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Biochimica et Biophysica Acta 1647 (2003) 200–205
BIOCHIMICA ET BIOPHYSICA ACTA
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The metal ion in the active site of the membrane glucose dehydrogenase of *Escherichia coli*

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Received 17 July 2002; accepted 30 September 2002

Abstract

All pyrroloquinoline quinone (PQQ)-containing dehydrogenases whose structures are known contain Ca^{2+} bonded to the PQQ at the active site. However, membrane glucose dehydrogenase (GDH) requires reconstitution with PQQ and Mg^{2+} ions (but not Ca^{2+}) for activity. To address the question of whether the Mg^{2+} replaces the usual active site Ca^{2+} in this enzyme, mutant GDHs were produced in which residues proposed to be involved in binding metal ion were modified (D354N-GDH and N355D-GDH and D354N-GDH/N355D-GDH). The most remarkable observation was that reconstitution with PQQ of the mutant enzymes was not supported by Mg^{2+} ions as in the wild-type GDH, but it could be supported by Ca^{2+}, Sr^{2+} or Ba^{2+} ions. This was competitively inhibited by Mg^{2+}. This result, together with studies on the kinetics of the modified enzymes have led to the conclusion that, although a Ca^{2+} ion is able to form part of the active site of the genetically modified GDH, as in all other PQQ-containing quinoproteins, a Mg^{2+} ion surprisingly replaces Ca^{2+} in the active site of the wild-type GDH.
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Keywords: Escherichia coli; Glucose dehydrogenase; Pyrroloquinoline quinone; Quinoprotein

1. Introduction

Glucose dehydrogenase (GDH) is a membrane-bound quinoprotein that requires pyrroloquinoline quinone (PQQ) as its prosthetic group [1–3]. It catalyses the oxidation of D-glucose to D-gluconate in the periplasm and transfers electrons to cytochrome oxidase through ubiquinone in the electron transport chain. GDH occurs as an apoenzyme in *Escherichia coli* because this organism is unable to synthesise PQQ; the active holoenzyme is readily reconstituted with PQQ and Mg^{2+} ions (but not Ca^{2+} ions). The N-terminal domain (154 amino acids) anchors the protein to the membrane [4] and the remaining amino acids (155–796) form a periplasmic superbarrel domain. This catalytic domain has been modelled on the X-ray structure of the α subunit of the quinoprotein methanol dehydrogenase (MDH) [5] in which PQQ is located at the centre of the superbarrel structure (Fig. 1) tightly coordinated to a Ca^{2+} ion by way of the C-5 carbonyl oxygen, the N-6 ring atom and one oxygen of the C-7 carboxylate of PQQ [6]. This model has been used successfully in interpreting the characteristics of many GDHs produced by site-directed mutagenesis [7–10]. In MDH, the Ca^{2+} ion plays a key role in the enzyme mechanism [2,11] and all PQQ-containing dehydrogenases whose structures have been determined also contain a Ca^{2+} ion bonded to the PQQ at the active site and are likely to have a similar mechanism of action [12]. There is no reason why the membrane GDH discussed here might be expected to be different. In the modelled GDH, PQQ is ligated to the metal ion in the same manner as in MDH and three residues from GDH form an additional four interactions with the metal ion; they are Asp-354, Asn-355 and Thr-424 (Fig. 1). This raises the question of the role of the Mg^{2+} ions that are essential for production of the active holo-GDH from apoenzyme. Either

Abbreviations: PQQ, pyrroloquinoline quinone; WT-GDH, glucose dehydrogenase encoded by the wild-type gene; N355D-GDH, glucose dehydrogenase in which Asn-355 has been altered to aspartic acid; D354N-GDH, glucose dehydrogenase in which Asp-354 has been altered to asparagine; D354N/N355D-GDH, glucose dehydrogenase in which Asn-355 has been altered to aspartic acid and Asp-354 has been altered to asparagine

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doi:10.1016/S1570-9639(03)00041-4

