Reconstitution of Membrane-Integrated Quinoprotein Glucose Dehydrogenase Apoenzyme with PQQ and the Holoenzyme's Mechanism of Action

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Received September 10, 1997; Revised Manuscript Received January 29, 1998

ABSTRACT: Membrane-integrated quinoprotein glucose dehydrogenase from *Acinetobacter calcoaceticus* was produced by heterologous expression of the gene for it in an *Escherichia coli* recombinant strain. The apoenzyme (lacking the cofactor pyrroloquinoline quinone, PQQ) was solubilized with Triton X-100 and purified to homogeneity. Reconstitution of the apoenzyme to full activity in the assay was achieved with a stoichiometric amount of PQQ in the presence of Mg^{2+}. Just as for other PQQ-containing dehydrogenases where Ca^{2+} fulfills this role, Mg^{2+} anchors PQQ to the mGDH protein and activates the bound cofactor. This occurs in a precise way since high anomer specificity was found for the enzyme toward the sugars tested. Although the steady-state-type kinetics were as expected for a dye-linked dehydrogenase (ping-pong) and the PQQ in it was present in oxidized form, addition of glucose to the holoenzyme resulted in a very slow but continuous production of gluconolactone; i.e., the reaction did not stop after one turnover, with O_2 apparently acting as an (albeit poor) electron acceptor by reoxidizing $PQQH_2$ in the enzyme. The surprisingly low reactivity with glucose, in the absence of dye, as compared to the activity observed in the steady-state assay appeared to be due to formation of an anomalous enzyme form, mGDH*. Formation of normal holoenzyme, mGDH, reducing added glucose immediately to gluconolactone (in one turnover), was achieved by treating mGDH* with sulfite, by reconstituting apoenzyme with PQQ in the presence of sulfite, or by applying assay conditions to mGDH* (addition of PMS/DCPIP). As compared to other quinoprotein dehydrogenases, mGDH appears to be unique with respect to the mode of PQQ-binding, as expressed by the special conditions for reconstitution and the absorption spectra of the bound cofactor, and the reactivity of the reduced enzyme toward O_2. The primary cause for this seems not to be related to a different preference for the activating bivalent metal ion but to the special way of binding of PQQ to mGDH.

Membrane-integrated, dye-linked glucose dehydrogenase (mGDH[1]) occurs in many Gram-negative bacteria, either as apoenzyme (without the cofactor pyrroloquinoline quinone, PQQ) or as holoenzyme (with PQQ) (*1*−*3*). Since the enzyme is linked to the respiratory chain via ubiquinone (*4*−*6*), the conversion of glucose into gluconolactone (which is hydrolyzed enzymatically or spontaneously to gluconic acid, depending on the presence of a lactonase and the prevailing pH, respectively) catalyzed by it can generate useful energy for the cell (*7*−*9*). In those bacteria producing the apoenzyme, the latter can be easily reconstituted to active holoenzyme by adding PQQ to the medium. Taking *Escherichia coli* as an example for the group of bacteria producing apo-mGDH but not PQQ, production of the apoenzyme seems useful since it has been shown that this organism exhibits chemotaxis for PQQ (*10*). Moreover, mutants lacking a functional phosphorylative route of glucose dissimilation can grow on glucose provided that PQQ is present in the medium (*2*), suggesting that the possession of a nonphosphorylative route for glucose dissimilation may be beneficial for survival of this organism under certain circumstances.

Most of the efforts which have been made so far to understand the enzymology of glucose oxidation by a PQQ-containing enzyme have focused on the so-called soluble glucose dehydrogenase from *Acinetobacter calcoaceticus* (sGDH, EC 1.1.99.17) (*11*−*16*). Although both s- and mGDH catalyze the same reaction and have the same cofactor, they differ in quite a number of properties: sGDH is a homodimeric enzyme with subunits of 50 kDa, while mGDH is a monomeric enzyme of 80 kDa; the amino acid sequence of mGDH shows some similarity to those of the PQQ-containing alcohol dehydrogenases, but that of sGDH not at all (except for a small stretch of amino acids, not directly involved in PQQ binding or catalysis (*17*)); just as with other PQQ-containing enzymes, reconstitution of apo-sGDH with PQQ is most efficient in the presence of Ca^{2+} (*12*) whereas this is Mg^{2+} in the case of mGDH (*18*); absorption spectra of the holoenzymes are different (*11*, *18*); and sGDH shows substrate inhibition with respect to glucose as well as artificial electron acceptor, but this has not been observed for mGDH (*3*). Although so far it has been

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[1] Abbreviations: mGDH, membrane-integrated quinoprotein glucose dehydrogenase reducible by glucose; mGDH*, the anomalous enzyme form which is not reducible by glucose; sGDH, soluble quinoprotein glucose dehydrogenase; MDH, methanol dehydrogenase; PQQ, pyrroloquinoline quinone (2,7,9-tricarboxy-1*H*-pyrrolo[2,3-*f*]quinoline-4,5-dione); PMS, *N*-methylphenazonium methyl sulfate; DCPIP, 2,6-dichlorophenol indophenol; Q_2, decyl ubiquinone; WB, Wurster's Blue.

S0006-2960(97)02261-7 CCC: $15.00
Published on Web 04/28/1998

implicitly taken for granted that both enzymes have the same mechanism of action, in view of the differences just mentioned it seemed questionable to us whether reconstituted sGDH and mGDH and their catalytic mechanisms would be the same. Previous work has shown how mGDH can be purified from *A. calcoaceticus*, but the yield obtained was rather low (*19*).

Since a recombinant *E. coli* strain has been constructed carrying the gene of *A. calcoaceticus* mGDH (*20*−21), in theory this provided a possibility to circumvent the problem. Thus, in the first instance investigations were carried out to determine whether this strain was suited for the production of the enzyme in adequate yield and of a quality identical to that of the authentic enzyme. By the use of the rational procedure commonly applied in reconstituting quinoprotein dehydrogenase apoenzymes with PQQ, a holoenzyme was obtained which appeared later to be an anomalous enzyme form, indicated here as mGDH*. Subsequently, it was discovered that mGDH* could be converted into an enzyme form showing normal behavior, mGDH, by treating a solution of mGDH* with N_2 gas. However, treatment of a Mg^{2+}-containing PQQ solution in this way indicated that this transformation was not due to O_2 removal but to formation of a PQQ−sulfite adduct which converts mGDH* into mGDH, the adduct probably formed from traces of SO_2 escaping from the O_2 trap used (containing a.o. sodium dithionite). This report describes the preparation and some of the properties of mGDH* and mGDH. To check the reliability of the methods used and to illustrate the uniqueness of mGDH, sGDH, considered to be a normal quinoprotein dehydrogenase, was taken as a reference.

