Structure and mechanism of soluble quinoprotein glucose dehydrogenase

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Soluble glucose dehydrogenase (s-GDH; EC 1.1.99.17) is a classical quinoprotein which requires the cofactor pyrroloquinoline quinone (PQQ) to oxidize glucose to gluconolactone. The reaction mechanism of PQQ-dependent enzymes has remained controversial due to the absence of comprehensive structural data. We have determined the X-ray structure of s-GDH with the cofactor at 2.2 Å resolution, and of a complex with reduced PQQ and glucose at 1.9 Å resolution. These structures reveal the active site of s-GDH, and show for the first time how a functionally bound substrate interacts with the cofactor in a PQQ-dependent enzyme. Twenty years after the discovery of PQQ, our results finally provide conclusive evidence for a reaction mechanism comprising general base-catalyzed hydride transfer, rather than the generally accepted covalent addition-elimination mechanism. Thus, PQQ-dependent enzymes use a mechanism similar to that of nicotinamide- and flavin-dependent oxidoreductases.
Keywords: glucose dehydrogenase/hydride transfer/PQQ/ reaction mechanism/X-ray structure

Introduction

Quinoproteins constitute a class of dehydrogenases distinct from the nicotinamide- and flavin-dependent enzymes (Duine and Frank Jzn, 1981). They use one of four different quinone cofactors (Salisbury *et al*., 1979; Janes *et al*., 1990; McIntire *et al*., 1991; Wang *et al*., 1996) to convert a vast variety of alcohols and amines to their corresponding aldehydes/lactones. Proteins containing the cofactor pyrroloquinoline quinone (PQQ; Figure 1) form the best-characterized and largest quinoprotein subclass (Duine, 1991), of which methanol dehydrogenase (MDH) and glucose dehydrogenase (GDH) are the classic examples (Duine *et al*., 1979, 1980; Salisbury *et al*., 1979). Two different GDHs have been identified in *Acinetobacter calcoaceticus*: a membrane-bound enzyme (m-GDH), which is also present in many other Gram-negative bacteria, and a soluble enzyme (s-GDH) (Cleton-Jansen *et al*., 1988; Matsushita *et al*., 1989). The two enzymes are completely different with respect to their amino acid sequences. m-GDH is partly homologous to MDH and

other PQQ-dependent enzymes. In contrast, no significant sequence identity of s-GDH to any characterized PQQ-dependent protein has been detected, although homologous sequences of uncharacterized proteins have been identified in the genomes of four other bacteria (Oubrie *et al*., 1999).

s-GDH is a basic (pI = 9.5), dimeric enzyme of identical subunits (Dokter *et al*., 1986). One monomer (50 kDa, 454 residues) binds one PQQ molecule and three calcium ions. One of the calcium ions is required for activation of the cofactor, the other two are needed for functional dimerization of the protein (Olsthoorn and Duine, 1998b; Oubrie *et al*., 1999). s-GDH oxidizes a wide range of mono- and disaccharides to the corresponding lactones (Matsushita *et al*., 1989), and it is able to donate electrons to various artificial electron acceptors, including *N*-methylphenazonium methyl sulfate (Dokter *et al*., 1986) and electroconducting polymers (Ye *et al*., 1993). The physiological electron acceptor of s-GDH is unknown, although electrons can be transferred from the reduced quinol form of PQQ (PQQH$_2$) to cytochrome b_{562} (Dokter *et al*., 1988).

s-GDH is a potent biocatalyst for the accurate monitoring of glucose in blood samples (Olsthoorn and Duine, 1996). For diabetics, such monitoring is crucial, because long-term complications of the disease can be controlled if the glucose concentration can be tightly regulated within the normal physiological range (DCCT research group, 1990). This has stimulated the development of s-GDH containing test strips (Hoenes, 1993, 1994; Hoenes and Unkrig, 1996). Nevertheless, the reliability of these strips might be improved if the protein can be engineered to be more glucose specific. For such a protein engineering project, knowledge of the three-dimensional structure of a complex of s-GDH with glucose is essential.

On the basis of the wealth of biochemical and kinetic data for MDH and s-GDH, two reaction mechanisms have been proposed for PQQ-containing enzymes (Figure 2). The first mechanism includes general base-catalyzed proton abstraction from the oxidizable hydroxyl group, followed by the formation of a covalent substrate–PQQ complex, and product elimination (Figure 2A) (Frank Jzn *et al*., 1988; Frank *et al*., 1989; Anthony, 1996; Olsthoorn and Duine, 1998b). The second possible reaction mechanism involves general base-catalyzed proton abstraction in concert with hydride transfer to PQQ, and subsequent tautomerization to PQQH$_2$ (Figure 2B) (Anthony, 1996; Zheng and Bruice, 1997; Olsthoorn and Duine, 1998b). It has not been possible to resolve which of these two mechanisms is correct, due to the lack of comprehensive structural information.