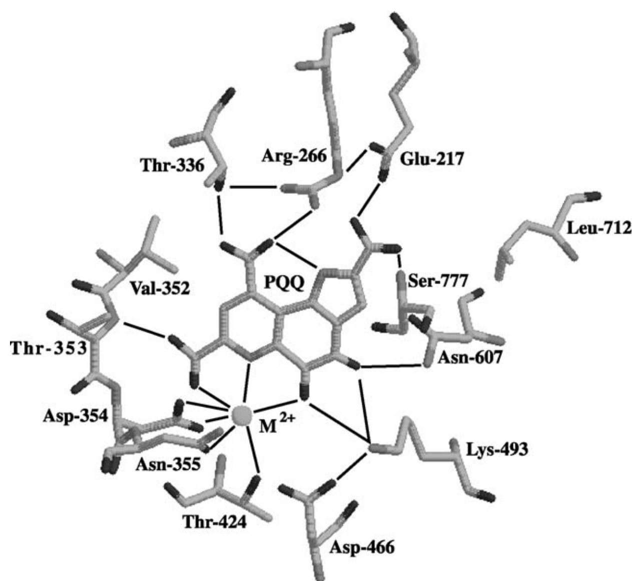


Fig. 1. The active site of the membrane glucose dehydrogenase of *E. coli*. This was modelled on the coordinates of MDH [5,6]. In MDH, the metal ion (M^{2+}) is Ca^{2+}.

the metal ion becomes incorporated into the active site of the holo-GDH, or perhaps it is required for some other purpose. To address this question and to confirm the proposed 'model' active site with respect to metal ion binding, site-directed mutants were produced in which the amino acids proposed to be involved in binding the metal ions were modified.

It is concluded that although a Ca^{2+} ion is able to form part of the active site of the genetically modified GDHs, as in all other PQQ-containing quinoproteins, a Mg^{2+} ion can surprisingly replace this in the active site of the wild-type enzyme.

2. Materials and methods

Unless otherwise stated, all materials and methods used in this work were exactly as described previously [9].

2.1. Growth of bacteria and site-directed mutagenesis

The plasmid pGEC1 containing the gene that codes for GDH was transformed into *E. coli* PP2418 (giving *E. coli* strain PPGEC1) to produce a constitutive overexpression system for GDH. The Stratagene Quickchange® method of mutagenesis was used to make the D354N, N355D and D354N/N355D mutations in GDH. The kit was obtained from Stratagene and the Sequenase® version 2.0 DNA sequencing kit was obtained from Amersham International (UK). Wizard SV DNA purification systems were supplied by Promega (Southampton) and all oligonucleotides used were supplied by Oswel (Southampton).

The plasmid pGEC1 was purified from strain PPGEC1 by using Wizard Plus SV minipreps DNA purification systems. The mutagenic primers used were as follows: for N355D, 5′-GGTTCAGTCACCGAT**GAC**TTCTCAACCCGCGAAACG-3′ and 5′-CGTTTCGCGGGTTGAGAA**GTC**ATCGGTGACTTGAACC-3′; for D354N, 5′-GGTTCAGTCACC**AAT**AACTTCTCAACCCGCGAAACG-3′ and 5′-CGTTTCGCGGGTTGAGAAGTT**ATT**GGTGACTTGAACC-3′; for D354N/N355D, 5′-GGTTCAGTCACC**AATGAC**TTCTCAACCCGCGAAACG-3′ and 5′-CGTTTCGCGGGTTGAGAA**GTCATT**GGTGACTTGAACC-3′ (mutated triplets are indicated in bold type). The mutagenesis reaction mixtures contained the following: 125 ng of primers, 20 ng of pGEC1, 1 μl of dNTP mixture (containing 10 mM dATP, 10 mM dCTP, 10 mM dGTP, 10 mM dTTP); 2.5 units of Pfu Turbo™ DNA polymerase and 10 μl reaction buffer were added to a final volume of 50 μl. Sixteen rounds of the polymerase chain reaction were used, each consisting of a denaturation step at 95 °C for 30 s, an annealing step at 55 °C for 1 min and an elongation step at 37 °C for 13 min. *Dpn*1 (10 units) was added to the PCR product to digest methylated parental DNA, leaving only mutant DNA. PCR products were visualised by DNA electrophoresis and transformed by electroporation into *E. coli* PP2418 (which has the chloramphenicol acetyltransferase gene inserted into the chromosomal *gcd* gene). All mutations were confirmed by DNA sequencing.

