Characterization of the membrane quinoprotein glucose dehydrogenase from *Escherichia coli* and characterization of a site-directed mutant in which histidine-262 has been changed to tyrosine

Gyles E. COZIER, Raff A. SALLEH and Christopher ANTHONY[1]

Division of Biochemistry and Molecular Biology, School of Biological Sciences, University of Southampton, Southampton SO16 7PX, U.K.

The requirements for substrate binding in the quinoprotein glucose dehydrogenase (GDH) in the membranes of *Escherichia coli* are described, together with the changes in activity in a site-directed mutant in which His^{262} has been altered to a tyrosine residue (H262Y-GDH). The differences in catalytic efficiency between substrates are mainly related to differences in their affinity for the enzyme. Remarkably, it appears that, if a hexose is able to bind in the active site, then it is also oxidized, whereas some pentoses are able to bind (and act as competitive inhibitors), but are not substrates. The activation energies for the oxidation of hexoses and pentoses are almost identical. In a previously published model of the enzyme, His^{262} is at the entrance to the active site and appears to be important in holding the prosthetic group pyrroloquinoline quinone (PQQ) in place, and it has been suggested that it might play a role in electron transfer from the reduced PQQ to the ubiquinone in the membrane. The H262Y-GDH has a greatly diminished catalytic efficiency for all substrates, which is mainly due to a marked decrease in their affinities for the enzyme, but the rate of electron transfer to oxygen is unaffected. During the processing of the PQQ into the apoenzyme to give active enzyme, its affinity is markedly dependent on the pH, four groups with p*K* values between pH 7 and pH 8 being involved. Identical results were obtained with H262Y-GDH, showing that His^{262} it is not directly involved in this process.

Key words: PQQ, pyrroloquinoline quinone.

INTRODUCTION

The membrane-bound glucose dehydrogenase (GDH) of *Escherichia coli* is a quinoprotein having pyrroloquinoline quinone (PQQ) as its prosthetic group [1–4]. It catalyses the oxidation of D-glucose to the lactone in the periplasm, the electron acceptor being ubiquinone in the membrane [5]. Under some growth conditions this makes an important contribution to the energy metabolism of these enteric bacteria [6,7]. GDH is an intrinsic monomeric membrane protein (about 87 kDa). The *N*-terminal domain (154 residues), having five transmembrane segments, is likely to contain the ubiquinone-binding site [8]. The remaining periplasmic region (641 residues) is similar to the α-subunit of methanol dehydrogenase, and it has been possible to model the GDH structure using the X-ray structure of this quinoprotein [9]. The prosthetic group (PQQ) of methanol dehydrogenase is held in place in the active site by stacking interactions between a tryptophan residue and a novel disulphide ring structure made up of adjacent cysteine residues [4,10]. In the GDH, the novel disulphide ring is replaced by a histidine residue (His^{262}) (Figure 1), consistent with a previous suggestion that a histidine residue is essential for binding PQQ in the GDH of *Pseudomonas fluorescens* [11]. The entrance to the active site is more open than in methanol dehydrogenase, and His^{262} is located at the entrance, together with a second histidine (His^{775}); viewed from the outside, the entrance to the active site is occluded to some extent by these two histidine residues (Figure 2). This is consistent with results using a mutant strain of *Gluconobacter oxydans* that had acquired the ability to oxidize the disaccharide maltose. A point mutation had led to a GDH in which this histidine residue (His^{787} in *G. oxydans*) had changed to an asparagine residue [12]. Remarkably little is known about the substrate specificity of the membrane

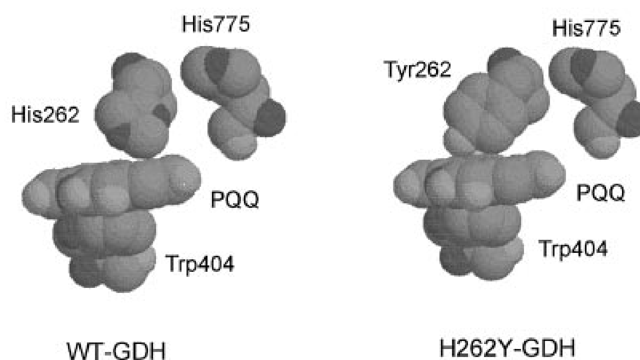

Figure 1 Active site of GDH

This is from the model structure [9] based on the co-ordinates of methanol dehydrogenase [10]. WT-GDH is the structure of the protein encoded by the WT *gcd* gene; H262Y-GDH is the structure of the protein encoded by the site-directed mutant described in the present paper. The structures were drawn using the programme Rasmol. A view from the surface of the molecule is shown in Figure 2.

Abbreviations used: PQQ, pyrroloquinoline quinone; WT-GDH, glucose dehydrogenase (GDH) encoded by the wild-type (WT) gene; H262Y-GDH, GDH in which His^{262} (H262) has been altered to tyrosine (Y); PES, phenazine ethosulphate; DCPIP, 2,6-dichlorophenol-indophenol; SA, specific activity.

[1] To whom correspondence should be addressed (e-mail c.anthony@soton.ac.uk).