Recently, we reported the three-dimensional structure of the dimeric apo-form of s-GDH at 1.7 Å (Oubrie *et al*., 1999). Here, we present the X-ray structures of a binary complex of s-GDH with PQQ at 2.2 Å resolution, and of

a ternary complex with reduced PQQ and glucose at 1.9 Å resolution. These structures reveal the active site structure of the enzyme, indicate how glucose binds on top of the cofactor in the active site, explain the broad substrate specificity and β-anomer preference of the enzyme and provide conclusive evidence that s-GDH catalyzes the oxidation of glucose through a direct hydride transfer mechanism. Moreover, they supply a structural framework for improvement of the substrate specificity of the enzyme by protein engineering.

Results

Structure determination and quality of the models
A complex of the enzyme with PQQ bound in the active site was obtained from a soaking experiment with the cofactor. As a result of the binding of PQQ, the space group changed from $P2_1$ to $C222_1$. The structure of the holo-enzyme was therefore determined by molecular replacement, using the apo-enzyme structure as a search model. The PQQ-containing model was refined at 2.2 Å
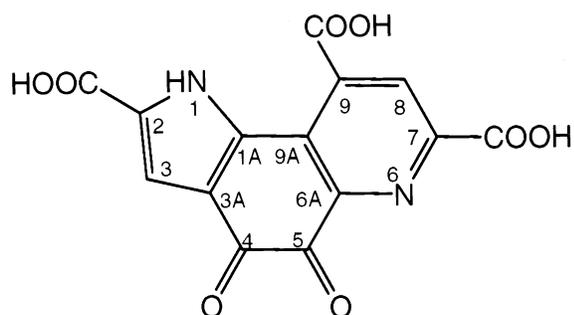
Fig. 1. Chemical structure of pyrroloquinoline quinone (PQQ). Atom nomenclature is indicated.

resolution to a crystallographic *R*-factor of 22.3% and a free *R*-factor of 28.6%. The model comprises two protein monomers (residues A1–A104, A111–A450, B1–B452), two PQQ cofactors, six calcium ions, 561 water molecules and three glycerol molecules.

The structure of the ternary s-GDH–PQQH$_2$–glucose complex was obtained by soaking a crystal of the apo-form in a PQQ-containing solution and subsequently in a solution with glucose. This crystal belonged to space group $P2_1$. Using the apo-structure as an initial model, the structure of the ternary complex was refined at 1.9 Å resolution to a crystallographic *R*-factor of 19.0% and a free *R*-factor of 22.6%. The final model comprises two protein monomers (residues A1–A104, A111–A450, B1–B104, B111–B452), two PQQH$_2$ molecules, six calcium ions, two glucose molecules and 600 water molecules.

Both structures have a good overall geometry (Table I). In each monomer of the two structures there are three systematic outliers in the Ramachandran plot (Ramakrishnan and Ramachandran, 1965): Pro248, Ser146 and Leu169. The backbone conformation of Pro248 allows the carbonyl groups of both Gly247 and Pro248 to be ligands of the active site calcium ion. The conformation of Ser146 is stabilized by several hydrogen bonds; its hydroxyl side chain is connected to the O4 atom of PQQ through a hydrogen bonding network involving Asn229. Leu169 is located at the protein surface, where it makes a hydrophobic interaction with the glucose molecule in the ternary complex. Pro266 and Tyr325 have a *cis*-peptide conformation.