2.2. Purification of apo-GDHs, reconstitution to holo-GDHs and measurement of activity

The purification protocol for WT-GDH and mutant GDHs was adapted from those described previously [4,9]. Pipes buffer was used throughout because of the need for subsequent experiments in the presence of Ca^{2+} and Ba^{2+} which would precipitate in the more usual phosphate buffer. After growth in Luria broth, cells were broken by ultrasonication and GDH solubilised from membranes with 0.5% Triton X-100 and purified by ion-exchange chromatography on DEAE-Sepharose.

Holoenzyme was formed from purified apoenzyme by incubating GDH (11 μg in a total of 100 μl solution) with 25 μM PQQ, 5 mM Mg^{2+} and 50 mM buffer (pH 6.5) at 25 °C for 20 min. In some experiments Ca^{2+}, Sr^{2+} or Ba^{2+} replaced Mg^{2+}. The reconstituted GDH was assayed spectrophotometrically (at 600 nm) in a dye-linked system containing phenazine ethosulfate (PES) and 2,6-dichlorophenolindophenol (DCPIP). The 1-ml assay mixture contained 20 mM Tris–HCl (pH 8.75), 40 mM D-glucose, 10 μl reconstituted GDH (0.55 μg), 100 μM DCPIP 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. Kinetic analysis of the results was performed using the program PRIZM™.

3. Results and discussion

3.1. Production, by site-directed mutagenesis of D354N-GDH, N355D-GDH and the double mutant D354N/N355D-GDH

D354N, N355D and D354N/N355D mutations were successfully produced on plasmid pGEC1, expressed in *E. coli* strain PP2418 and the altered GDHs purified as described in the Materials and methods section. Triton X-100 was used for solubilisation of GDH from membranes and, as found previously [4], this caused some inhibition of WT-GDH (40%). Remarkably, all the mutant GDHs were less sensitive to the inhibitory effect of the detergent, the concentration (0.5%) used to solubilise mutant GDHs, not causing any inhibition. After purification by ion-exchange chromatography, the yields were 25–48%, the purification was 10- to 20-fold and the enzymes were more than 80% pure.

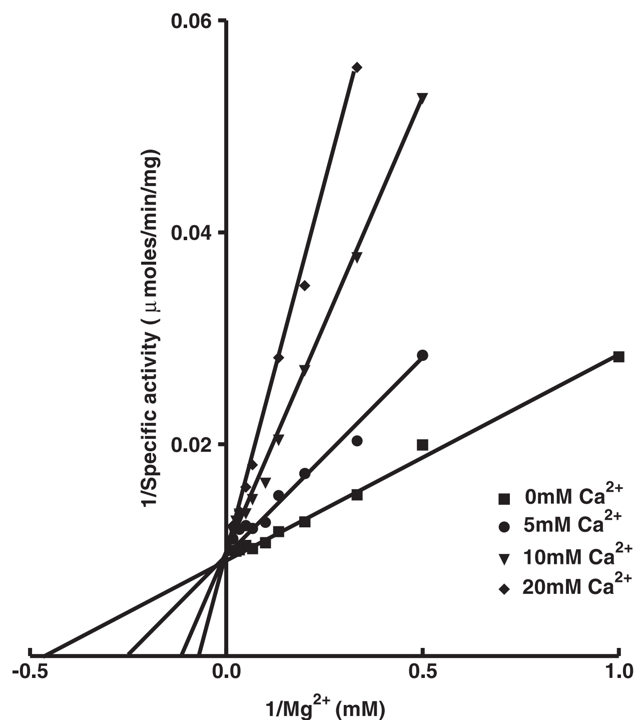
3.2. The metal ion requirements for reconstitution of WT-GDH

The results summarised in Table 1 show that the thermal stability of D354N-GDH and of the double mutant enzyme was similar to that of WT-GDH, in all cases, the stability being greater for the reconstituted, active holoenzyme. The N355D mutation, however, caused a significant increase in thermal stability of both the apo-GDH and the holo-GDH.