MATERIALS AND METHODS

Materials. The materials used were from the following sources (in parentheses): Bacto agar, casamino acids, tryptone and yeast extract (Difco Laboratories); superoxide dismutase from bovine erythrocytes, ampicillin, and the gluconic acid determination kit (Boehringer Mannheim); thiamine (Fluka); DEAE and CM Toyopearl 650S ion exchangers (TosoHaas Co); Nova-Pak C_{18} (3.9 × 150 mm) HPLC column (Waters); PD-10 gel filtration and Superdex200 columns (Pharmacia); Centricon-30 concentrator (with membranes of 30 kDa cutoff) (Amicon, Inc.); decylubiquinone (Q_2) (Sigma); DNAse (Fluka); DCPIP and PMS (Merck); PES (Sigma). Wurster's Blue (WB) was prepared as described (*22*). Piericidin A was kindly provided by Dr. S. Albracht. All other materials were of analytical grade and were obtained from commercial sources. Soluble glucose dehydrogenase from *A. calcoaceticus* (sGDH, EC 1.1.99.17), in apo as well as holo form, was prepared as described (*12*).

Bacterial Strain and Growth Conditions. The recombinant *E. coli* strain (PP 2419 + pGP 469) containing the mGDH gene from *A. calcoaceticus* was kindly provided by Dr. N. Goosen. It was grown on Luria broth containing 40 μg/mL ampicillin and 0.5 μg/mL thiamine at 30 °C, as previously described (*23*). The cells were harvested at the end of the exponential growth phase.

Apoenzyme Purification and Reconstitution. Purification of apo-mGDH was done with a procedure adapted from the existing one for holoenzyme (*19*). All steps were performed at about 4 °C, unless indicated otherwise. Buffer exchange or protein concentration was done with the membrane concentrator. Harvested cells were washed with 50 mM potassium phosphate buffer, pH 7.0, and suspended in the same buffer. The suspension was passed through a French pressure cell at 28 MPa, and DNAse and $MgCl_2$ (to 4 mM final concentration) were added. The mixture was centrifuged at 2000*g* for 25 min to remove intact cells and cell debris.

The supernatant was centrifuged at 150000*g* for 90 min to sediment the membrane fraction. The pellets were suspended in 10 mM potassium phosphate buffer, pH 6.0, containing 0.2% Triton X-100, to a final concentration of 10 mg of protein/mL. After a 30-min incubation on ice, the suspension was centrifuged at 150000*g* for 90 min. The pellets were resuspended in 50 mM potassium phosphate buffer, pH 7.0, and the solution was brought to a final concentration of 1% Triton X-100 and 0.3 M KCl. After incubating for 30 min on ice, the solution was centrifuged at 150000*g* for 90 min. The supernatant was dialyzed overnight against a 50-fold volume of 0.1% Triton X-100.

The dialysate was centrifuged to remove any aggregates, and the supernatant was applied to a DEAE-Toyopearl column equilibrated with 50 mM potassium phosphate buffer, pH 8.0, containing 0.1% Triton X-100 (1 mL of bed volume per 1−2 mg of protein applied). The column was washed with the same buffer at a flow rate of 2.5 mL/min. The enzyme passed through and the active fractions were pooled and applied to a CM-Toyopearl column equilibrated with 50 mM potassium phosphate buffer, pH 6.0, containing 0.1% Triton X-100. The column was eluted with a gradient of 0.1% Triton X-100 to 50 mM potassium phosphate buffer, pH 6.0, containing 1 M NaCl and 0.1% Triton X-100 at a flow rate of 1.0 mL/min. Active fractions were pooled and stored at −20 °C.

To reconstitute apoenzyme to mGDH*, the sample was mixed with an equal volume of 0.1 M $MgCl_2$ and subsequently with a solution containing at least a stoichiometric amount of PQQ, the amount calculated from the molar absorbance (*23*). Preparation of mGDH was done by mixing 180 μL apoenzyme (2 μM) in 20 mM MOPS buffer, pH 7.0, with 20 μl of the same buffer containing 20 μM PQQ, 1 mM $MgCl_2$, and 0.4 mM Na_2SO_3. Holo-mGDH formation was completed after 30-min incubation. Conversion of mGDH* into mGDH was carried out by bringing an mGDH* solution (17 μM) to 1 mM *N*-methylphenazonium methyl sulfate (PMS) and 50 μM 2,6-dichlorophenol indophenol (DCPIP). Removal of low molecular weight compounds from mGDH was done by gel filtration on a PD10 column, equilibrated with 50 mM potassium phosphate buffer, pH 7.0. To achieve the appropriate enzyme concentration for the assay, samples were diluted with 50 mM potassium phosphate buffer, pH 7.0, containing 0.1% Triton X-100.

Enzyme Assay and Kinetics. Enzyme activity was determined spectrophotometrically at 600 nm, following the reduction of DCPIP (ϵ = 21000 M^{-1} cm^{-1}) in time with PMS as primary electron acceptor. The 1-mL assay mixture consisted of 50 mM glycine/NaOH buffer, pH 8.5, containing 50 mM glucose, 50 μM DCPIP, and 1 mM PMS. The assay was carried out at room temperature, and about 1 pmol of enzyme was used. One activity unit (U) corresponds with the amount of enzyme converting 1 μmol of glucose or

DCPIP per minute under the assay conditions specified above.

To determine the steady-state kinetic parameters, varying concentrations in the range of 1−10 mM substrate (except for glucose, 1−25 mM) and 12.5−200 μM PMS or PES (*N*-ethylphenazonium ethyl sulfate), 5−200 μM Q_2 (decyl ubiquinone), or 25−70 μM WB (Wurster's Blue) were used. In cases where electron acceptors other than PMS were used, the buffers were (for PES/DCPIP and Wurster's Blue) 50 mM glycine, pH 8.5, and (for PMS/DCPIP, DCPIP, and Q_2/DCPIP) 50 mM potassium phosphate, pH 6.0. To calculate the apparent kinetic parameter values (with their error margins), the program LEONORA, Analysis of Michaelis Menten Data, version 1.0 (1994), written by Dr. A. Cornish-Bowden (*24*), was used.