Molecular architecture
Details of the structure have been described elsewhere (Oubrie *et al.*, 1999). Briefly, s-GDH is a dimeric enzyme consisting of identical subunits (Figure 3). Each monomer
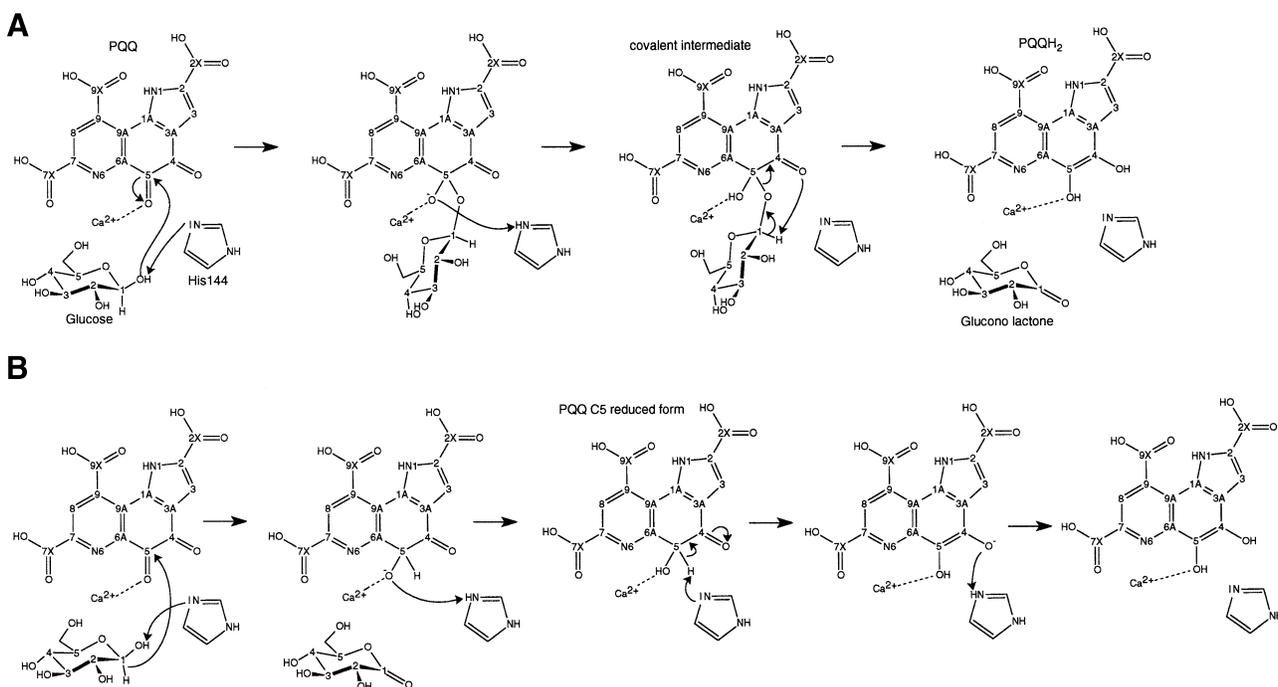
Fig. 2. The two possible reaction mechanisms for s-GDH. (**A**) The addition-elimination mechanism comprises general base-catalyzed proton abstraction followed by covalent addition of the substrate and subsequent elimination of the product. (**B**) Mechanism comprising general base-catalyzed proton abstraction in concert with direct hydride transfer from substrate to PQQ, and tautomerization to PQQH$_2$.

has a β-propeller (or superbarrel) fold composed of six 4-stranded anti-parallel β-sheets (Figure 4). These 24 β-strands form the structural core of the enzyme. The β-strands are labeled according to the β-sheet in which they occur (Figure 4, 1–6). Within each four-stranded β-sheet, the β-strands are labeled from the inside to the outside of the molecule (Figure 4, A–D). The polypeptide chain enters the β-sheet at strand A, and follows the β-sheet topology in building up a sheet from the inside

Table I. Data collection and structure refinement statistics

	Soaking experiment	
	PQQ	PQQ + glucose
Data		
X-ray source	D2AM	BW7A
Temperature (K)	100	100
Resolution (Å)	2.13	1.75
Number of unique reflections	48 649	92 032
Completeness (%)	80.4	99.8
R_{merge}[a]	6.7	6.4
Structure refinement		
Resolution (Å)	2.2	1.90
Number of unique reflections	46 479	68 659
(in test set)	(4720)	(3442)
R_{crys} (%)[b]	22.3	19.0
R_{free} (%)[c]	28.6	22.6
R.m.s. deviation from ideal bond lengths (Å)	0.007	0.008
R.m.s. deviation from ideal bond angles (°)	2.5	3.0

[a]$R_{merge}(I) = \Sigma_{hkl}\Sigma_i |I_i(hkl) - \langle I(hkl)\rangle| / \Sigma_{hkl}\Sigma_i I_i(hkl)\times100$.
[b]$R_{crys} = \Sigma_{hkl} |F_{obs, work}| - k|F_{calc}| /\Sigma_{hkl}|F_{obs, work}|\times100$.
[c]$R_{free} = \Sigma_{hkl} |F_{obs, test}| - k|F_{calc}| /\Sigma_{hkl}|F_{obs, test}|\times100$.

to the outside. The peptide chain, coming out of the outer β-strand nD, is then led via a loop into the inner β-strand (Figure 4A) of sheet n + 1. The sole exception to this rule is β-sheet 6, whose A, B and C β-strands emerge into the C-terminus, whereas the D β-strand is provided by the N-terminal region.

The β-propeller fold has been found in an ever-growing family of structurally related proteins, including neuraminidase (Varghese *et al.*, 1983), G-proteins (Wall *et al.*, 1995; Sondek *et al.*, 1996), the N-terminal domain of the heavy chain of clathrin (ter Haar *et al.*, 1998), nitrite reductase (Fülöp *et al.*, 1995) and MDH (Xia *et al.*, 1992; Ghosh *et al.*, 1995).