Because *E. coli* is unable to synthesise PQQ, GDH is produced as an apo-enzyme, the holoenzyme being produced by reconstitution with PQQ and Mg^{2+} ions. Before measurement of reconstitution, apo-GDH was incubated with 100 mM EDTA for 15 min at 25 °C, the chelating agent being removed by gel filtration. The rate of reconstitution with WT-GDH was very rapid; 80% of maximum activity was reached within 2 min and 100% achieved within 10 min. The K_d values for Mg^{2+} and PQQ were 1.5 mM and 0.28 μM, respectively, and, over the pH range 5.5–8.0, there was no significant difference in the K_d value for Mg^{2+} (all were between 1.0 and 1.9 mM). Reconstitution of WT-GDH with Mg^{2+} concentrations higher than 20 mM inhibited the enzyme, indicating that there was a

Table 1
The thermal stability of WT-GDH and mutant GDHs

	Temperature causing 50% inactivation after incubation for 10 min (°C)		Time required for 50% inactivation at 40 °C (min)	
	Apo-GDH	Holo-GDH	Apo-GDH	Holo-GDH
WT-GDH	40.8	51	14.3	Not denatured
N355D-GDH	43.2	55	Not denatured	Not denatured
D354N-GDH	40.5	49	13.3	Not denatured
D354N-GDH/ N355D-GDH	41.0	48.6	15.0	Not denatured



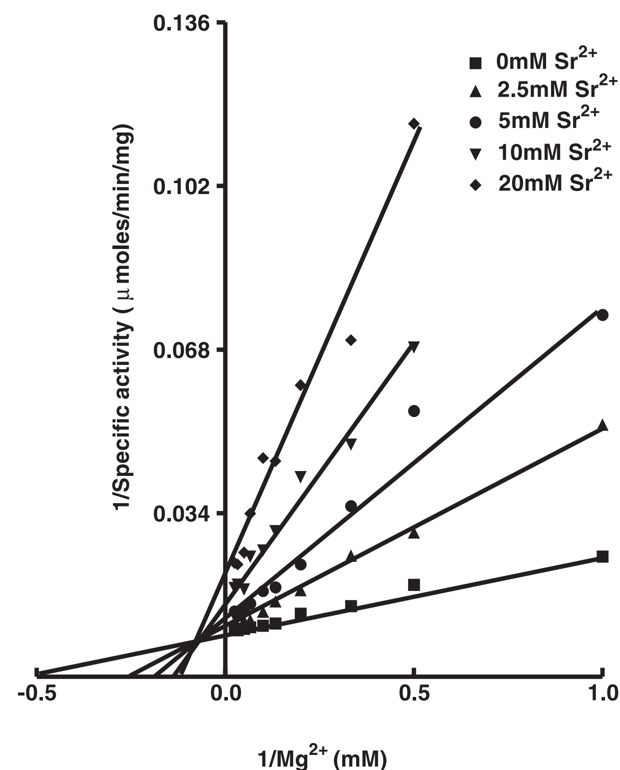


Fig. 2. The effect of Mg^{2+} on reconstitution of WT-GDH previously incubated with PQQ plus Ca^{2+} or Sr^{2+} ions. The double reciprocal plots are for the rate of GDH activity measured after reconstitution of GDH with Mg^{2+} for 20 min after prior incubation (20 min) with Ca^{2+} or Sr^{2+} ions. Identical results were obtained when the GDH was first incubated with Mg^{2+} (20 min) followed by further incubation (20 min) with Ca^{2+} or Sr^{2+} ions before assay of activity.