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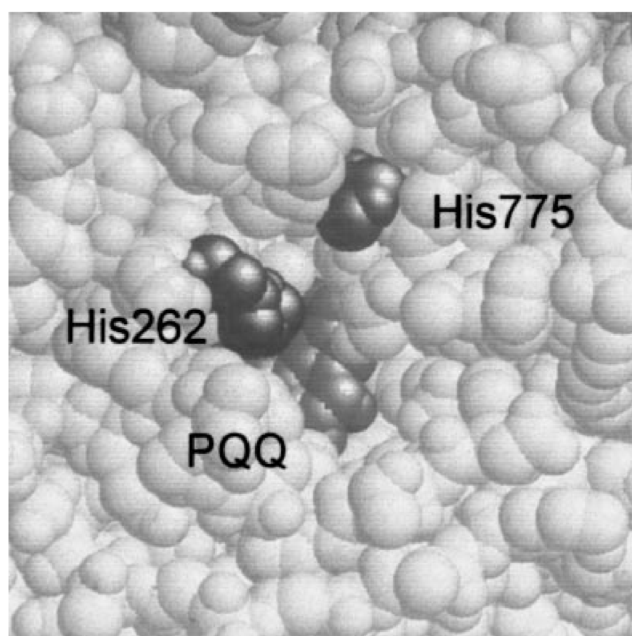

Figure 2 A surface view of the entrance to the active site of GDH

GDH, although an early qualitative study showed that it is also able to oxidize D-mannose, D-galactose, D-fucose and D-xylose at more than 25% of the rate with D-glucose [2].

Compared with methanol dehydrogenase, there are fewer equatorial interactions between the protein and PQQ, perhaps explaining why it is possible to effect the reversible dissociation of PQQ from the GDH but not from methanol dehydrogenase [9]. *E. coli* is unable to synthesize PQQ; in the absence of PQQ in the growth medium the GDH is therefore always produced as the apoenzyme, active enzyme being reconstituted by incubation with PQQ and Mg^{2+} [1,2,7]. The structure of the PQQ-containing quinoproteins is a superbarrel made up of eight β-pleated sheets arranged radially. Because the holoenzyme can be formed so readily from apoenzyme by addition of PQQ, it is likely that the apoenzyme is in an almost completely folded form, the final step in production of the active enzyme being the binding of PQQ followed by its insertion.

The present paper describes a thorough investigation of the activity of GDH of *E. coli*, together with a characterization of a site-directed mutant in which His^{262} has been replaced by tyrosine. This mutant was produced to investigate the possibility that His^{262} is involved in binding PQQ in the active site, and to investigate its influence on substrate binding and specificity.

MATERIALS AND METHODS

Chemicals and reagents

Analytical-grade chemicals were obtained from either Sigma Chemical Co. (Poole, Dorset, U.K.) or BDH Ltd. (Poole, Dorset, U.K.). Enzymes for cloning, and Wizard™ Minipreps (DNA purification system) were obtained from Promega (Southampton, U.K.). The QIAEX II Gel Extraction Kit was obtained from Qiagen Ltd., Dorking, Surrey, U.K. The Sequenase version 2.0 DNA sequencing kit was obtained from United States Biochemicals, Cleveland, OH, U.S.A. The T_4 polymerase kit was obtained from Bio-Rad (Hemel Hempstead, Herts., U.K.). The Long Ranger™ acrylamide solution (A. T. Biochem, Malvern, PA, U.S.A.) was used for preparation of sequencing gels.

Growth and maintenance of bacterial strains and plasmids

The bacteria and plasmids used in the present study are listed in Table 1. Bacteria were maintained as glycerol stocks at −20 °C, as nutrient agar stabs or on L-agar plates stored at 4 °C for short periods. The F′-carrying strains (MV1190, JM101) were maintained on solid minimal medium supplemented with thiamin to ensure retention of the plasmid. *E. coli* was grown in Luria broth with shaking, or on agar, at 37 °C. Antibiotics or supplements were added as required, with all the heat-labile components being filter-sterilized through a 0.22 μm-pore-size filter and added after the media had been autoclaved and cooled to about 60 °C. The following were included in growth media as required: ampicillin (100 μg/ ml); chloramphenicol (15–100 μg/ml); isopropyl β-D-thiogalactoside ('IPTG') (1 mM); thiamin (1 μg/ml); yeast extract (500 μg/ml); 5-bromo-4-chloroindol-3-yl β-D-galactopyranoside ('X-gal') (40 μg/ml).

Methods used in mutagenesis

Three methods for transformation were used: the heat-shock method [13], the freeze–thaw method [14] and the '5 min transformation system' [15]. There is no significance in which method was used; in the later stages of the work the '5 min transformation system' was used and replaced the other two methods that were used earlier. Agarose-gel electrophoresis of DNA was performed as described previously [16], the DNA fragments being purified using the QIAEX Gel Extraction Kit.

For the production of single-stranded DNA, *E. coli* strain MV1190 was transformed with the appropriate plasmid and incubated with untransformed bacteria on solid media. The resulting phage stocks were stored at 4 °C and were used to produce the single-stranded DNA by infecting strain MV1190 (for sequencing) or CJ236 (for production of the uracil-containing templates). The single-stranded DNA was purified from the phage by using Wizard Minipreps, with single-stranded binding resin (15 mg/ml diatomaceous earth, acid-washed, calcined; from Sigma, in 7 M guanidinium chloride) instead of the normal Miniprep resin. DNA sequencing was carried out using the Sequenase® version 2.0 kit.

For site-directed mutagenesis, the *Sal*I/*Xma*I fragment of pGEC1 was cloned into M13mp18 and the resulting phage (M13GEC1) was used for mutagenesis using the Kunkel protocol [17,18] with the Bio-Rad T_4 polymerase kit. The mutagenic primer used was 5′-TCT TTC CAG **TACGTA** ACC TGC-3′ (the mutated triplet is indicated in **bold** and the new *Sna*BI restriction site produced by mutagenesis is underlined). The H262Y mutation (in M13GEC2) was confirmed by restriction digestion using *Sna*BI and by sequencing of M13GEC2. The mutated fragment was cloned back into pGEC1 using *Sal*I and *Age*I and transformed into *E. coli* PP2418, giving PPGEC2.