Calcium binding sites

s-GDH requires calcium for dimerization and function (Olsthoorn *et al.*, 1997). The present structures confirm the presence of three calcium binding sites per monomer (Oubrie *et al.*, 1999). They have *B*-factors comparable to the immediate protein environment, and seven ligating atoms in a pentagonal bipyramidal coordination at distances between 2.2 and 2.6 Å. Two calcium ions are located in the 4CD loop, keeping together sequentially distant parts of this loop. The requirement of calcium for dimerization may be explained by the necessity to rigidify this loop, which constitutes the majority of the dimerization interface (Figure 3). The third calcium ion is located in the active site, and its coordination and function will be discussed in more detail below.

PQQ binding

The cofactor resides in a deep, broad, positively charged cleft at the top of the barrel near the pseudo-symmetry
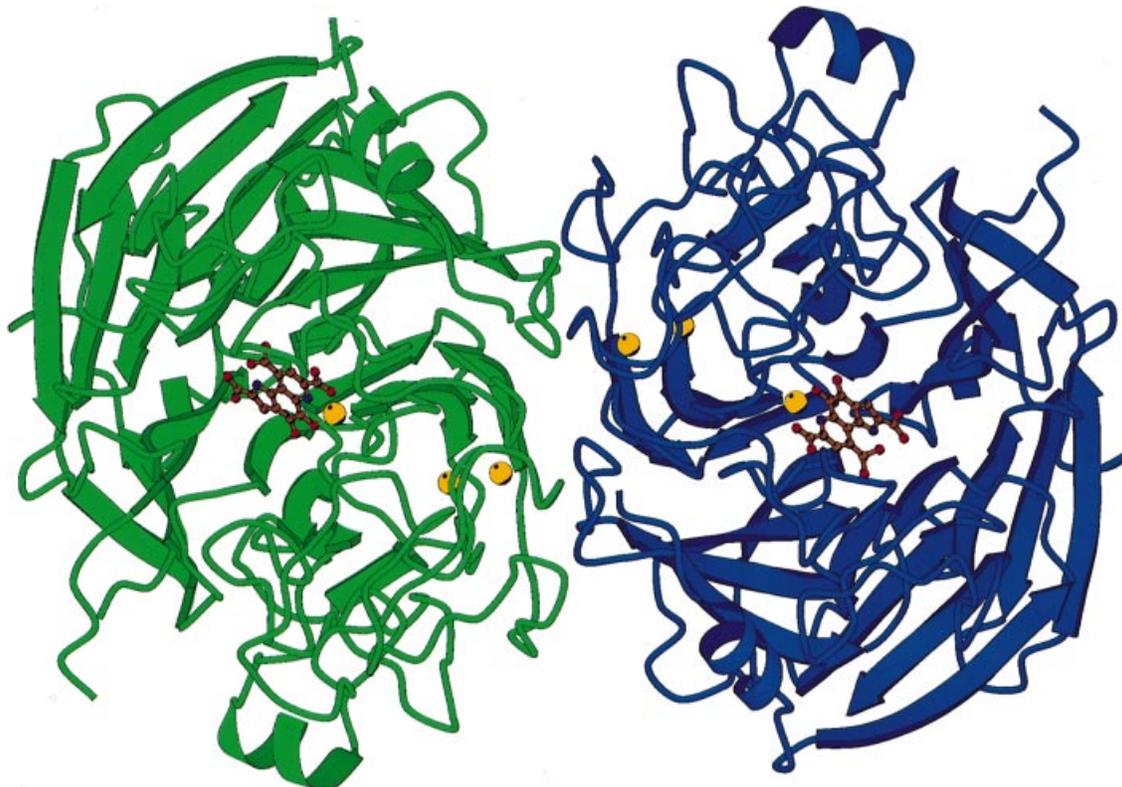