Pre-steady-state kinetic measurements were performed with a stopped-flow reaction analyzer (SX-18MV) from Applied Photophysics using 2.3 μM holoenzyme in 50 mM potassium phosphate buffer, pH 7.0, containing 0.1% Triton X-100 and 10−100 μM glucose in one syringe and 40 μM PMS with 40 μM DCPIP in the other.

Analytical Procedures. The protein content was determined by the modified Bradford method (*25*, *26*), using salt-free bovine serum albumin as a standard.

The stereospecificity of the enzyme was investigated for several sugars by measuring its change in activity over time, starting with freshly prepared sugar solutions, taking samples from these at regular time intervals, and assaying the samples in the standard assay. The rate of change determined from this was compared with the reported rate of anomerization of the sugar used.

To determine the tightness of binding of PQQ to mGDH*, gel filtration chromatography of holoenzyme was carried out on a Superdex200 column in 50 mM potassium phosphate buffer, pH 7.0, containing 0.1% Triton X-100 at a flow rate of 0.5 mL/min. The eluate was monitored with a UV detector at 280 nm and with a fluorescence detector (excitation at 365 nm, emission at 460 nm).

For cofactor extraction, a solution containing 5.5 nmol of holoenzyme was brought to 40% methanol and 1 M HCl. Aggregated protein was removed by a 5-min centrifugation at 12000*g*. The supernatant was analyzed by HPLC as previously described (*27*).

To determine the turnover number of the enzyme with O_2, glucose (1000-fold excess) was added to 30 nmol of mGDH* in 50 mM potassium phosphate buffer, pH 7.0, containing 0.1% Triton X-100 . Enzyme and product were separated by membrane filtration, and the amount of gluconic acid formed was determined in the filtrate with the assay kit.

Electrophoresis was done with the Pharmacia Phast System with protein standards, SDS-containing polyacrylamide gels, and protocols provided by the same manufacturer.

Fluorescence and absorption spectra were recorded with a Shimadzu RF-5001PC and a Hewlett-Packard 8453 diode-array spectrophotometer, respectively.

RESULTS

Isolation and Characteristics of Recombinant Apo-mGDH. Reproducible growth of the *E. coli* recombinant strain and adequate production of mGDH was observed (yield: 0.47 mg of enzyme/g of cell paste). Although about 40% higher levels of enzyme were produced in the presence of the inducer isopropyl β-D-1-thiogalactopyranoside, the costs of this compound prohibited its application in large-scale cultivations. Since mGDH activity in the cell homogenate was only observed when reconstitution was carried out with PQQ, apparently the enzyme is produced in its apo form. This is in line with the observation that *E. coli* strains do not produce PQQ (*2*). When the cell homogenate was centrifuged, all mGDH activity was present in the pellet. The activity could be solubilized by extracting the pellet with buffer containing Triton X-100. Since the amino acid sequence predicts mGDH to be a very hydrophobic protein (*20*) and activities remained constant in the presence of Triton X-100, buffers used in the purification steps were supplemented with this detergent.

Table 1: Purification of Apo-mGDH from the *E. coli* Recombinant Strain Using 36 g of Cell Paste

purification step	protein (mg)	act. (U)	sp act. (U/mg)	yield (%)	fold purification
cell-free extract	7000	41 600	6	100	1
membrane fraction	1600	36 000	22	86	4
washed membranes	1000	35 000	35	84	6
solubilized membrane	500	22 700	45	54	8
DEAE-Toyopearl	87	20 000	228	48	41
CM-Toyopearl	17	10 400	612	25	108

The enzyme could be isolated in reasonable yield (Table 1). SDS−PAGE of the final preparation showed only one band with protein staining, the position of the band in a calibrated gradient gel corresponding to a protein of 80 kDa. This value is similar to that calculated (83 kDa) from the amino acid sequence derived from the gene sequence (*21*). Since the specific activity (Table 1; ref *19*, the absorption spectrum (Figure 1; ref *18*), and the substrate and electron acceptor specificity (Tables 2 and 3; ref *3*) of the reconstituted, recombinant enzyme were comparable to those of the authentic holoenzyme, the two seem to be identical, implying that correct processing to mature enzyme occurred in the recombinant strain.

Binding of PQQ. Reconstitution of apoenzyme with PQQ to activity in the assay required the presence of Mg^{2+}, in accordance with the observations reported for authentic apoenzyme (*18*). The holoenzyme produced by this procedure is further indicated here as mGDH*. EDTA (10 mM) prevented reconstitution when it was present before the addition of Mg^{2+} but did not reverse it, as judged from the absence of inhibition when it was added after the reconstitution. As shown by the titration curve in Figure 2, a linear relationship existed between the amount of PQQ added and the activity measured. It was calculated from this that the dissociation constant for PQQ from mGDH* is 270 nM and that 0.98 mol of PQQ binds per mol of apoenzyme. Only small differences were observed between the absorption spectrum of mGDH* and that of free PQQ in the presence of Mg^{2+} (Figure 1A). mGDH* exhibited fluorescence, the emission as well as the excitation spectra (Figure 3) being similar to those of free PQQ (*28*) (NB, the fluorescence of a PQQ-containing solution, derives from PQQ covalently hydrated at the C_5-position, which is in a temperature-dependent equilibrium with the nonfluorescing PQQ). Moreover, a temperature decrease enhanced the fluorescence intensity of the enzyme (Figure 4), just as for free PQQ (28).