Purification of GDH

The purification protocol was adapted from those described previously [8]. All buffers were based on 10 mM potassium phosphate buffer, pH 7.0. Typically 2 litres of a strain over-expressing GDH (PPGEC1) were grown with shaking to late-exponential phase, harvested by centrifugation, washed twice with 0.85% NaCl, and suspended in buffer containing 5 mM $MgCl_2$. After sonication, cell debris was removed by centrifugation and membranes collected by ultracentrifugation at

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Table 1 (a) Bacterial strains, (b) plasmids and (c) bacteriophages

(a)

E. coli strains	Genotype	Source/description
MV1190	Δ(*lac-pro*AB), *thi*, *sup*E, Δ(*srl-rec*A) 306::Tn10 (tet^r) [F′:*tra*D36, *pro*AB+, *lac*I^{q}ZΔM15]	Good plasmid and M13 host; suitable for blue/white selection
CJ236	*dut*-1, *ung*-1, *thi*-1, *rel*A1; pCJ105(Cm^r F′)	Strain for production of uracil-substituted DNA
JM101	*sup*E, *thi*-1, Δ(*lac-pro*AB) [F′ *tra*D36, *pro*AB, *lac*I^{q}ZΔMI5]	Stratagene
PP2418	*pts*I, *thi*, *gal*P, *cat* insertion in *gcd* gene	Dr. N. Goosen
PPGEC1	PP2418 containing pGEC1	The present study
PPGEC2	PP2418 containing pGEC2	The present study

(b)

Plasmid	Description	Source
pBluescript KS$^+$	Amp^r, cloning vector containing a multiple cloning site, *lacZ*, and an f1 origin of replication.	Stratagene
pGP478	pBR322 cloning vector with *gcd* gene cloned into the *Bam*HI site.	Dr. N. Goosen
pGEC1	Amp^r, *Sal*I–*Eco*RI *gcd* from pGP478 cloned into pBluescript in the direction of the *lacZ* gene	The present study
pGEC2	Amp^r, pGEC1 (H262Y *gcd*)	The present study

(c)

Bacteriophage	Description	Source
M13mp18	Multipurpose cloning vector, *lacZ*	The present study
M13GEC1	*Sal*I–*Xma*I fragment of *gcd* from pGEC1 cloned into M13mp18 against *lacZ* gene	The present study
M13GEC2	M13GEC1 with H262Y mutation	The present study

40000 rev./min for 1 h. They were suspended in buffer containing 5 mM $MgCl_2$ and 0.1 % Triton X-100 and incubated on ice for 30 min. Following collection by centrifugation, they were suspended in buffer containing 5 mM $MgCl_2$, 0.2 M KCl and 0.5 % Triton X-100; they were incubated on ice for 30 min to solubilize the GDH from the membranes, which were removed by centrifugation at 40000 rev./min for 1 h. The supernatant, containing GDH, was dialysed overnight against buffer containing 0.1 % Triton X-100, before being applied to a DEAE-Sepharose column (approx. 50 ml column vol.) that had been previously equilibrated with buffer containing 0.5 % Triton X-100. This was then washed with 10 bed vol. of the same buffer, followed by 10 bed vol. of buffer containing 0.1 % Triton X-100. The GDH was eluted with a linear gradient of 0–100 mM KCl in buffer containing 0.1 % Triton X-100. Active fractions were pooled, concentrated, and then dialysed overnight as described above to remove the KCl.

Measurement of protein, GDH reconstitution and GDH activity

For SDS/PAGE the Laemmli buffer system was used [19], protein being detected with the Coomassie Blue stain [20]. Protein was determined using the bicinchoninic acid ('BCA') assay [21] adapted for microtitre plates [22].

Reconstitution of holoenzyme from apoenzyme was achieved by incubating purified enzyme in buffer containing PQQ and Mg^{2+} ions. A mixture of enzyme solution (15 μg in a total of 100 μl solution), 5 mM $MgSO_4$,$7H_2O$, 25 μM PQQ and 50 mM potassium phosphate, pH 6.5, were incubated at 25 °C for 15 min. A sample of this was used in the assay for the enzyme activity.

GDH activity was measured spectrophotometrically using a dye-linked assay involving phenazine ethosulphate (PES) and 2,6-dichlorophenol-indophenol (DCPIP). PES accepts electrons from GDH and passes them on to DCPIP; the reduction of DCPIP was monitored by decreasing absorbance at 600 nm. The assay mixture (total volume 1 ml) contained 20 mM Tris/HCl buffer (pH 8.75), 40 mM D-glucose, 100 μM DCPIP, 10 μl (1.5 μg) of reconstituted enzyme and 660 μM PES. One unit of enzyme activity was defined as the amount of enzyme that catalyses the reduction of 1 μmol of DCPIP/min under the assay conditions between 10 and 70 s after the start of the reaction. Kinetic analysis of the results from the dye-linked assays was performed using the program Graphpad PRISM™.

The coupling of the GDH to the electron transport chain in membranes was determined at 37 °C in an oxygen electrode at pH 7.0 in 20 mM Pipes buffer containing 0.1M D-glucose, after reconstitution of active GDH from apoenzyme and PQQ as described above.

Measurement of glucose using the glucose oxidase system

Because GDH was shown to have a low affinity for some substrates, the glucose concentration in potential substrates was determined by using the Trinder glucose oxidase assay system (purchased from Sigma and used according to their instructions). Except for maltose, the highest concentration of glucose in any potential substrate was 0.4 %, which was insufficient to account for any results described here. Maltose contained about 3 % D-glucose, which was sufficient to account for the apparent oxidation of this substrate by GDH. It was confirmed that no sugar being tested was able to inhibit the glucose oxidase determination using glucose as substrate.

