfer, as deduced from the fact that the semiquinone radical form of POQ was not detected in half-reduced preparations with EPR spectroscopy. Since the kinetic performance of sGDH at low and high substrate concentrations is clearly different, as shown by the results of steady-state kinetics (15) and here with those of pre-steady-state kinetics of glucose-1-d, substrate-occupation-dependent subunit interaction (i.e., occupation of a subunit by substrate affects the kinetic behavior of the other subunit) is the most likely mechanism responsible for the observed negative cooperativity. Whether this concerns a real Michaelis Menten complex of oxidized enzyme subunits with substrate or a situation in which one of the subunits has already reacted to an intermediate stage (e.g., the fluorescing intermediate) is not clear. However, the first possibility seems more likely since the second possibility would probably have been detected in the stopped-flow experiments with glucose-1-d.

Chemical Mechanism. POQ reacts with a large number of nucleophiles, including alcohols and aldehydes, to a fluorescing adduct at the 5-position (26) (Figure 1C). Thus, when a fluorescing enzyme species was detected in the reaction of methanol dehydrogenase with (deuterated) methanol, a catalytic catalysis mechanism in which this adduct plays a central role was proposed for the enzyme (7) (see a recent publication for possibilities to explain the chemistry leading to product formation from the adduct (27)). The results of studies on the model reaction between a POQ ester, methanol, and Ca²⁺ in an organic solvent support this view, one of the roles of the bivalent cation being ascribed to assisting in adduct formation (28). The results reported here for sGDH are compatible with such a mechanism: a reversible step(s) leading to the formation of a fluorescing intermediate in the catalytic cycle, having an absorption spectrum (Figure 6) compatible with that of POQ adducts (26), the steps being rather insensitive to the deuterium isotope effect exerted by glucose-1-d; a subsequent, irreversible step in which the transfer of reducing equivalents takes place, being prone to the deuterium isotope effect. Also other properties are in line with this: the very low turnover number for glucose of Holo-X, an enzyme form of sGDH in which the "assisting Ca²⁺ ions" are lacking (6), but for which no fluorescing intermediate is observed in the catalytic cycle (A. Dewanti, unpublished results), indicating that the low turnover of this enzyme form could be due to the rate-limiting step of adduct formation between POQ and substrate; the insensitivity of the maximal turnover rate and the UV–Vis spectrum of sGDH (oxidized or reduced) to substitution of Ca²⁺ by Sr²⁺, Cd²⁺, or Mn²⁺ (6), indicating that although POQ is a ligand for the metal ions, the variation in properties of the metal ions does not affect the properties of POQ in sGDH. Although the catalytic catalysis mechanism is generally accepted, the crucial evidence for it is still lacking: structural data for a POQ–substrate adduct in the dehydrogenases are unavailable (the only indication in favor for this is the similarity of the fluorescence spectra of the intermediate with those of the genuine adducts formed between POQ and a nucleophile in solution); it is unknown in which step product release takes place. In view of the uncertainty, could an alternative chemical mechanism be compatible with the kinetic mechanism?