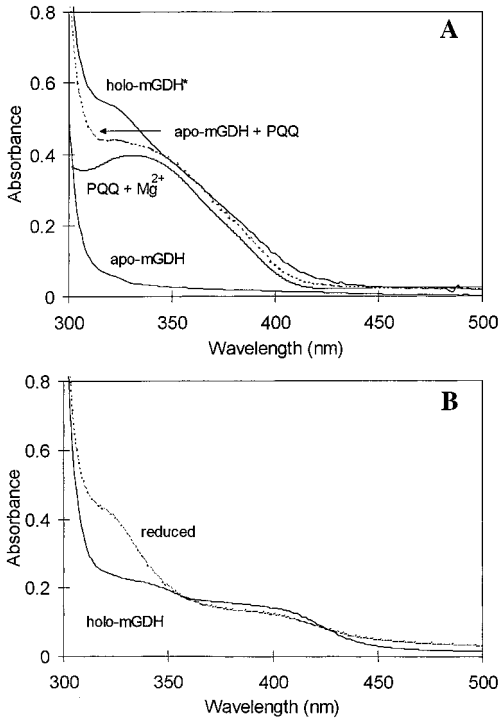

FIGURE 1: Absorption spectra of mGDH* and its constituents and mGDH. (A) Spectra of 20 μM holo-mGDH*, 20 μM apo-mGDH, apo-mGDH + PQQ (both 20 μM), and PQQ (20 μM) + $MgCl_2$ (1 mM) measured in 50 mM potassium phosphate buffer, pH 7.0, containing 0.1% Triton X-100. (B) Absorption spectra of 20 μM holo-mGDH, prepared by reconstitution in the presence of Na_2SO_3 (0.4 mM), and after reduction by glucose (100 μM).

To determine whether the fluorescence originated from mGDH*-bound or from free PQQ (dissociated from the enzyme), titrations of PQQ were performed with increasing amounts of apoenzyme. As shown in Figure 5, a plateau was obtained, indicating that the fluorescence derives from enzyme-bound PQQ but that the intensity is (20%) lower than that corresponding with the same amount of free PQQ. Values for the dissociation constant and the stoichiometric ratio of PQQ to enzyme were calculated from the titration that were similar to those indicated above for the titration of apoenzyme with PQQ.

On gel filtration of mGDH*, PQQ appeared to be bound to the protein, as judged from the coincidence of the absorbance and fluorescence traces representative for PQQ with that of the absorbance trace representative for the protein (results not shown). However, it was estimated from the peak areas and from the specific activities from the fractions that only 50% of the original amount of PQQ remained bound to mGDH* under the chromatography conditions applied (PQQ in sGDH is not detached under these conditions (*16*)). In view of the relatively high dissociation constant value of mGDH* and the practically immeasurable value for sGDH (*16*), this is not unexpected. When Mg^{2+} was absent during the reconstitution process, no PQQ was detected in the protein peak trace.

When the reconstitution took place in the presence of Na_2SO_3, the holoenzyme that formed, further indicated as mGDH, showed a somewhat different absorption spectrum as compared to that of mGDH* (Figure 1, panels A and B) and no fluorescence was observed. Treatment of mGDH* with PMS and DCPIP, followed by gel filtration, yielded a preparation with the same spectroscopic properties as mGDH. Similarly, treatment of mGDH* with Na_2SO_3 also yielded mGDH.

Kinetic Investigations. mGDH* showed normal behavior in the assay with PMS/DCPIP, as judged from the linear traces representing DCPIP reduction and from the proportionality existing for the amount of enzyme used and the activity measured. However, at low PMS concentrations, a short lag time was observed before the reaction started. Using the same amounts (in units) of PMS and enzyme, this lag phase was not seen in the case of sGDH (results not shown). Steady-state kinetics were performed with a variety of sugars, the plots showing parallel lines and no indications for substrate inhibition, confirming previous observations (*3*). The kinetic parameter values of mGDH* for several aldose sugars and artificial electron acceptors as well as for Q_2 are given in Tables 2 and 3. They show that the specificity constant value for a certain electron acceptor ($V_m/K_{M,B}$) does not vary for different sugars. In the reverse case, for unknown reasons a somewhat larger spread is seen in the value for the specificity constant of the sugar ($V_m/K_{M,A}$) when the electron acceptor is varied. Ignoring this spread, the data indicate ping-pong kinetic behavior of the enzyme. Piericidin appeared to be an inhibitor for mGDH* activity in assays where PMS or Q_2 was used as electron acceptor, but not in the case of sGDH with PMS as electron acceptor. Using the standard assay with PMS, the presence of 10 μM piericidin caused 90% inhibition of mGDH*. The inhibition was overcome by increasing either the glucose or the PMS concentration (Figure 6), but the pattern was too complicated to allow conclusions to be made.

To determine whether O_2 was able to act as electron acceptor for mGDH*, mixtures were probed with an electrode to detect any O_2 consumption. The result was positive for the standard assay mixture. Moreover, a 6-fold decrease in the DCPIP reduction rate was observed when superoxide dismutase (400 U) was added to the standard assay mixture. However, no O_2 consumption was detected when PMS was lacking in this mixture. When the assay was carried out at pH 7.0 (50 mM potassium phosphate buffer), no O_2 consumption was detected and addition of superoxide dismutase had no effect. The same results were obtained when sGDH was used instead of mGDH*.

Pre-steady-state kinetics, determined with a stopped-flow spectrophotometer by rapid mixing of a solution of mGDH* plus glucose with a solution containing PMS plus DCPIP, showed that the reduction of the enzyme started after mixing, as judged from the linear decrease of the DCPIP absorbance in time (Figure 7). However, when mGDH was used, an initial burst was seen in the trace (Figure 7), indicating that reduced enzyme was already present before the mixing with PMS/DCPIP took place. Judging from the size of the burst (with a corresponding decrease in the DCPIP concentration in the mixture of 0.95 μM) and the amount of enzyme used (1.15 μM in the mixture), the enzyme was catalytically fully active (83% in the experiment shown). The parallel traces shown in Figure 7 indicate that mGDH* and mGDH have the same specific activity.