RESULTS AND DISCUSSION

Production of the H262Y-GDH mutant from GDH by site-directed mutagenesis

The gene coding for GDH in *E. coli* (*gcd*) was kindly provided by Dr. N. Goosen (Laboratory of Molecular Genetics, University

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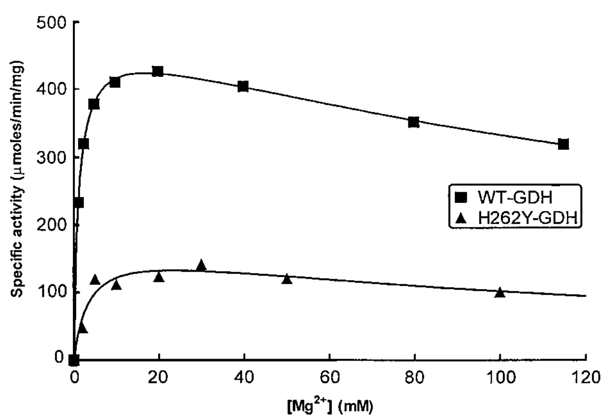
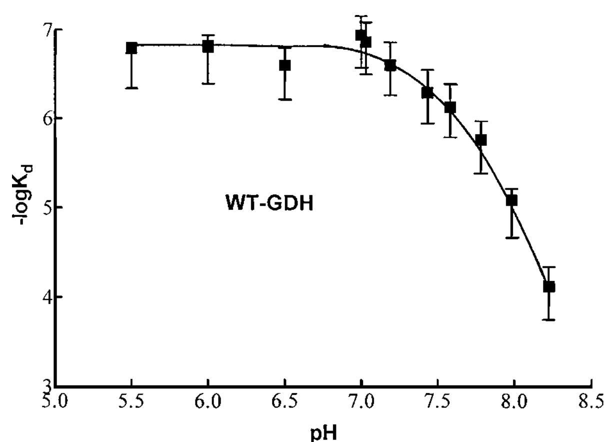


Figure 3 The effect of [Mg^{2+}] on the reconstitution of GDH and H262Y-GDH

The enzyme was reconstituted at pH 6.5 (0.1 M KPO_4 buffer) and incubated at 25 °C for 20 min with a range of Mg^{2+} concentrations, using a PQQ concentration of 37.5 μM. The resulting enzyme was assayed under normal conditions and the results expressed as specific activity (SA). The equation used for the best-fit line was:

$$SA = (SA_{max}[S])/[K_d + [S](1 + [S]/K_i)]$$

where SA_{max} is the highest SA acheived with the reconstituted enzyme. ■, results with WT-GDH; the kinetic constants are: SA_{max}, 490 μmol/min per mg; K_d, 1.4 mM; K_i, 219 mM. ▲, results with H262Y-GDH; the kinetic constants are: SA_{max}, 173 μmol/min per mg; K_d, 3.6 mM; K_i, 151 mM.



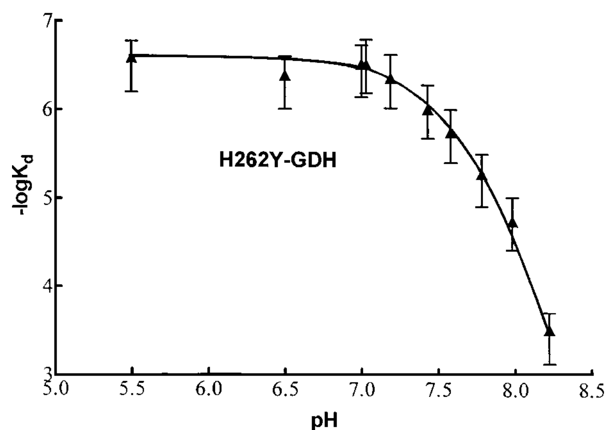


Figure 4 Effect of pH on the affinity for PQQ of WT-GDH and H262Y-GDH

The K_d values for PQQ were determined for reconstitution in the presence of 5 mM Mg^{2+} at 25 °C using 235 nM enzyme. Activity was measured in the standard assay system after complete reconstitution in a range of different buffers (100 mM) (after about 30 min). The buffers used were: pH 5.5, Mes; pH 6.0, BisTris; pH 6.5, phosphate; pH 7.0–8.0, Tris. The K_d values are the averages of three separate determinations; the error bars indicate 95% confidence limits.

of Leiden, Leiden, The Netherlands) in a low-copy-number plasmid (pGP478) [23]. The *Sal*I/*Eco*RI fragment of pGP478 was cloned into the high-copy vector, pBluescript KS^+, giving pGEC1, which was then transformed into *E. coli* PP2418 [23], which has the *cat* (chloramphenicol acetyltransferase) gene inserted into the chromosomal *gcd* gene (giving PPGEC1) as described in the Materials and methods section. This strain, in which the GDH is constitutively produced, was used for production of the membrane GDH and for production of a site-directed mutant of GDH in which His^{262} was replaced by tyrosine. The position of His^{262} (in WT-GDH, the GDH encoded by wild-type bacteria) and Tyr^{262} (in H262Y-GDH) are shown in Figures 1 and 2.

Purification of GDH and reconstitution of active enzyme from apo-GDH and PQQ

The enzymes from WT bacteria and from the site-directed mutant (H262Y-GDH) were solubilized from membranes and purified by anion-exchange chromatography as described in the Materials and methods section. For both enzymes the yield was 35–45%, purification was about 40-fold, and the purified enzymes were more than 80% pure, as indicated by SDS/PAGE.