Based on stereoelectronic considerations, the anomeric specificity of aldose oxidoreductases has been predicted (29). Assuming that hydride removal from the C₁-atom of an aldopyranose takes place, the theory predicts that an optimally efficient catalyst should have an absolute specificity for the β-anomer of glucose, as is the case for NAD-dependent glucose dehydrogenase (30) and glucose oxidase (31). Since it appears now that sGDH is no exception to this rule, the question can be posed whether the aforementioned assumption also applies to this enzyme. It is generally accepted that oxidation of a CH(OH) moiety in a substrate by NAD(P)-dependent dehydrogenases proceeds via hydride transfer from the C₁-atom to the electrophilic site in the coenzyme, i.e., the 4-position in NAD(P). In one of the hypotheses, the catalytic Zn²⁺ is presumed to facilitate deprotonation of the OH group, enabling hydride transfer to take place. Extrapolating such a mechanism to quinoprotein dehydrogenases, Ca²⁺ could fulfill the assisting role (either by deprotonation of the OH at the 1-position or by polarizing the oxygen of the C₁-carbonyl group) so that hydride transfer can occur to the most electrophilic site in POQ, i.e., the C₂-atom [without bringing this in line with experimental results, such a step has already been put forward by Anthony et al. (32) as a possible mechanism for methanol dehydrogenase]. Applying this to sGDH, the C₃-reduced POQ (Figure 1D) should be equivalent to the observed fluorescing intermediate species, and the rate-limiting step should concern irreversible taumORIZATION of the C₃-reduced POQ to the energetically more favorable reduced form, POQH₂ (in view of the structural resemblance to the POQ–H₂O adduct (Figure 1C (R=H)) and 4,5-dihydro-POQH₂ (POQH₂) (Figure 1E), the similarity of the fluorescence spectra of C₃-reduced POQ to those of POQ adducts is not unlikely; to explain the formation of POQH₂ upon reducing POQ with NaBH₄, the assumption that C₃-reduced POQ exists is also required (33)]. Since the rate-limiting step in the hydride transfer mechanism does not concern the actual oxidation of substrate but the taumorization of C₃-reduced POQ to POQH₂, this mechanism can easily explain why the maximal oxidation rates for the sugars are the same. In the catalytic catalysis mechanism, including the one proposed in which the hydride is transferred to the oxygen of the C₁-carbonyl group (27), it has to be assumed that the nature of the sugar in the adduct complex is irrelevant for the rate-limiting step in the internal redox chemistry with which the complex is decomposed into aldololactone and POQH₂. On the other hand, in the hydride transfer mechanism, the low deuterium isotope effect exerted on the process in which actual hydride removal from the substrate takes place is somewhat curious. To explain this, it has to be assumed that hydride removal is not rate-limiting but another step in this process is. Summarizing, the kinetic mechanism as reported here for sGDH is compatible with both chemical mechanisms discussed above, and no strong arguments in favor of one of them can be put forward at the moment.
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Structural and mechanistic aspects of soluble quinoprotein glucose dehydrogenase from *Acinetobacter calcoaceticus*

**Summary**

This thesis describes investigations on the structural and catalytic properties of the soluble glucose dehydrogenase (sGDH) from *Acinetobacter calcoaceticus*. This enzyme contains the cofactor pyrroloquinoline quinone (PQQ) and thus belongs to the quinoproteins. It catalyses the oxidation of glucose and other aldose sugars to the corresponding aldono-δ-lactones, with a variety of artificial electron acceptors. Interest in sGDH stems on the one hand from its dependence on the uncommon cofactor PQQ and on the other hand from its potential applicability in the determination of glucose (which is of importance in the fields of medicine, biotechnology, and food technology).

The availability of a recombinant *Escherichia coli* strain containing the sGDH-gene from *A. calcoaceticus* allowed for the isolation of the enzyme in the apo-form (i.e. without the cofactor PQQ), as described in Chapter 2. The apoenzyme is a homo-dimer, like the active holoenzyme, which is readily formed from the apoenzyme upon addition of PQQ in the presence of calcium ions. Dissociation of the apoenzyme to the monomeric form is achieved by incubation at temperatures above 35°C in the presence of a chelating agent, and is reversed by the subsequent addition of Ca²⁺. In the absence of free Ca²⁺, PQQ binds to the dimeric apoenzyme (but not to the monomer) to yield an enzyme form which exhibits spectral properties differing from the normal holo-enzyme, and which becomes reduced only very slowly upon addition of substrate and is apparently inactive in the standard assay. This enzyme form can subsequently be converted into active holo-sGDH by the addition of Ca²⁺, requiring 1 calcium ion per subunit (see Chapter 3). Thus it is concluded, that Ca²⁺ fulfills two different roles, one in dimerization and one in enzyme activation. The Ca²⁺ involved in activation has a distinctive effect on the absorbance spectrum of the enzyme, suggesting that it coordinates to PQQ in a similar fashion as found for PQQ-dependent methanol dehydrogenase. Although titration of monomer with Ca²⁺ in the presence of a stoichiometric amount of PQQ and atomic absorption spectrometry of holo-sGDH initially appeared to indicate that two Ca²⁺-ions per subunit are required for the formation of fully active holo-sGDH (see Chapter 3), re-evaluation of the determination of the enzyme concentration suggests that this value may be too low (see Chapter 5). Cd²⁺, Mn²⁺ and Sr²⁺ also cause dimerization as well as activation. The replacement of Ca²⁺ by these other metal ions affects the catalytic behaviour of the enzyme in the standard assay only slightly.