Fig. 3. Ribbon diagram of the overall structure of the dimer of s-GDH from *A.calcoaceticus*. The two monomers are shown in blue and green. Calcium ions are shown as yellow spheres. PQQ is shown in ball-and-stick representation. This picture was produced with MOLSCRIPT (Kraulis, 1991).
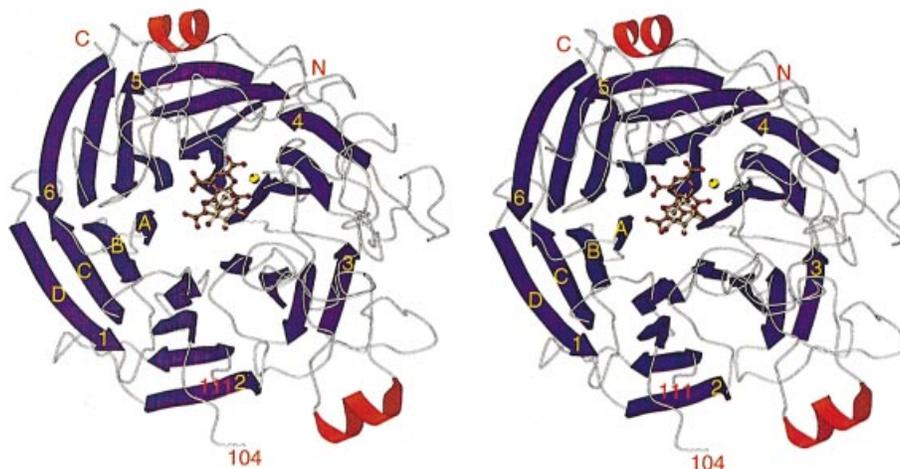
Fig. 4. Stereo view of one s-GDH monomer. β-strands are shown in blue and α-helices in red. β-sheets are numbered one to six, β-strands are labeled A to D from the inside to the outside of the protein. The N- and C-terminal residues of the model are indicated, as well as the two residues preceding and following a missing loop. Calcium ions are shown as yellow spheres. PQQH₂ and glucose are shown in ball-and-stick representation. This picture was created with MOLSCRIPT (Kraulis, 1991).
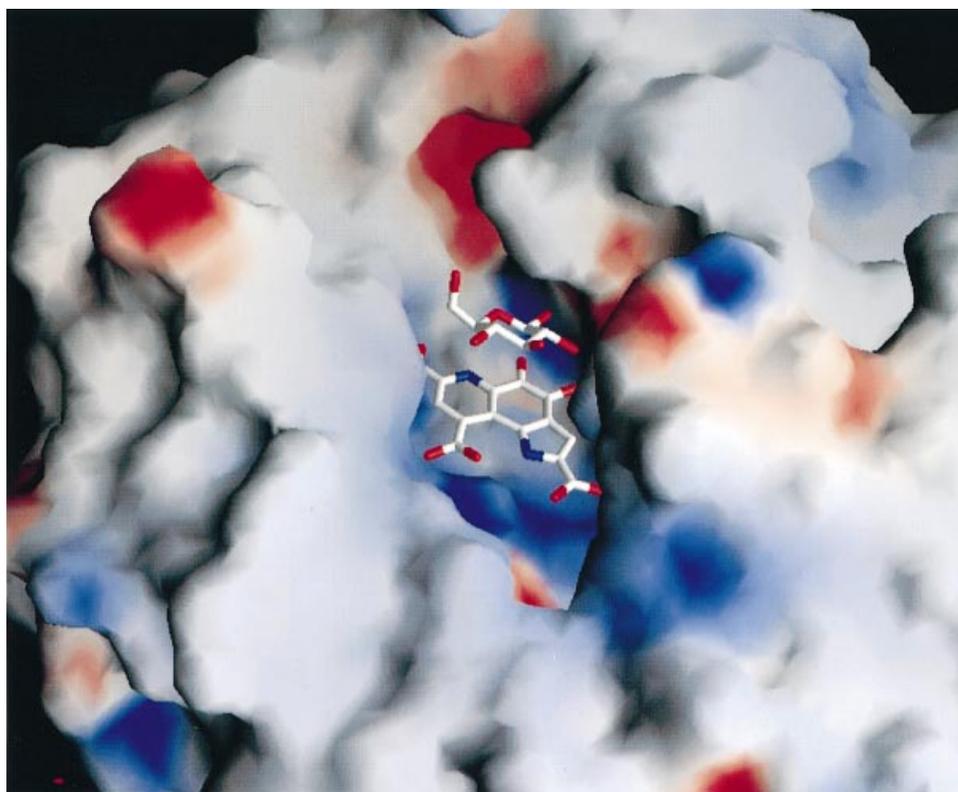
Fig. 5. Representative view of the binding of PQQH₂ and glucose to s-GDH. Surface drawing of s-GDH created using the program GRASP (Nicholls *et al.*, 1991). PQQH₂ and glucose are shown in ball-and-stick representation.