As previously described, reconstitution of active GDH from apoenzyme and PQQ required Mg^{2+} [1]. The rate of reconstitution was very rapid, and was similar in WT-GDH and H262Y-GDH, completion always occurring within about 5 min (80% within 2 min) at 25 °C in 50 mM buffers. The K_d values for Mg^{2+} and PQQ were determined by plotting the final activity achieved against the concentrations of Mg^{2+} and PQQ. Figure 3 shows that, in the presence of excess PQQ (25 μM), the K_d for Mg^{2+} was similar for WT-GDH (1.4 mM) and H262Y-GDH (3.6 mM). The value of 1.4 mM for WT-GDH was the average of values measured with enzyme purified from three separate batches of cells; all values were between 1 and 3 mM, and these were independent of pH between pH 5.5 and 7.5. There is no obvious reason for the difference between our values, and the only previously published value for binding Mg^{2+} (K_m about 30 μM) [24]. At higher concentrations of Mg^{2+}, binding at a second site led to inhibition, the K_i values for the two enzymes being similar (219 mM for WT-GDH; 151 mM for H262Y-GDH) (Figure 3).

In the presence of 5 mM Mg^{2+}, both enzymes showed a similar very high affinity for PQQ at pH 6.5 (apparent K_d 249 nM for WT-GDH; 105 nM for H262Y-GDH). These values are similar to those measured previously (about 100 nM) [24]. For routine reconstitution (e.g. for enzyme assays), 50 mM phosphate buffer, pH 6.5, was used with excess Mg^{2+} (5 mM) and PQQ (25 μM).

Effect of pH on the affinity for PQQ of WT-GDH and H262Y-GDH

The rates of reconstitution were similar in WT-GDH and H262Y-GDH and were slightly higher at pH 5.5 than at pH 7.5. At all pH values tested the rates of reconstitution were too high for accurate measurement, but the variation of affinity for PQQ could be measured accurately over a range of pH values. Figure 4 shows the relationship of $-\log K_d$ and the pH value of the reconstitution buffer. The affinity of WT-GDH for PQQ was high and independent of pH between pH 5.5 and 7.0. Between

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Table 2 Substrate specificity of WT-GDH and H262Y-GDH

The kinetic constants were determined using at least two batches of enzyme (values in parentheses are standard errors); kinetic constants for the mutant enzyme were less reproducible than for WT because of the low affinity for substrate; estimated V_{max} values were all 25–50% of those measured with D-glucose and the WT enzyme.

	WT-GDH				H262Y-GDH
Substrate	K_m (mM)	V_{max} (μmol/min per mg)	$10^{-3}\times$ Catalytic efficiency ($M^{-1}\cdot s^{-1}$)*	K_i (mM)	K_m (mM)
Hexoses and derivatives					
D-Glucose	2.1±0.2	404±8	279	–	460 (±33)
6-Deoxy-D-glucose	1.3±0.1	409±5	456	–	High‡
2-Deoxy-D-glucose	1.6±0.2	403±9	365	–	32 (±5)
D-Allose	2.5±0.3	493±13	286	–	810 (±148)
D-Fucose (6-deoxy-D-galactose)	8.3±1.1	555±20	97	–	> 1600‡
2-Amino-D-glucose (glucosamine)	9.5±3.0	830±192	123	–	nd†
3-Deoxy-D-glucose	10.8±1.7	484±34	65	–	High‡
D-Melibiose (6-galactosyl-D-glucose)	17.7±5	383±38	31.4	–	> 1000‡
D-Galactose	39±5	516±23	19.2	–	> 1000‡
D-Mannose	78±8	516±22	9.6	–	> 1600‡
3-*O*-Methyl-D-glucose	79±15	267±15	4.9	–	nd†
Pentoses					
D-Xylose	22±4	403±19	26.6	–	> 1600‡
L-Xylose	–	–	–	92±9	
L-Arabinose	46±8	525±32	16.5	–	> 1000‡
D-Arabinose	–	–	–	90±9	
L-Lyxose	100±19	239±17	3.5	–	> 1000‡
D-Lyxose	–	–	–	70±8	
D-Ribose	110±30	297±30	3.9	–	> 1600‡
L-Ribose	–	–	–	nd§	

* The catalytic efficiency was calculated assuming that the enzyme was 100% pure.
† nd, not determined.
‡ These values could not be determined accurately because, at the concentration quoted, the values were in the linear portion of the Michaelis–Menten curve.
§ Not determined; insufficient data to determine accurately (90 mM gave about 50% inhibition with 2 mM D-glucose).

pH 7 and 8.3 the affinity for PQQ decreased, the shape of the curve indicating that the decrease was due to deprotonation of three or four groups in the free apoenzyme or in PQQ, all having p*K* values between 7 and 8 (for a discussion of the interpretation of this sort of curve, see [25]). The dissociable groups thus shown to be involved in binding are likely to be positively charged at pH 7. It is possible that one of these is the pyrrole nitrogen group of the PQQ itself, which may bind to a charged carboxylate residue on the apoenzyme. This proposal is consistent with the previous demonstration that methylation of the pyrrole nitrogen on PQQ decreased its affinity for apo-GDH during reconstitution [26]. As the cysteine residues all form disulphide bridges, the other groups on the dehydrogenase that could give rise to the observed p*K* values are histidine, arginine or lysine residues. These might include Arg^{266}, which bonds (in the model structure) to the 9-carboxylate of PQQ, consistent with the observation that the affinity of the enzyme for the PQQ analogue lacking this carboxylate (or the 2-carboxylate or 7-carboxylate) has a very much lower affinity for GDH than does the normal PQQ [26]. However, it should be noted that the groups on the dehydrogenase giving rise to the observed p*K* values do not necessarily interact directly with the PQQ; they may be involved in determination of folded states with different affinities for PQQ. It might be expected that one of these groups would be His^{262}, but the results with the H262Y-GDH indicate that this is not the case, because the response to changes in pH of the affinity of PQQ for this mutant enzyme was almost identical with that observed for WT-GDH (Figure 4).