Previous investigations of the kinetics of sGDH have indicated that its catalytic action proceeds via a ping-pong mechanism, involving oxidation of substrate accompanied by reduction of the PQQ to the quinol-form and release of the oxidized product prior to reoxidation of the enzyme by the electron acceptor, which is accompanied by mutual substrate inhibition. Chapter 4 describes steady state kinetic experiments performed under conditions that yield high turnover rates and disfavour the occurrence of substrate inhibition as much as possible. It is found that the kinetic behaviour of sGDH, while basically obeying the kinetic mechanism
described above, is further complicated by pronounced negative cooperativity with respect to the concentration of the sugar substrate, which is possibly due to interactions between the subunits. The observed behaviour is adequately described by an equation accounting for biphasic cooperativity, yielding two sets of apparent kinetic parameters, $V_1$ & $K_1$ and $V_2$ & $K_2$, representing the enzyme's $V_{max}$ and $K_M$ under non-cooperative and cooperative conditions, respectively. The cooperativity effect dramatically influences the performance of sGDH, as reflected by the $V_2$ and $K_2$ values for glucose, that are about 10-fold and 100-fold higher than the $V_1$ and $K_1$ values, respectively. Comparison of the kinetic parameters found with various sugars shows that $V_1$ is fairly independent of the identity of the substrate, suggesting that substrate structure has only a minor influence on the turnover process under non-cooperative conditions. Substitution of Ca$^{2+}$, involved in activation of the enzyme, by Sr$^{2+}$, leads to an increase in $K_2$ but has only a small effect on the other kinetic parameters.

The change of the absorbance spectrum of sGDH, that occurs when it is reduced by substrates, was exploited to study the substrate specificity of sGDH and the transient kinetics of the enzyme-reducing half-reaction (see Chapter 5). sGDH is found to react with only the $\beta$- anomers of a wide range of aldoses exhibiting a $\text{C}_4$-chair conformation, i.e. $\beta$-D-glucose and its structural homologs. Specificity constants obtained for various substrates indicate that enzyme-substrate-interaction is considerably influenced by the nature and configuration of substituents at the 2-position of the aldose molecule, but not at the 3-position, while equatorial pyranose-groups at the 4-position only hinder but do not prevent binding in the reaction of the enzyme with disaccharides.

The high catalytic activity of sGDH precluded a more detailed study of the enzyme-reducing half-reaction by transient kinetic measurements with normal aldoses. This could however be achieved by using C1-deuterated-glucose, due to a significant deuterium isotope effect on the maximum turnover rate. The reaction is found to proceed via the transient formation of a fluorescent intermediate, followed by an irreversible step, leading to the reduced enzyme, that involves C-H bond breaking and determines the overall rate of the complete reaction under non- cooperative conditions. Unfortunately, also with C1-deuterated glucose, the rates occurring under cooperative conditions were too fast to be measured by stopped flow measurements. The observed transient kinetics are compatible with two different mechanisms of action originally proposed for PQQ-dependent methanol dehydrogenase: either a mechanism involving the formation of a C5-hemiketal adduct between PQQ and the substrate, or one comprizing hydride transfer to the PQQ-C5 atom followed by enolisation to the quinol form. Additional research is needed to discriminate between these two possibilities.
Structurele en mechanistische eigenschappen van niet-membraan-bonden chinoproteïne glucose dehydrogenase uit *Acinetobacter calcoaceticus*