axis (Figures 4 and 5). The binding of PQQ to the protein is predominantly governed by polar interactions (Figure 6A). The C2, C7 and C9 carboxyl groups of PQQ (nomenclature explained in Figure 1) make ion-pair interactions with Arg408, Lys377 and Arg406, respectively. The *ortho*-quinone O4 and O5 atoms are bound by Asn229 and Arg228, respectively. The N6, O7A and O5 atoms of PQQ are ligands for the active site calcium ion. The other calcium ligands are provided by the two main-chain carbonyl oxygen atoms of Gly247 and Pro248, and two water molecules. The ring structure of the cofactor stacks onto a flat surface area, which is largely built up from the

side chains of Ala350, Gln246, Leu376 and Gln23. Above the plane of PQQ, no protein main and/or side chains are present to bury and shield it from the solvent.

Redox states of the cofactor in the present structures

Owing to the absence of substrates in the experimental conditions, the cofactor is present in the oxidized state (PQQ; see Figure 2) in the binary s-GDH–PQQ complex. Although *ab initio* calculations have suggested that the conformation of PQQ may not be completely planar (Zheng and Bruice, 1997), this possibility could not be
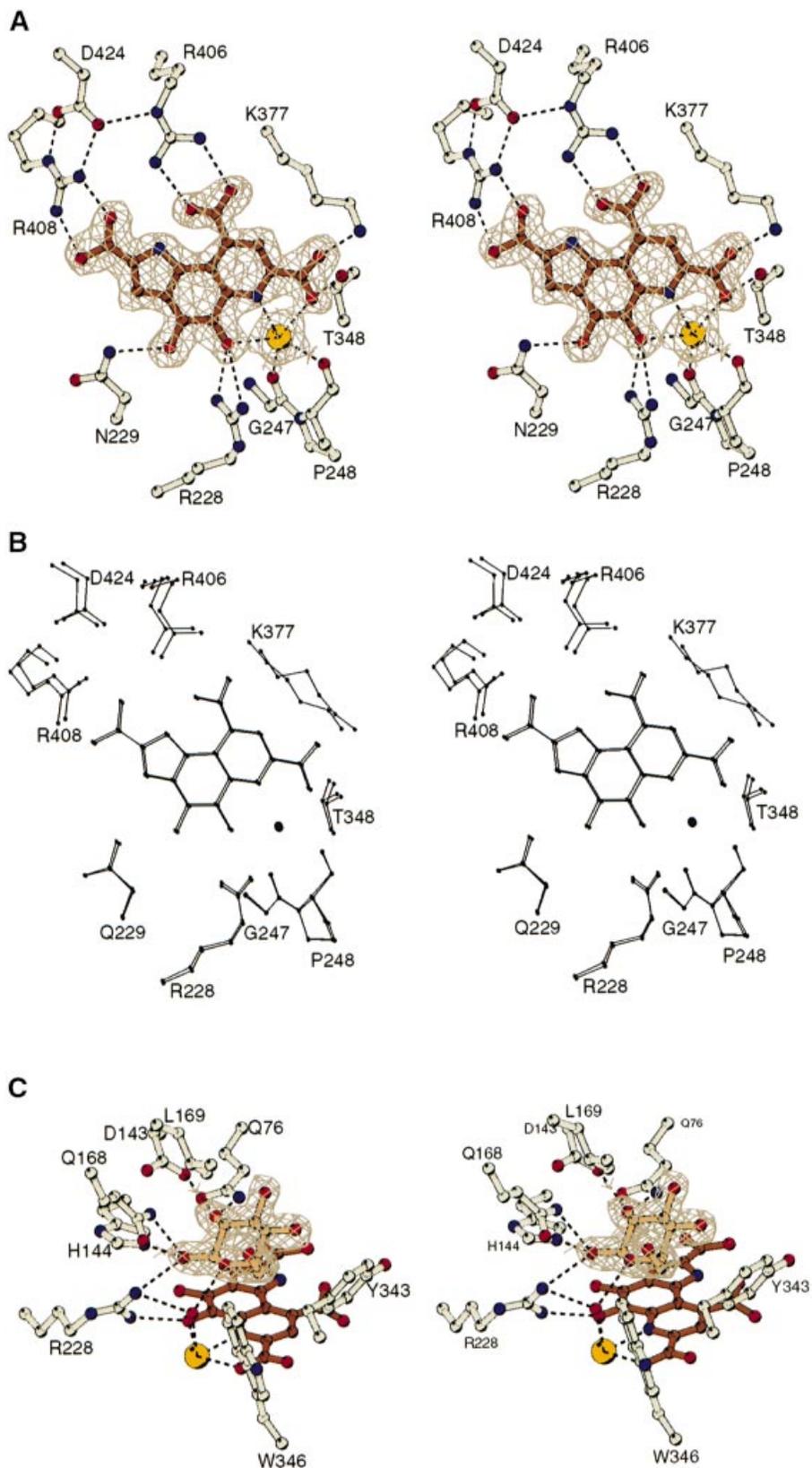
Fig. 6. (**A**) Stereo view of the binding of PQQ/PQQH$_2$ and calcium to s-GDH. The final σ_A-weighted (Read, 1986) $2F_o$–F_c electron density map, contoured at 1.3 σ, is shown for PQQH$_2$, present in the glucose complex. (**B**) Superimposition of PQQ and PQQH$_2$ in the active site of holo-s-GDH and the ternary s-GDH–PQQH$_2$–glucose complex, respectively. The superposition was done with the program O (Jones *et al.*, 1991). (**C**) Stereo view of the binding of glucose to s-GDH. The final σ_A-weighted (Read, 1986) $2F_o$–F_c electron density map, contoured at 1.3 σ, is shown for glucose. The calcium ion is shown in yellow. Oxygen and nitrogen are shown in red and blue, respectively. Carbon atoms of amino acid side chains, PQQH$_2$ and glucose are depicted in white, brown and light brown, respectively. Hydrogen bonds are marked by a dotted line. This picture was produced with BOBSCRIPT (Esnouf, 1997).