The results presented here are consistent with a previous comment that the extent of reconstitution was decreased under alkaline conditions using the *E. coli* enzyme [1,2], and the suggestion that the lower reconstitution rates measured with the *Acinetobacter calcoaceticus* enzyme under slightly alkaline conditions could indicate the involvement of a histidine residue in this process [27].

Substrate specificity of WT-GDH

Table 2 summarizes the results of kinetic studies of the substrate specificity of the WT-GDH; the structures of most of the relevant compounds are given in Figure 5. The disaccharides maltose, lactose, trehalose and sucrose were not oxidized, nor was the trisaccharide raffinose, nor D-fructose, *myo*-inositol, methanol or ethanol (all tested at concentrations up to 0.5 M). None of these compounds (at 0.5 M) inhibited the oxidation of glucose (2 mM), so they were presumably unable to bind at the active site. It should be noted that, initially, maltose appeared to be a substrate, but the maltose sample was shown to contain about 3% glucose, which was sufficient to account for its apparent oxidation by GDH.

Oxidation of hexoses

None of the L-hexoses tested was able to act as a substrate (0.5 M); these included L-glucose, L-mannose, L-allose and L-rhamnose. None acted as an inhibitor (at 0.5 M), suggesting that L-hexoses are unable to bind at the active site. Their only common feature is that they all have a C-6 hydroxymethyl (or

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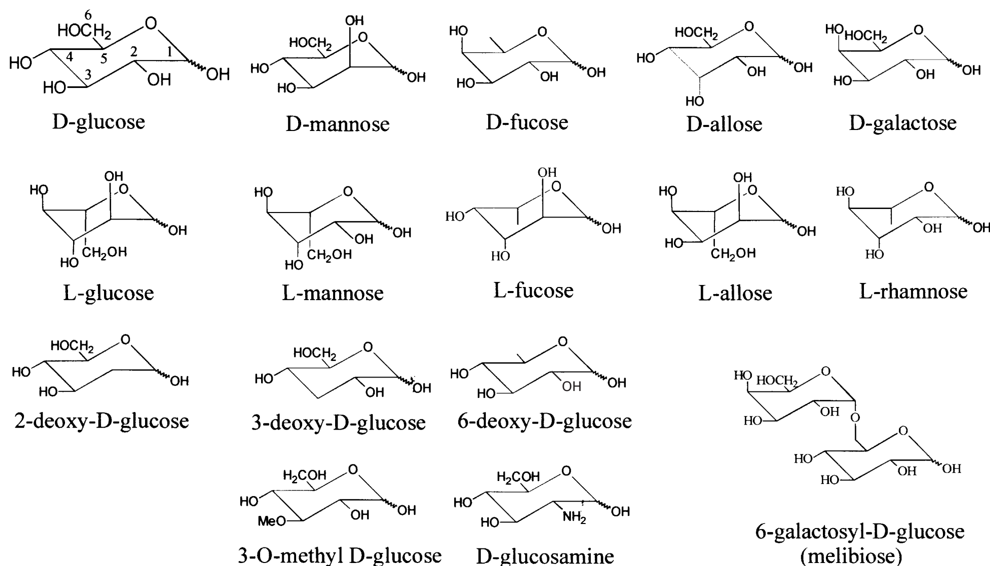
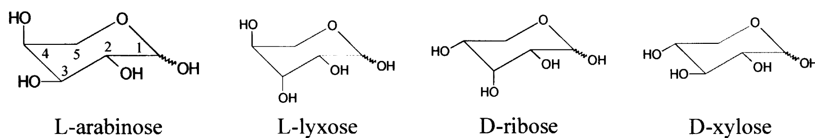
Aldohexoses



Aldopentoses

Substrates



Competitive inhibitors

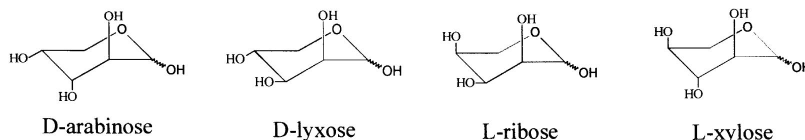


Figure 5 Structures of potential substrates for GDH

The kinetic constants for oxidation of these substrates are given in Table 2. All the D-hexoses shown here were substrates; L-hexoses were not oxidized and were not competitive inhibitors. All pentoses were either substrates or competitive inhibitors.

methyl) group below the plane of the ring, suggesting that this prevents binding by steric hindrance.

All of the D-hexoses tested were able to act as substrates for GDH. The V_{max} values did not vary greatly, the catalytic efficiency (k_{cat}/K_m) being determined mainly by the affinity of the enzyme for its substrate (Table 2).

The C-6 hydroxymethyl group itself is not essential for binding to GDH, because the affinity was not markedly changed when it was replaced by a methyl group (in 6-deoxy-D-glucose and 6-deoxy-D-galactose) or by a galactosyl group (in the disaccharide melibiose). The activity with pentose sugars (which lack a C-6 group) also supports this conclusion. Replacement of the 6-hydroxymethyl group by a 6-methyl group (in deoxygalactose) led to an increased affinity, suggesting that the hydroxy group at this position causes some steric hindrance. The relatively low affinity for melibiose is also probably due to steric hindrance from the second saccharide moiety at the C-6 position.

The orientation of the C-4 hydroxy group is not critical for binding, as indicated by the oxidation of galactose, which differs from glucose only at the C-4 position. That the active site is more constricted in this region than it is near the C-6 position is shown by the failure to bind the disaccharides in which the second saccharide is bonded at the C-4 of glucose (lactose, maltose and trehalose).