Samenvatting

Dit proefschrift beschrijft onderzoek aan de structurele en mechanistische eigenschappen van het niet membraan-bonden glucose dehydrogenase (sGDH) uit *Acinetobacter calcoaceticus*. Dit enzym bevat de cofactor pyrrolochinoline chinon (PQQ) en behoort derhalve tot de groep der chinoproteïnen. Het katalyseert de oxidatie van glucose en andere aldose-suikers tot de bijbehorende aldono-δ-lactons, met verschillende artificiële electron-acceptoren. Interesse in de eigenschappen van sGDH stoelt enerzijds op de afhankelijkheid van dit enzym van de ongewone cofactor PQQ, en anderzijds op de mogelijke toepasbaarheid ervan in methoden ter bepaling van glucose concentraties (welke hun toepassing vinden in gezondheidszorg, biotechnologie en levensmiddelentechnologie).

De beschikbaarheid van een *Escherichia coli* stam voorzien van het gen voor sGDH uit *A. calcoaceticus* maakte het mogelijk het enzym in de apo-vorm (d.w.z. zonder de cofactor PQQ) te verkrijgen, op de wijze beschreven in hoofdstuk 2. Dit apo-enzym is een homo-dimeer, evenals het actieve holo-enzym, wat zich bij toevoeging van PQQ in de aanwezigheid van calcium ionen hieruit spontaan vormt. Dissociatie van het apo-enzym tot monomeer treedt op bij incubatie bij temperaturen boven 35°C in de aanwezigheid van chelator en reassociatie tot dimeer vindt vervolgens plaats bij toevoeging van Ca²⁺. PQQ bindt, in de afwezigheid van vrij Ca²⁺, aan het dimere apo-enzym (maar niet aan de monomere vorm), onder vorming van een enzym vorm met spectrale eigenschappen die afwijken van die van het normale holo-enzym. Deze afwijkende vorm wordt slechts zeer traag gereduceerd bij toevoeging van substraat en vertoont geen meetbare activiteit in de standaard assay. Toevoeging van Ca²⁺ hierna leidt tot vorming van actief holo-sGDH, waarvoor 1 calcium-ion per subunit nodig is (zie hoofdstuk 3). Hieruit volgt dat Ca²⁺ in sGDH twee verschillende rollen vervult, één bij de dimerisatie en één bij enzymactivering. De spectrale veranderingen waarmee de omzetting naar de actieve vorm gepaard gaan, suggereren dat de hierbij betrokken Ca²⁺ coördineert aan PQQ, op een vergelijkbare manier als gevonden in PQQ-afhankelijk methanol dehydrogenase. Hoewel verschillende bepalingen aanvankelijk duidden op een totaal aantal van 2 calcium-ionen per subunit (zie hoofdstuk 3) wees een andersoortige bepaling van de enzymconcentratie erop dat deze waarde mogelijk te laag is (zie hoofdstuk 5). Cd²⁺, Mn²⁺ en Sr²⁺ kunnen Ca²⁺ vervangen bij zowel de dimerisatie als de activering, zonder dat dit leidt tot grote veranderingen in katalytische activiteit van het enzym in de standaard assay.