resolved in this 2.2 Å resolution complex. Therefore, PQQ was refined as a planar molecule.

s-GDH oxidizes glucose to gluconolactone with concomitant reduction of PQQ to PQQH$_2$ (see Figure 2) (Olsthoorn *et al.*, 1997). High concentrations of glucose inhibit the reduced enzyme (Olsthoorn and Duine, 1998a). To locate the substrate binding site, we soaked s-GDH–PQQ crystals in an excess of glucose and in the absence of an electron acceptor. Therefore, we conclude that these crystals contain a ternary s-GDH–PQQH$_2$–glucose complex. The planar conformation of the cofactor in this complex is in agreement with the presence of PQQH$_2$, being a fully aromatic compound. In view of the minimal structural differences of the cofactor in the present structures of s-GDH–PQQ and s-GDH–PQQH$_2$–glucose (Figure 6B), we infer that the present s-GDH–PQQH$_2$–glucose complex mimics a reactive s-GDH–PQQ–glucose complex.

Glucose binding

The glucose-binding site is a wide and solvent accessible crevice, which is located directly above PQQH$_2$ (Figure 5). This crevice is constituted by the 1D2A, 2D3A, 3BC, 4D5A and 6BC loops, which protrude from the β-propeller structure. Glucose docks onto the PQQH$_2$ surface, making extensive hydrophobic interactions (Figures 5 and 6C). Other, less extensive hydrophobic interactions are made between Leu169 and the glucose C1 atom, and Tyr343 and Trp343 form a hydrophobic patch that is within van der Waals distance of the glucose O4 hydroxyl and C5 hydroxymethyl groups. Hydrogen bonds are made between the glucose O2 atom and the side chains of Gln76 and Asp143, and also between the glucose O1 atom and the side chains of His144, Gln168 and Arg228. The interactions of the protein with the glucose O1 hydroxyl group are only possible if it is in an equatorial position and thus explain the absolute β-anomer preference of the enzyme. The O3, O4 and O6 hydroxyl groups of glucose do not make any hydrogen-bonding interaction with the protein, which explains the lack of specificity of s-GDH towards these positions (Matsushita *et al.*, 1989; Olsthoorn and Duine, 1998b).

Discussion

Reaction mechanism

Closer inspection of the binding of glucose to s-GDH shows that His144 is the only base close to the glucose O1 atom and that the glucose C1 atom is positioned directly above the PQQH$_2$ C5 atom (Figure 6C). Therefore, His144 can act as the general base that abstracts a proton from the glucose O1 atom. The involvement of a functional group with a pK_a of ~6 in the reaction (J.A.Duine, personal communication) supports this notion. The primary catalytic role of the active site calcium in s-GDH is probably the polarization of the PQQ C5–O5 bond (Zheng and Bruice, 1997; Olsthoorn and Duine, 1998b), thus improving the reactivity of the PQQ C5 atom towards nucleophiles.

For the addition-elimination reaction to occur (Figure 2A) after base-catalyzed proton abstraction, three favorable interactions of the then negatively charged O1 atom (with Arg228, His144 and Gln168) would have to be broken and major rearrangements would have to take place to form a covalent bond between the glucose O1 atom and the C5 atom of PQQ (see Figure 6C). This seems energetically expensive and the addition-elimination mechanism is therefore unlikely in s-GDH.

In contrast, the orientation of glucose in the active site of s-GDH is ideal for direct hydride transfer from the glucose C1 atom to the PQQ C5 atom. The distance between the two latter atoms is only 3.2 Å in the s-GDH–PQQH$_2$–glucose complex. Since the C1 hydrogen atom is axially positioned in β-glucose, it is expected to point down towards the PQQ C5 atom. A hydride ion thus has to be transferred over a distance of only 1.2 Å for covalent addition to the C5 atom. Moreover, the H(C1)–C5–C4, H(C1)–C5–O5 and H(C1)–C5–C6A angles are estimated to lie between 80 and 100°. This optimal arrangement of the two reactants makes a fast reaction possible. Kinetic experiments indeed indicate that the oxidation of glucose proceeds through an initial fast step, leading to a fluorescing intermediate that could be the C5 reduced form, and a subsequent rate-determining step which could be the tautomerization of the intermediate to PQQH$_2$ (Figure 2B) (Olsthoorn and Duine, 1998b). Hence, two decades after the discovery of PQQ as a coenzyme in bacterial dehydrogenases (Salisbury *et al.*, 1979), the ternary s-GDH–PQQH$_2$–glucose complex presents conclusive evidence for a reaction mechanism that comprises general base-catalyzed hydride transfer, followed by tautomerization to PQQH$_2$.