Table 2
The inhibition by Ca^{2+}, Sr^{2+} or Ba^{2+} of reconstitution of WT-GDH with Mg^{2+}

Concentration (mM)	Inhibiting ion					
	Ca^{2+}		Sr^{2+}		Ba^{2+}	
	A_{max} for Mg^{2+} (μmol/min/mg)	K_d for Mg^{2+} (mM)	A_{max} for Mg^{2+} (μmol/min/mg)	K_d for Mg^{2+} (mM)	A_{max} for Mg^{2+} (μmol/min/mg)	K_d for Mg^{2+} (mM)
0	104.6	1.6	104.6	1.6	104.6	1.6
2.5	–	–	100.6	3.9	90.1	10.9
5	94.2	3.0	89.3	5.7	88.8	20.0
10	94.8	6.8	62.6	6.4	55.1	30.9
20	99.2	12.5	57.3	12.0	14.5	36.6

EDTA-treated GDH was incubated for 20 min with 25 μM PQQ and various concentrations of Ca^{2+}, Sr^{2+} or Ba^{2+}. Then Mg^{2+} (1–60 mM) was added and activity measured after incubation at 25 °C for a further 20 min. The A_{max} and K_d values shown are for Mg^{2+} binding. These values were calculated using the data from Fig. 2.

second (inhibitory) binding site for Mg^{2+} (K_i, 190 mM). The activity of reconstituted GDH was lost by dialysis in 10 mM buffer (pH 7.0) containing 0.1% Triton X-100; subsequent addition of 25 μM PQQ had no effect but addition of 25 μM PQQ plus 5 mM Mg^{2+} generated fully active GDH. These results for WT-GDH were all consistent with those previously published [9].

When Cu^{2+}, Fe^{2+}, Ni^{2+} and Mn^{2+} replaced Mg^{2+} ions in the reconstitution system, GDH was inactivated and activity could not be restored by the addition of Mg^{2+} ions.

Ca^{2+}, Sr^{2+} and Ba^{2+} ions were all reversible inhibitors of Mg^{2+}-supported reconstitution. Ca^{2+} had no effect on A_{max} (the maximum achievable activity with saturating PQQ), but it increased the measured K_d for Mg^{2+}, showing that Ca^{2+} is a competitive inhibitor (Fig. 2). By contrast, Sr^{2+} and Ba^{2+} also caused an increase in the K_d for Mg^{2+} but also caused the A_{max} to decrease (Fig. 2, Table 2), indicating that mixed inhibition had occurred. That is, Sr^{2+} and Ba^{2+} can bind to the active site (the Mg^{2+} binding site) and to a second, low-affinity, inhibitory site which is possibly the same site that leads to inhibition by higher concentrations of Mg^{2+}. The K_i values for Ca^{2+}, Sr^{2+} and Ba^{2+} ions were 3.4, 3.5 and 0.45 mM, respectively; the K_{ii} values (for the second site) for Sr^{2+} and Ba^{2+} ions were 12 and 3.5 mM, respectively.

These results indicate that the active site is able to bind all the divalent metal ions (Mg^{2+}, Ca^{2+}, Sr^{2+} or Ba^{2+}) but that the enzyme is only active when Mg^{2+} is bound.

3.3. The reconstitution of D354N-GDH, N355D-GDH and D354N/N355D-GDH

All mutant enzymes were produced as the apoenzyme and required reconstitution with PQQ for activity, the affinity for this prosthetic group being 1.5–15 times lower than that measured with WT-GDH (Table 3). The rates of reconstitution were very similar to those observed with WT-GDH but the highest activities achieved were lower, being 9–25% as active (with 1.5 M D-glucose) as when measured with WT-GDH (Table 3).

Remarkably, unlike WT-GDH, mutant GDHs could only be reconstituted with Ca^{2+}, Sr^{2+} or Ba^{2+} ions; Mg^{2+} ions

Table 3
The kinetic properties of WT-GDH and the mutant GDHs

Enzyme	Activity with no added metal ion (%)[a]	Activity compared with WT-GDH (%)[b]	K_d for Mg^{2+} (mM)	K_d for Ca^{2+} (mM)	K_d for Sr^{2+} (mM)	K_d for Ba^{2+} (mM)	K_d for PQQ (μM)	
WT-GDH	1	100	1.5	na	na	na	0.28	
N355D-GDH	4	25	na	3.8	1.4	1.2	Ca^{2+}	0.41
							Sr^{2+}	1.48
							Ba^{2+}	0.48
D354N-GDH	67	9	na	nd	nd	nd	Ca^{2+}	4.2
							Sr^{2+}	3.2
							Ba^{2+}	2.9
N355D/D354N-GDH	48	10	na	nd	nd	nd	Ca^{2+}	0.74
							Sr^{2+}	1.0
							Ba^{2+}	0.94

The relative activities were measured in the dye-linked assay system containing 1.5 M glucose after reconstitution in the presence of 25 μM PQQ and 5 mM Mg^{2+} or Ca^{2+} ions.

na, no activity observed; nd, the values for K_d could not be measured because of the high activity that was present in the absence of added metal ions.