The C-3 hydroxy group is not critical for binding, as shown by the relatively high affinity for 3-deoxy-D-glucose and for allose (having the C-3 hydroxy group below the plane of the ring). Methylation of the C-3 hydroxy group of glucose (to give 3-*O*-methyl-D-glucose) led to a 40-fold decrease in binding, presumably due to steric hindrance above the plane of the ring near the C-3 position.

The C-2 hydroxy group is also not critical for binding, as shown by the high affinity for 2-deoxyglucose and 2-amino-D-glucose (glucosamine). The 40-fold lower affinity for D-mannose,

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having the C-2 hydroxy group above the plane of the ring, is presumably due to steric hindrance.

In summary, no particular hydroxy group is absolutely essential for binding of hexose substrates to glucose dehydrogenase. The hydroxy groups on C-2, C-3 and C-4 can be in either orientation but any substituent on the C5-carbon atom must be above the plane of the ring; the nature of the substituent (methyl, hydroxymethyl or a second saccharide) only affects affinity and not the rate of reaction.

The oxidation of pentoses

As with hexoses, the form of pentose that predominates in solution is the pyranose form, but pentoses lack a hydroxymethyl substituent at the C-5 position (Figure 5). As this substituent is not essential for binding hexoses, it is not surprising that some pentoses are good substrates (Table 2). Because the major distinction between the D- and L-isomers of the hexoses (the orientation of the C-6 hydroxymethyl) does not apply in the pentoses, it might be expected that they would all be able to bind to the enzyme, and the results in Table 2 indicate that this does occur. All pentoses were either substrates or competitive inhibitors; the K_m values were all in the range 20–110 mM and the K_i values for competition with glucose as substrate were in the same range. The action of GDH on pentoses inexplicably differs, however, from its action on the hexoses. Those hexoses that were not substrates were not competitive inhibitors and so were presumably unable to bind to the active site; by contrast, all pentoses were able to bind, but only one of the steroisomers of each substrate was oxidized. The difference in activity between pentoses cannot therefore be interpreted in terms of binding alone. The only common feature of those pentoses that were substrates is that the C-2 hydroxy group is below the plane of the ring, whereas in the inhibitory pentoses it is above the plane. This is consistent with the conclusion that the orientation of its C-2 hydroxy group above the plane of the ring must lead to some steric hindrance with the hexose substrates.

Finally, It should be noted that, when the straight-chain or furanose structures of hexoses and pentoses are considered as possible substrates, there is no more correlation between structure, binding and activity than has been argued here on the basis of the pyranose forms being the preferred substrate.

Altered enzyme activity of H262Y-GDH

The main difference between WT-GDH and H262Y-GDH was the poor substrate binding in the mutant enzyme, the K_m values being at least 200 times higher in the H262Y-GDH (Table 2). The one exception was 2-deoxyglucose, the K_m value of which increased only 20-fold, by contrast with the 230-fold increase for glucose, although these two substrates have almost identical affinities for WT-GDH. The results with H262Y-GDH suggest that the mutation has led to an alteration of the configuration of the active site that now causes some steric hindrance near the hydroxy group at the C-2 position in glucose.

The mutation of His^{262} to tyrosine had very little effect on the coupling of the GDH to the electron-transport chain in membranes. The rates of oxygen consumption by way of the complete electron-transport chain involving quinol oxidation was 10–20 % of the rate when PES was also present (this mediates direct oxidation of the enzyme by oxygen). The same relative rates were obtained with membranes isolated from WT or mutant bacteria. This shows that His^{262} is not essential for electron transfer between PQQ and membrane ubiquinone, but it does not rule out the possibility that electron transport does occur by way of His^{262} and that tyrosine in this position can also fulfil this function.

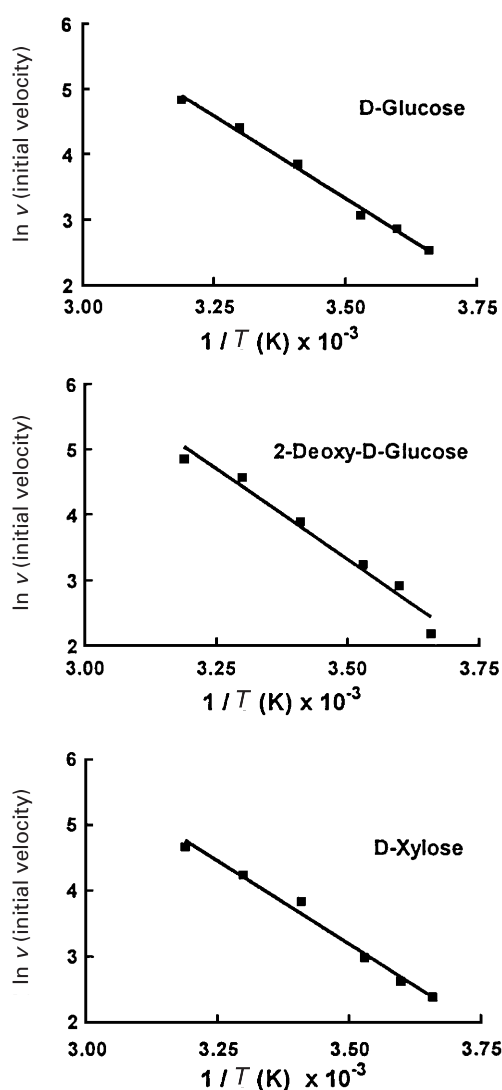

Figure 6 Activation energy for the reactions of GDH

WT-GDH (8 μg) was reconstituted and assayed over a range of temperatures in the standard system containing 40 mM glucose, 40 mM 2-deoxy-D-glucose or 670 mM D-xylose. The lines are the best-fit curves, using the GraphPad Prism program using the formula relating rate (v) and activation energy (E):

$$v = A \cdot \exp(-E/\boldsymbol{R}T)$$

where A is a constant that qualitatively expresses the frequency of collisions and the requirement for specific orientation between colliding molecules, $\boldsymbol{R}$ is the gas constant and T is the temperature.