Conversion Experiments. When an excess of glucose was added to mGDH*, followed by immediate separation of low molecular weight compounds from the enzyme by membrane filtration of the mixture and subsequent assay of the filtrate,

Table 2: Kinetic Parameter Values of mGDH for Several Substrates and PMS or PES, Determined with Steady-State Kinetics

substrate	V_m (U/mg)	$K_{M,A}$[a] (mM)	$K_{M,B}$[b] (μM)	$V_m/K_{M,A}$ (U/mg/M)	$V_m/K_{M,B}$ (U/mg/M)
			Electron Acceptor PMS		
D-glucose	720 ± 39	4 ± 0.4	178 ± 12	$(180 \pm 9) \times 10^3$	$(40 \pm 1) \times 10^5$
2-deoxyglucose	1619 ± 272	22 ± 4.3	362 ± 70	$(74 \pm 2) \times 10^3$	$(45 \pm 2) \times 10^5$
D-fucose	1017 ± 225	12 ± 3.2	220 ± 56	$(85 \pm 7) \times 10^3$	$(46 \pm 2) \times 10^5$
L-arabinose	459 ± 89	18 ± 4.2	118 ± 30	$(26 \pm 2) \times 10^3$	$(39 \pm 3) \times 10^5$
D-xylose	715 ± 164	12 ± 3.3	156 ± 45	$(60 \pm 5) \times 10^3$	$(46 \pm 4) \times 10^5$
D-galactose	377 ± 54	19 ± 3.6	64 ± 17	$(20 \pm 1) \times 10^3$	$(59 \pm 11) \times 10^5$
			Electron Acceptor PES		
D-glucose	540 ± 42	4 ± 0.7	278 ± 29	$(135 \pm 15) \times 10^3$	$(19 \pm 1) \times 10^5$
D-xylose	448 ± 17	7 ± 0.5	268 ± 17	$(64 \pm 3) \times 10^3$	$(17 \pm 1) \times 10^5$
D-fucose	486 ± 43	5 ± 0.9	291 ± 34	$(97 \pm 9) \times 10^3$	$(17 \pm 1) \times 10^5$
L-arabinose	416 ± 22	19 ± 2.0	292 ± 26	$(22 \pm 1) \times 10^3$	$(14 \pm 1) \times 10^5$

[a] $K_{M,A}$ is the Michaelis constant for the substrate. [b] $K_{M,B}$ is the Michaelis constant for the electron acceptor.

Table 3: Kinetic Parameter Values of mGDH for Several Electron Acceptors with Glucose as Substrate

electron acceptor (pH)	V_{max} (U/mg)	$K_{M,A}$ (mM)	$K_{M,B}$ (mM)	$V_{max}/K_{M,A}$ (U/mg/M)	$V_{max}/K_{M,B}$ (U/mg/M)
PMS/DCPIP (8.5)	720 ± 39	4.0 ± 0.4	178 ± 12	$(180 \pm 9) \times 10^3$	$(4.04 \pm 0.06) \times 10^6$
PES/DCPIP (8.5)	540 ± 37	4.1 ± 0.6	280 ± 26	$(135 \pm 15) \times 10^3$	$(1.94 \pm 0.05) \times 10^6$
WB (8.5)	1405 ± 894	5.9 ± 5.8	138 ± 143	$(238 \pm 43) \times 10^3$	$(10.18 \pm 1.35) \times 10^6$
PMS/DCPIP (6.0)	364 ± 49	3.9 ± 1.4	560 ± 124	$(93 \pm 32) \times 10^3$	$(0.65 \pm 0.05) \times 10^6$
DCPIP (6.0)	28 ± 3	0.23 ± 0.11	29 ± 7	$(122 \pm 88) \times 10^3$	$(0.97 \pm 1.81) \times 10^6$
Q_2/DCPIP (6.0)	45 ± 6	0.31 ± 0.09	2.1 ± 1.4	$(145 \pm 34) \times 10^3$	$(21.43 \pm 34.66) \times 10^6$

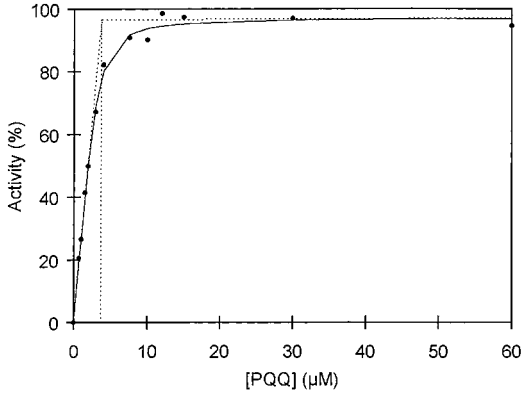

FIGURE 2: Titration of apo-mGDH with PQQ. To apo-mGDH (3.3 μM) in 50 mM potassium phosphate buffer, pH 7.0, containing 0.1% Triton X-100 and 33 mM $MgCl_2$ were added aliquots of PQQ in the same buffer. After a 60-min incubation, the activity of the samples was determined with the standard assay. The data were fitted with the following equation:

$$\text{activity} = {}^1/_2 C\{([\text{apo}] + [\text{PQQ}] + K_d) - \sqrt{([\text{apo}] + [\text{PQQ}] + K_d)^2 - 4[\text{apo}][\text{PQQ}]}\}$$

in which C is a constant and K_d is the dissociation constant.

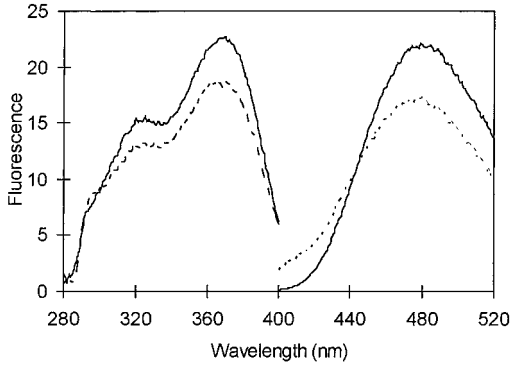

FIGURE 3: Fluorescence spectra of holo-mGDH* and free PQQ. Excitation (emission at 469 nm) and emission (excitation at 365 nm) spectra were measured for holo-mGDH* (2 μM, broken lines) and PQQ (2 μM, solid lines) in 50 mM potassium phosphate buffer, pH 7.0, containing 0.1% Triton X-100 and 1 mM $MgCl_2$.

no gluconolactone (gluconic acid) was detected (Figure 8). This was not due to an inadequate experimental design since sGDH yielded a stoichiometric amount (one turnover, i.e., approximately 2 gluconolactone molecules formed per enzyme molecule) under the same conditions. mGDH behaved similarly to sGDH, also showing immediate formation of gluconolactone in one turnover with glucose (i.e. approximately 1 gluconolactone molecule formed per enzyme molecule). On the other hand, when mGDH* and glucose were incubated longer, gluconolactone was detected, the amount determined even exceeding the stoichiometric ratio after some time. This indicated that mGDH* is able to react with glucose and that O_2 is an electron acceptor for mGDH* but not for sGDH. The low rate (0.06 turnover/min) could be due either to the slow reaction of mGDH* with glucose or to the slow reoxidation of reduced enzyme with O_2. The latter appears to be the case since mGDH gave results similar to those from mGDH* during long incubations with glucose.

mGDH* preferred the β-anomer of D-glucose, D-galactose, D-mannose, and D-xylose and the α-anomer of L-arabinose, as judged from the activity changes shown by the freshly made sugar solutions occurring in time in the assay. Since the anomerization rates determined from this appeared to be very similar to those determined in another way (*29*) (Table 4), the preference is high, if not absolute.