[a] Relative activity compared with the maximum achieved after reconstitution with the 'preferred' metal ion.

[b] Relative activities compared with the maximum activity obtained with WT-GDH reconstituted with Mg^{2+} ions.

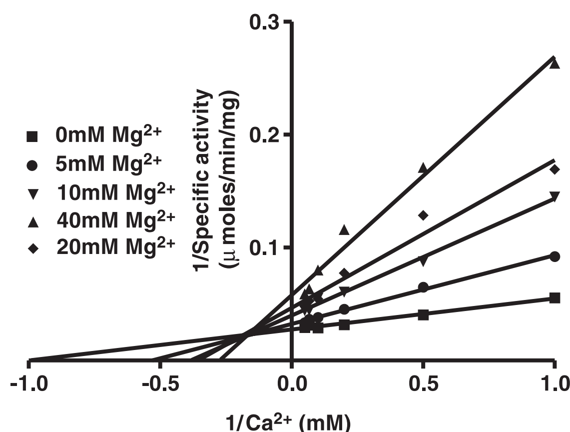


Fig. 3. The effect of Ca^{2+} on reconstitution of N355D-GDH previously incubated with PQQ plus Mg^{2+} ions. The double reciprocal plots are for the rate of GDH activity measured after reconstitution of GDH with Ca^{2+} for 20 min after prior incubation (20 min) with Mg^{2+} ions. Identical results were obtained when the GDH was first incubated with Ca^{2+} (20 min) followed by further incubation (20 min) with Mg^{2+} ions before assay of activity.

did not support reconstitution. The K_d values measured with N355D-GDH (3.8, 1.4 and 1.2 mM for Ca^{2+}, Sr^{2+} and Ba^{2+}, respectively) were similar to the K_d value for Mg^{2+} measured with WT-GDH (1.5 mM) and these values were similar between pH 5.5 and 8.0. The K_i for a second, inhibitory site for Ca^{2+} was 124 mM. Mg^{2+} was a mixed inhibitor, increasing the K_d for reconstitution with Ca^{2+}, and decreasing the A_{max} value (Fig. 3). The K_i and K_{ii} values for Mg^{2+} binding were 11.4 and 39.6 mM, respectively.

The other two mutant enzymes (D354N-GDH and D354N/N355D-GDH) usually had 30–70% of their maximum activities when reconstituted with PQQ alone. Treatment with 100 mM EDTA or EGTA followed by gel filtration could not remove all contaminating metal ions that presumably supported this activity. Addition of 5 mM Ca^{2+}, Sr^{2+} or Ba^{2+} (but not Mg^{2+}) ions to the reconstitution mixture gave fully active enzymes. Incubation with Mg^{2+} ions led to loss of activity obtained after prior reconstitution with Ca^{2+} ions, but full kinetic studies of the reconstitution process could not be performed because of the activity that was always observed in the absence of added metal ions. Incubation of Ca^{2+}-reconstituted D354N-GDH and D354N/N355D-GDH with 20 mM Mg^{2+} ions decreased the activity by 50%.

3.4. The substrate specificity of mutant GDHs

The N355D, D354N and D354N/N355D mutations all markedly decreased the apparent affinity of GDH for glucose, as indicated by the higher K_m values (Table 4). With N355D-GDH, the metal ions used for reconstitution also had an effect on the affinity for substrates, the K_m values for D-glucose with Ca^{2+}, Sr^{2+} and Ba^{2+} being 949, 15.4 and 15.3 mM, respectively. This suggests that the oxidation of substrates is more effective with larger metal ions bound to the active site. N355D-GDH oxidised the same substrates as WT-GDH (Table 4) but the apparent affinity for substrates decreased by 5- to 340-fold and the V_{max} values were also decreased, to 4–40% of WT-GDH values. As previously shown [9], no particular hydroxyl group in the substrate is absolutely essential for substrate binding but, if present, the C6 hydroxymethyl groups of hexose sugars must not be below the plane of the ring if they are to be oxidised.