Activation energy for oxidation of hexoses and pentoses

In order to determine the activation energy for the GDH reaction, its rate of reaction in the standard dye-linked assay system was determined between 5 and 40 °C in the presence of high substrate concentrations. The results (Figure 6) show that the activation energies were in the usual range for enzyme-catalysed reactions and were almost identical for the two hexoses D-glucose (−42 kJ/mol) and 2-deoxy-D-glucose (−46 kJ/mol) and for the pentose D-xylose (−42 kJ/mol). This is consistent with the

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conclusion that the mechanism for oxidation of pentoses is essentially the same as that for hexoses. It was not possible to determine the activation energy for the reaction catalysed by the mutant H262Y-GDH because it was relatively unstable, becoming denatured during the course of the reaction above 20 °C.

GENERAL DISCUSSION

This paper describes the first thorough investigation of substrate specificity of the membrane GDH and a study of reconstitution of active enzyme from apoenzyme and PQQ. Our results are consistent with previous qualitative observations published with the first descriptions of this enzyme, which reported that it is able to oxidize a number of D-hexoses and also the pentose D-xylose [2]. Preliminary studies of the enzymes from *Gluconobacter suboxydans* [28] and from a *Pseudomonas* species [29] have indicated a similar range of substrates. Our measured K_m values for some substrates [24,30] and for PQQ [24] during the reconstitution process are all consistent with previously available measurents. However, inexplicably, our measured value of the K_m for Mg^{2+} was much higher than previously recorded [24].

The work presented here has demonstrated that the substrate specificity of the membrane GDH is even wider than previously reported, thus extending the range of substrates that should be considered a source of metabolic energy for enteric bacteria during growth in some situations, and drawing attention to the advantages and disadvantages of using this enzyme, and mutants derived from it, for biotechnological purposes. Although it shows a wide range of catalytic efficiencies, the maximum rates achieved were fairly similar for all substrates, the major differences in activity being in the affinity for the different substrates. Although this study has not shed direct light on the catalytic mechanism of the enzyme, it has raised an interesting question relating to the oxidation of hexoses and pentoses; we are unable to explain why all those hexoses that bind are also oxidized, whereas some pentoses are able to bind as competitive inhibitors but are not themselves substrates.

Although mutation of His^{262} to tyrosine had almost no effect on the reconstitution process (see below), it had a major effect on substrate binding; the affinity for most substrates was decreased at least 200-fold, consistent with the proposed location of His^{262} at the entrance to the active site (Figure 2). Remarkably, not one of the eight mutant enzymes described by Yamada et al. [24] was altered in its binding of D-glucose (no substrate other than glucose was investigated). His^{262} was not investigated, but it was shown that mutation of the nearby His^{775} to arginine led to a decrease in V_{max} to about 3% of that of WT-GDH (the K_m was unchanged). Mutation of the same residue to alanine had no affect on either substrate binding or activity. It was therefore concluded that the arginine mutation, but not the alanine mutation, may disturb the conformation of the active site, leading to a decreased turnover. This same residue was mutated to asparagine (H775N-GDH) and aspartate (H775D-GDH) by Sode and Kojima [30] as part of a programme of site-directed mutagenesis aimed at improving GDH for use as an analytical tool [30–35]. Remarkably, in this case, the changes in activity of the mutant enzymes were mainly due to changes in binding of substrate. The affinity for glucose of H775N-GDH was almost unchanged but its affinity for other substrates decreased. The affinity of H775D-GDH for most substrates was also markedly decreased, and the K_m for glucose increased about 25-fold. That study, with a rather narrow range of substrates, led those authors to speculate that His^{775} is hydrogen-bonded to glucose at its C-6 hydroxymethyl group. However, this is inconsistent with our conclusion, based on the study of many substrates, that this group is not essential for binding glucose, and it is more likely that the mutation of the histidine to the carboxylate led to to a more general distortion of the active site.

We have shown that although His^{262} is probably involved in direct interaction with PQQ, as indicated in the model structure [9], changing this to tyrosine has no effect on the affinity of apoenzyme for PQQ during reconstitution. At least three residues on the enzyme with pK values between 7 and 8 were shown to be involved in this process, and an obvious suggestion is that one of these is His^{775}, which is located very close to PQQ in the structure (Figures 1 and 2). This conclusion is supported by a recent extensive site-directed-mutagenesis analysis of the *E. coli* enzyme by Yamada and colleagues, who concluded that His^{775} plays an important role in binding PQQ during reconstitution [24]. Mutation to alanine or arginine decreased the affinity of apoenzyme for PQQ 30-fold and 230-fold respectively. They suggested that His^{775} might bond to the C-2 carboxy group of PQQ instead of the serine (Ser^{777}) proposed in our model structure [9]. However, it would be very difficult to model the His^{775} sufficiently close to the PQQ [in the present model it is about 0.8 nm (8 Å) distant], and their alternative suggestion is an attractive one; the substitutions may lead to movement of the position of Ser^{777} to weaken its hydrogen bond to PQQ. Mutation of other residues near the active site ($Ser^{357} \rightarrow$ Leu and $Gly^{689} \rightarrow$ Asp) also led to a 5–7-fold-decreased affinity for PQQ; these mutations also led to production of enzyme with a very low activity (3% of WT-GDH). These residues clearly could not contribute directly to the pK values observed in our work.

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