Redox State of PQQ in the Enzyme Forms. To detect whether the cofactor in mGDH* was really in its oxidized form (mGDH contains several cysteinyl residues (*20*−*21*), so that PQQ might become reduced in the reconstitution process), the cofactor was extracted from the enzyme and the extract was chromatographed under acidic conditions to prevent oxidation of any extracted $PQQH_2$ (*27*). $PQQH_2$ was absent, indicating that the reconstituted enzyme is in the fully oxidized form. This is in line with the observation that DCPIP was not reduced when a PMS/DCPIP mixture was added to a mGDH* (or mGDH) preparation.

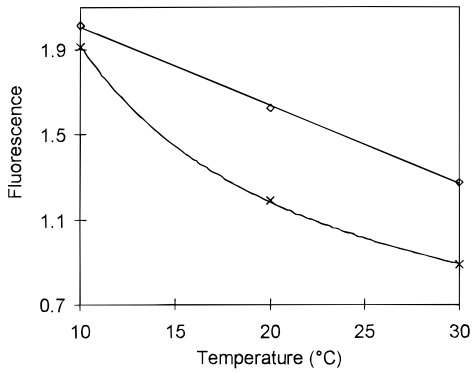

FIGURE 4: Temperature dependence of the fluorescence intensity. The fluorescence intensity (excitation at 364 nm, emission at 472 nm) as a function of the temperature was measured for holo-mGDH* (10 μM, $\times$) and for free PQQ (10 μM, $\diamond$), both in 50 mM potassium phosphate buffer, pH 7.0, containing 0.1% Triton X-100 and 1 mM $MgCl_2$.

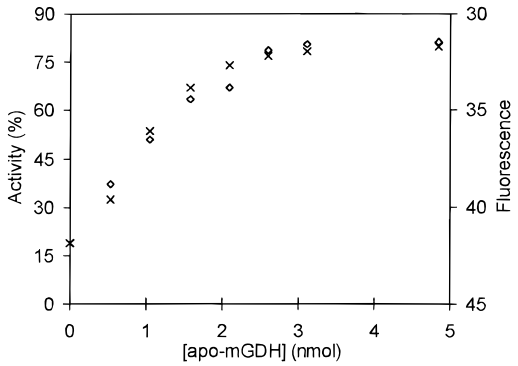

FIGURE 5: Titration of PQQ with apo-mGDH. To PQQ (2.7 nmol) in 50 mM potassium phosphate buffer, pH 7.0, containing 0.1% Triton X-100 and 1 mM $MgCl_2$ (1 mL), were added aliquots of apo-mGDH in the same buffer. After a for 60-min incubation, the activity ($\diamond$, in the standard assay) as well as the fluorescence intensity ($\times$, excitation at 365 nm, emission at 472 nm) were measured.

Surprisingly, when glucose was added to mGDH*, neither the absorption nor the fluorescence spectrum changed (results not shown). On the other hand, when dithiothreitol was added to mGDH*, a red-shifted (10 nm), a "$PQQH_2$-like" spectrum (*27*) was obtained. Such a spectrum was also obtained when glucose was added to mGDH (Figure 1B), and cofactor extraction showed indeed that formation of $PQQH_2$ takes place when glucose is added.

However, the spectral change in mGDH induced by glucose did not occur in the presence of piericidin. When mGDH was prepared by reconstituting apoenzyme in the presence of Ca^{2+} instead of Mg^{2+}, the spectra of oxidized and reduced enzyme were similar to those of normal enzyme (results not shown).

DISCUSSION

Production of Apo-mGDH. The *E. coli* recombinant strain used here produced *A. calcoaceticus* mGDH apoenzyme in good yield. As judged from SDS−PAGE and protein staining, the final enzyme preparation was homogeneous. The recombinant enzyme appeared to be identical to the authentic enzyme according to similarity with respect to specific activity, substrate specificity, and molecular mass.

Reconstitution to Holoenzyme and Characteristics of Bound PQQ(H_2). Titration of apo-mGDH with PQQ (Figure 2) in the absence and presence of Mg^{2+}, titration of PQQ with apoenzyme, and gel filtration chromatography of mGDH* showed that Mg^{2+} is required for binding of PQQ and for activity, and that one PQQ is bound per enzyme molecule but that binding of PQQ to the protein is weaker as compared to sGDH. Thus, just as for Ca^{2+} in sGDH (*12*, *16*, *18*), the role of Mg^{2+} seems to be confined to anchoring the PQQ to the protein and functionalizing the bound PQQ. Once the holoenzyme has been formed, the Mg^{2+} ion is locked up in a strong complex not accessible to chelator, as judged from the fact that EDTA prevented reconstitution but did not affect activity once the holoenzyme was formed.

The titration and gel filtration experiments indicated that PQQ is firmly bound to the apoenzyme but that the dissociation constant of PQQ for mGDH* is measurable.