The affinities of D354N-GDH and D354N/N355D-GDH for all substrates tested were very low (K_m values more than 3 M) and so could not be determined accurately; activities were linear with sugar concentration irrespective of the metal ion used to reconstitute holoenzyme.

4. General discussion

The competition experiments suggest that GDH has two sites for binding divalent metal ions; these are the active site

Table 4
The substrate specificity of WT-GDH and N355D-GDH

Substrate	Mg^{2+}-WT-GDH			Ca^{2+}-N355D-GDH			Sr^{2+}-N355D-GDH			Ba^{2+}-N355D-GDH		
	K_m (mM)[a]	V_{max}[b]	V_{max}/K_m	K_m (mM)	V_{max}	V_{max}/K_m	K_m (mM)	V_{max}	V_{max}/K_m	K_m (mM)	V_{max}	V_{max}/K_m
D-Glucose	2.8	116	41.4	949	44	15.4	15.4	21.7	1.4	15.3	25.9	1.7
2-Deoxy-D-glucose	3.5	137	39.1	803	45.3	0.06	170	25.1	0.15	489	40.3	0.08
L-Arabinose	31	122	3.9	>3000	nd	nd	139	10.9	0.08	197	8.9	0.04
D-Xylose	17	61	3.6	>3000	nd	nd	219	15.1	0.07	80	2.0	0.025
D-Galactose	17.5	48	2.7	1650	2.1	0.0013	1370	13.5	0.01	2270	19.5	0.008
D-Mannose	116	137	1.2	>3000	nd	nd	106	5.2	0.05	135	5.7	0.04
3-*O*-methyl-D-glucose	79	77	0.97	>3000	nd	nd	2130	15.4	0.007	2105	15.6	0.007
D-Ribose	166	41	0.25	>3000	nd	nd	1033	3.2	0.003	2140	6.2	0.003

N355D-GDH was reconstituted under standard conditions with 5 mM metal ions (Ca^{2+}, Sr^{2+} or Ba^{2+}) and 25 µM PQQ. Activity was measured with the standard dye-linked assay containing various concentrations of substrate. The values for WT-GDH were almost identical to those published previously [9]. L-Glucose, D-arabinose and D-maltose were not oxidised.

[a] K_m values are in millimolar.

[b] V_{max} values are in micromoles per minute per milligram; nd values not determined.

in which the ion is bound to amino acids and to PQQ, and a second inhibitory site. Both sites can bind all four divalent metal ions tested in this work but only when Mg^{2+} is bound in the active site of the wild-type GDH is an active enzyme formed. Ca^{2+}, Sr^{2+} and Ba^{2+} ions all compete for this same site but lead to inactive enzyme.

All the results obtained using the purified genetically modified enzymes were consistent with them playing the role in metal ion binding predicted in the model GDH. The most remarkable observation was that reconstitution of active holoenzyme from PQQ and N355D-GDH, D354N-GDH or D354N/N355D-GDH preferred Ca^{2+}, Sr^{2+} and Ba^{2+} for reconstitution instead of Mg^{2+}. This demonstration that mutation of amino acids Asp-354 and Asn-355 changed the specificity for metal ions in the reconstitution process supports the proposal that these metal ions become incorporated into the active site. The conclusions that the mutations had occurred in amino acids closely involved at the active site were consistent with the observation that the affinity for substrates had markedly decreased in all cases, presumably because of some distortion of the active site when larger metal ions had replaced the smaller Mg^{2+} ion.

It can thus be concluded that, although a Ca^{2+} ion is able to form part of the active site of the genetically modified GDHs, as in all other PQQ-containing quinoproteins, a Mg^{2+} ion surprisingly replaces this in the active site of the wild-type enzyme.

Acknowledgements

We thank BBSRC (UK) and The Wellcome Trust for financial support.

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