A similar proximity (3.2 to 3.5 Å) and geometry of substrate and cofactor reactive groups has been observed in several nicotinamide- and flavin-dependent oxidoreductases (Karplus and Schulz, 1989; Ramaswamy *et al.*, 1994; Mattevi *et al.*, 1996, 1997), which also make use of a hydride transfer mechanism. Thus it appears that PQQ is the third organic cofactor that favors hydride transfer in enzymatic reactions.

Conclusions

The present X-ray structures reveal for the first time the active site of s-GDH and identify the amino acid residues involved in binding of the cofactor PQQ and the substrate glucose, and postulate His144 as the general base. Moreover, based on the constellation of reactive groups in the active site, the catalytic mechanism is proposed to proceed through general base-catalyzed proton abstraction in concert with direct hydride transfer from the substrate to the cofactor. At present we are investigating the function of the proposed general base by mutation analysis. In addition, the enzyme–substrate complex is used for the development of novel protein material with an improved specificity towards glucose.

Materials and methods

Crystallization

Recombinant s-GDH was obtained in a purified, lyophilized state. The crystallization of the apo-form of s-GDH has been described elsewhere (Oubrie *et al.*, 1999). Briefly, crystals were grown with the vapor-diffusion hanging drop method at room temperature from 20–23% (w/v) polyethylene glycol (PEG) 6000, 120 mM NaCl, 3 mM CaCl$_2$ in 50 mM Tris–Gly pH 9.2. They belong to space group $P2_1$ with cell dimensions $a = 61.9$ Å, $b = 94.2$ Å, $c = 86.5$ Å, $\beta = 104.9°$ at room temperature. There are two monomers per asymmetric unit.

PQQ soaking experiment

Crystals of PQQ-bound s-GDH were obtained by transferring crystals of the apo-enzyme to a stabilizing solution containing 1 mM PQQ, 23% (w/v) PEG6000, 120 mM NaCl, 3 mM CaCl₂ and 50 mM CHES buffer pH 9.2. In the course of the 5-h soaking time, crystals changed color from colorless to blue, and the space group changed from $P2_1$ to $C222_1$, with cell dimensions at cryo temperature of $a = 60.6$ Å, $b = 158.7$ Å, $c = 221.4$ Å. The cryo-protectant consisted of 15% (v/v) glycerol, 25% (w/v) PEG6000, 120 mM NaCl, 3 mM CaCl₂ and 50 mM CHES buffer7 pH 9.2. A data set was collected at 100 K to a resolution limit of 2.13 Å at the D2AM beam line at the ESRF, Grenoble, France. Data were processed and reduced with a special version of XDS (Kabsch, 1988), adapted for the CCD detector used at this beam line. Data collection statistics are summarized in Table I.

Structure determination and refinement of the holo-enzyme

The crystal structure of the holo-enzyme was solved by molecular replacement using as a search model the structure of the dimeric apo-enzyme. Rotation and translation functions were calculated with the CCP4 version (CCP4, 1994) of the program AMoRe (Navaza, 1994) using data from 8 to 4 Å, resulting in a clear solution with a correlation coefficient of 61.4% and an R-factor of 36.3%. This solution was then subjected to rigid body refinement with AMoRe, improving the solution to a correlation coefficient of 71.1% and an R-factor of 31.1% for data from 8 to 4 Å. After building of the PQQ cofactor, this model was refined by simulated annealing with X-PLOR, followed by the automated refinement procedure of ARP (Lamzin and Wilson, 1993). A final round of positional and individual B-factor refinement was done using the bulk solvent correction in X-PLOR (Brünger et al., 1987), resulting in a final R-factor of 22.3% with a free R-factor of 28.6%. Further information on the refinement statistics and quality of the model are given in Table I. The structure factors and model coordinates have been submitted to the Protein Data Bank at Rutgers University, NJ (accession code 1C9U).

Glucose soaking experiment

A crystal of the glucose–s-GDH complex was obtained by soaking a crystal of the apo-enzyme overnight in a stabilizing solution containing 1 mM PQQ, 23% (w/v) PEG6000, 120 mM NaCl, 3 mM CaCl₂ and 50 mM CHES buffer pH 9.2, and subsequently for 30 min in a similar solution containing 1% (w/v) glucose. The crystal belonged to space group $P2_1$ with similar cell dimensions as the apo-enzyme. The cryo-solution contained 25% (w/v) glucose, instead of the 15% (v/v) glycerol used for the holo-enzyme.

Refinement of the glucose complex

After building of the PQQH₂ cofactor and the glucose substrate, this model was refined by the automated refinement procedure of ARP (Lamzin and Wilson, 1993). A final round of refinement was done using the bulk solvent correction and anisotropic scaling in X-PLOR, resulting in a final R-factor of 19.0% with a free R-factor of 22.6%. Further information on the refinement statistics and quality of the model are given in Table I. The structure factors and model coordinates have been submitted to the Protein Data Bank at Rutgers University, NJ (accession code 1CQ1).

Acknowledgements

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