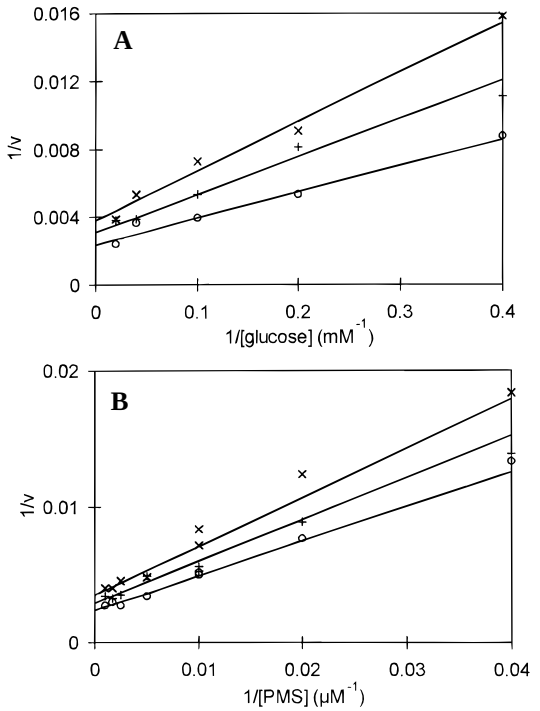

FIGURE 6: Inhibition of mGDH with piericidin. The inhibition effect of piericidin at concentrations of 0 ($\bigcirc$), 5 (+), and 10 ($\times$) μM was determined with varying concentrations of glucose (A) or with varying concentrations of PMS (B).

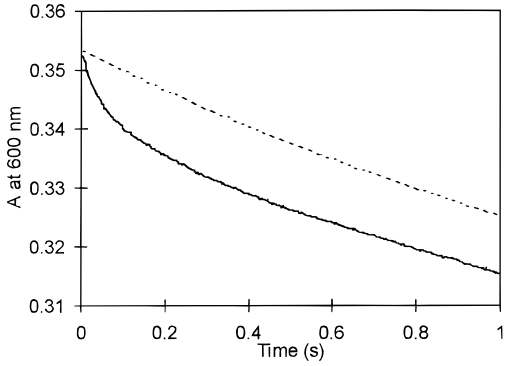

FIGURE 7: Pre-steady-state kinetics of mGDH* and mGDH. The stopped-flow apparatus contained a solution of 2.3 μM holo-mGDH* (broken line) or holo-mGDH (solid line) and 50 μM glucose in 50 mM potassium phosphate buffer, pH 7.0, containing 0.1% Triton X-100, in one syringe and a solution of 40 μM PMS and 40 μM DCPIP in the same buffer in the other syringe. After mixing, the reduction of DCPIP was followed over time at 600 nm.

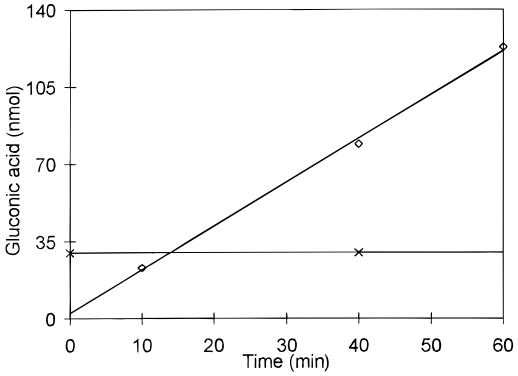

FIGURE 8: Conversion of glucose by mGDH* and sGDH. To mGDH* (30 nmol) (◇) sGDH (15 nmol) (×) in 50 mM potassium phosphate buffer, pH 7.0, containing 0.1% Triton X-100, was added glucose (5 mM). Gluconic acid was determined either immediately or after a certain incubation time in the filtrate fraction with the assay kit.

Table 4: Anomer Specificity of MGDH for Several Sugars

substrate	anomer preference	K^a (10^{-4} s^{-1})	K^b (10^{-4} s^{-1})
α-D-glucose	β	2.8	3.8
β-D-glucose	β	2.6	3.8
α-D-galactose	β	6.3	
β-D-mannose	β	11.5	10.7
α-D-xylose	β	16.5	12.5
β-L-arabinose	α	15.3	

[a] K is the rate of anomerization calculated from the activities determined with the standard assay, using the following equation (*29*): activity $= aA_0 + (b - a)B_e(1 - \exp(-Kt))$ in which a and b are the first-order rate constants for the oxidation of the α- and β-anomers, A_0 is the concentration of the α-anomer at the start, and B_e is the equilibrium concentration of the anomer mixture. [b] Anomerization rate values given in ref *29*.

According to the PQQ-like absorption spectrum exhibited by mGDH* (Figure 1A), interaction of the cofactor with the protein scarcely affects those moieties of PQQ (in particular, the aromatic rings) directly responsible for light absorption in the part of the spectrum measured. Also, interaction with the C_5-carbonyl group seems to be absent since PQQ in the oxidized enzyme was fluorescing, the intensity of it being affected by temperature in the same way as for free PQQ, suggesting that the group is freely accessible to H_2O.

Since mGDH is nonfluorescing, it might be argued that PQQ in the form of the hydrate or any other adduct is absent in the active site. However, preliminary investigations showed that PQQ in the solution used for reconstituting apoenzyme was also nonfluorescing, although the absorption spectrum was very similar to that of the PQQ hydrate. This could mean that PQQ forms a nonfluorescing adduct with sulfite which enables formation of mGDH, whereas PQQ and PQQ−H_2O are unable to do so. However, the role of sulfite seems dispensable in this since transformation of mGDH* into mGDH also takes place when mGDH* is treated with PMS/DCPIP or in the assay with a variety of artificial electron acceptors. Although the mechanism in the latter case is also unclear, a mediating effect of a specific (contaminating) compound seems unlikely. The rate of transformation seems rather slow in this case, as suggested by the lag time observed for the assay of mGDH* when low PMS concentrations were used. Since the mGDH prepared by the sulfite method or by incubation with PMS/DCPIP showed identical absorption spectra and specific activities, it is highly unlikely that the PQQ−sulfite adduct postulated above is still present after reconstitution to mGDH has taken place. Reconstitution in vivo occurs rapidly, as illustrated by the immediate start of glucose-mediated respiration when PQQ is added to cells containing mGDH in the apo-form (*1*). Whether formation of holo-mGDH is assisted by some component present in the periplasm or is due to the presence of the natural electron acceptor ubiquinone is presently unknown. However, the latter possibility is in line with the fact that mGDH* is active in the assay with Q_2.