Eerder kinetisch onderzoek aan sGDH heeft uitgewezen, dat het katalytische proces verloopt via een ping-pong mechanisme, waarbij eerst oxidatie van het substraat plaatsvindt gepaard gaand met reductie van het enzymgebonden PQQ tot de chinolvorm (PQQH₂), gevolgd (na dissociatie van het product) door reductie van de electron-acceptor gepaard aan reoxidatie van het PQQH₂. Verder treedt wederzijdse inhibietie op door substraat en electron-acceptor. Metingen van de steady state kinetiek, onder condities waarbij een hoge omzettingssnelheid wordt bereikt
en waarbij substraat-inhibitié zich zo min mogelijk manifesteert, staan beschreven in hoofdstuk 4. Hieruit komt naar voren dat het kinetische gedrag van sGDH, hoewel globaal verlopend via het boven beschreven mechanisme, wordt gecompliceerd door een duidelijke negatieve coöperativiteit, met betrekking tot de afhankelijkheid van de suiker-substraat concentratie, wat mogelijk wordt veroorzaakt door het optreden van interactie tussen de subunits. Het waargenomen gedrag kan adequaat worden beschreven met een vergelijking voor bifasische coöperativiteit, welke twee sets van apparent kinetische parameters levert, respectievelijk \( V_1 \) en \( K_1 \) en \( V_2 \) en \( K_2 \), die de maximale omzettings snelheid \( V_{\text{max}} \) en Michaelis-constante \( K_M \) van het enzym vertegenwoordigen onder respectievelijk non-coöperatieve en coöperatieve omstandigheden. Het coöperativiteits-effect heeft een drastische invloed op de prestaties van sGDH, wat wordt weerspiegeld door het respectievelijk 10- en 100-voudig groter zijn van \( V_2 \) en \( K_2 \) ten opzichte van \( V_1 \) en \( K_1 \). Vergelijking van kinetische parameters bepaalde met verschillende suikers toont aan dat \( V_1 \) vrij onafhankelijk is van de identiteit van het substraat, wat suggereert dat de verschillen in de structuren van deze substraten maar een geringe invloed hebben op de omzettings-stap onder non-coöperatieve omstandigheden. Substitutie van \( \text{Ca}^{2+} \), betrokken bij activering van het enzym, door \( \text{Sr}^{2+} \), leidt tot een verhoging van \( K_2 \), maar heeft slechts een gering effect op de andere parameters.

De verandering van het absorptie-spectrum van sGDH, welke optreedt wanneer het enzym wordt gereduceerd door toevoeging van substraat, is in hoofdstuk 5 benut om de kinetiek van de enzym-reducerende halfreactie nader te bestuderen en om de substraat-specificiteit van sGDH te bepalen. sGDH reageert met de \( \beta \)-anomere vormen van aldoses met een \( { }^4\text{C}_{1} \)-chair conformatie, d.w.z. met \( \beta \)-D-glucose en andere suikers met een homologe structuur. Vergelijking van de specificiteitsconstanten voor verschillende substraten tonen aan dat de interactie tussen enzym en substraat sterk wordt beïnvloed door de aard en configuratie van zitgroepen op de 2-positie van het aldose molecuul, maar niet op de 3-positie, terwijl equatoriale pyranosyl-groupen op de 4-positie geen specifieke invloed hebben op de interactie van het enzym met disaccharides.

De hoge katalytische activiteit van sGDH (zelfs onder non-coöperatieve condities) verhinderde diepgaandere analyse van de enzym-reducerende halfreactie via meting van de transiënt kinetiek met gewone substraten. Dit bleek echter wel mogelijk met C1-gedeutereerd glucose, vanwege het optreden van een significat isoop-effect op de maximale omzettingssnelheid. De reactie blijkt te verlopen via de transiënte vorming van een fluorescente intermediair, gevolgd door een irreversible stap naar het gereduceerde enzym, waarbij een C-H-binding wordt verbroken en die de overall omzettingssnelheid van de complete reactie onder niet-coöperatieve omstandigheden bepaalt. Ook met C1-gedeutereerd glucose zijn de snelheden onder coöperatieve condities te snel voor bepalingen van de transiënt kinetiek. Het waargenomen gedrag kan worden verklaard met twee verschillende reactie-mechanismes, welke oorspronkelijk zijn voorgesteld voor PQQ-afhankelijk methanol-dehydrogenase: hetzij een mechanisme verlopend via de vorming van een C5-hemi-ketaal-adduct van PQQ met substraat, of een waarbij hydride-transfer van de substraat-C1 naar de PQQ-C5 plaatsvindt, gevolgd door enolisatie tot PQQH₂. Welke van de twee de juiste is, dient te worden bepaald door verder onderzoek.
Curriculum vitae

Nawoord

Het tot stand komen van dit proefschrift is voor mij uiteraard zeer belangrijk. Wel ben ik me bewust van het feit dat in het geheel van de wetenschap deze bijdrage niet meer is dan een heel klein blaadje aan een reusachtige boom of een enkele druppel in het water van de wereldzeeën. Als het deze kwalificering al waard is!

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