On the basis of the three-dimensional structure of methanol dehydrogenase (MDH) (*30*, *31*), it has been proposed that PQQ is present in a hydrophobic cavity, close to a disulfide ring (formed by two vicinal cysteinyl groups) and the ring of a tryptophan residue at the bottom of the cavity. In addition, interactions (mediated by Ca^{2+}) were proposed to exist between amino acid residues and the carboxylic acid groups and the two carbonyl groups of PQQ. The oxidized and reduced forms of MDH show large red shifts of their absorption maxima as compared to those of free PQQ and $PQQH_2$, respectively (*32*). The reduced form of sGDH shows a red shift (33 nm) similar to that observed for the reduced form of MDH, whereas PQQ in the oxidized enzyme form is not fluorescing (*16*). Since mGDH* has spectral features more reminiscent of those of free PQQ, and since the absorption maximum of $PQQH_2$ in reduced mGDH exhibits only a small red shift (10 nm), the optical properties of the cofactor in mGDH are quite different from those in MDH and sGDH. This is related to the unique properties of the active site of mGDH and not to the fact that it has Mg^{2+} as the activating metal ion instead of Ca^{2+}, because replacement of Mg^{2+} by Ca^{2+} in mGDH did not affect the spectral properties.

Vicinal cysteinyl groups are lacking in sGDH as well as mGDH. Since amino acid sequence similarity between sGDH and MDH is absent, it cannot be determined whether the special tryptophyl in the cavity is present or not. Alignment of the amino acid sequence of mGDH with that of MDH (*17*) indicated that the special tryptophyl residue and the hydrophobic lining of the cavity in which PQQ is residing in MDH are most probably lacking, too, in mGDH. Thus, from the comparison of primary structures, it is not surprising that PQQ in mGDH is bound in a different way as compared to that in MDH and sGDH.

PQQ and Ca^{2+} in sGDH are very firmly bound, the accessibility of Ca^{2+} in the protein to chelator requiring dissociation of the subunits of holo-sGDH by heat (*16*), and PQQ being fully bound to the protein under all chromatographic conditions, in line with the fact that the dissociation constant of sGDH is so small that a reliable value could not be calculated from the titration curve of apoenzyme with PQQ (*12*).

The binding of Mg^{2+} to mGDH* seems rather strong, as suggested by the observation that addition of EDTA to holo-mGDH did not affect its activity in the assay. As compared to sGDH, the binding of PQQ is not strong since dissociation constant from mGDH* is rather high (270 nM), in line with the release of PQQ from the enzyme upon gel filtration. This rather loose binding of the cofactor does not affect the selectivity of the enzyme, the high (if not absolute) anomer specificity of mGDH indicating that substrate binding occurs in a precise way. On the other hand, it may affect the

catalytic fitness of the enzyme since the turnover number of mGDH (540 s^{-1}) is much lower than that of sGDH (6200 s^{-1}) (*12*) (as determined with the standard assays).

Mechanistic Behavior. The kinetic behavior of mGDH* under steady-state conditions strongly suggested a ping-pong mechanism. It was surprising to find, therefore, that the addition of glucose to mGDH* did not change its fluorescence or absorption spectra, and that no (immediate) production of gluconolactone or $PQQH_2$ was detected, although extraction showed that the cofactor in mGDH* was in the fully oxidized state. The latter was in line with the observation that mGDH* in the absence of glucose did not react with PMS and that addition of dithiothreitol changed the absorption spectrum into that of a red-shifted $PQQH_2$-like spectrum. The discrepancy can be explained now from the foregoing: the inactivity of mGDH* toward glucose and its activity in the assay is due to the transformation of inactive mGDH* into mGDH in the assay. The kinetic behavior, together with the fact that mGDH in the oxidized form reacts with glucose in the absence of electron acceptor, indicates that, just like that of many other dye-linked dehydrogenases, the catalytic cycle of mGDH proceeds according to a ping-pong mechanism.

Reaction of O_2 with the Reduced Enzyme. The fact that slow but continuous formation of gluconolactone was observed when mGDH was incubated with glucose indicates that O_2 reacts with the reduced enzyme (either directly or with dissociated $PQQH_2$). Inhibition of the respiratory chain by piericidin has been ascribed to its binding with the ubiquinone binding site (*33*). Since ubiquinone is the natural electron acceptor for mGDH (*5*) and PMS (and O_2) might react at the same site, inhibition studies were performed with piericidin. Unfortunately, mixed inhibition was observed which could be due to interaction of piericidin with reduced as well as oxidized mGDH. The latter is in line with the observation that piericidin was able to inhibit reduction of mGDH by glucose.

It has been reported that O_2 inhibits the activity of methanol dehydrogenase (the assay carried out with the PMS/DCPIP couple). The inhibition was ascribed to reaction of O_2 with $PQQH_2$ present in the reduced enzyme form, yielding PQQ and superoxide, the latter concluded from the observation that the presence of superoxide dismutase enhanced the inhibition (*35*). The authors did not observe this phenomenon in the case of the TTQ-containing quinoprotein, methylamine dehydrogenase, from which they concluded that methanol dehydrogenase is unique in this respect. However, methylamine dehydrogenase is assayed with PES/DCPIP as electron acceptor at pH 7.5 (*36*), and methanol dehydrogenase, at pH 9.0 (*35*), which could mean that the pH of the assay is the cause for the different behavior and the phenomenon observed. When we investigated the effect for mGDH as well as sGDH, it appeared that inhibition of DCPIP reduction in the assay by O_2 occurred for both enzymes at pH 8.5 but not at 7.0. Since no O_2 consumption was observed for sGDH when incubated with glucose (Figure 8; ref 12), at neither pH 8.5 nor 7.0, the inhibition must be a general phenomenon, i.e. an efficient competition of O_2 with DCPIP at high pH for a reduced PMS species, perhaps yielding superoxide. This implies that the poor electron acceptor function of O_2 for mGDH is unique among the quinoprotein dehydrogenases and that the conclusion that O_2 reacts with reduced methanol dehydrogenase (*35*) could be incorrect. The extent which the unique binding mode of PQQ to the protein, its anchoring and activation by Mg^{2+}, or easy accessibility of the active site is responsible for the susceptibility of reduced mGDH for O_2 remains to be investigated. However, the spectral similarity of mGDH in which Mg^{2+} was replaced by Ca^{2+} to normal mGDH and the dissimilarity between mGDH and sGDH suggest that differences in binding of PQQ are the primary cause for this.

ACKNOWLEDGMENT

We thank Dr. S. Albracht and Dr. N. Goosen for providing us with piericidin and the *E. coli* recombinant strain, respectively.

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BI9722610