Purification and characterization of quinoprotein glucose dehydrogenase from *Acinetobacter calcoaceticus* L.M.D. 79.41

Paul DOKTER, Johannes FRANK, Jzn. and Johannis A. DUINE

Delft University of Technology Laboratory of Microbiology and Enzymology, Julianalaan 67, 2628 BC Delft, The Netherlands

Quinoprotein glucose dehydrogenase (EC 1.1.99.17) from *Acinetobacter calcoaceticus* L.M.D. 79.41 was purified to homogeneity. It is a basic protein with an isoelectric point of 9.5 and an M_r of 94000. Denaturation yields two molecules of PQQ/molecule and a protein with an M_r of 48000, indicating that the enzyme consists of two subunits, which are probably identical because even numbers of aromatic amino acids were found. The oxidized enzyme form has an absorption maximum at 350 nm, and the reduced form, obtained after the addition of glucose, at 338 nm. Since double-reciprocal plots of initial reaction rates with various concentrations of glucose or electron acceptor show parallel lines, and substrate inhibition is observed for glucose as well as for electron acceptor at high concentrations, a ping-pong kinetic behaviour with the two reactants exists. From the plots, K_m values for glucose and Wurster's Blue of 22 mM and 0.78 mM respectively, and a V_{max} of 7.730 μ mol of glucose oxidized/min per mg of protein were derived. The enzyme shows a broad substrate specificity for aldose sugars. Cationic electron acceptors are active in the assay, anionic acceptors are not. A pH optimum of 9.0 was found with Wurster's Blue and 6.0 with 2,6-dichlorophenol-indophenol. Two types of quinoprotein glucose dehydrogenases seem to exist: type I enzymes are acidic proteins from which PQQ can be removed by dialysis against EDTA-containing buffers (examples are found in *Escherichia coli*, *Klebsiella aerogenes* and *Pseudomonas* sp.); type II enzymes are basic proteins from which PQQ is not removed by dialysis against EDTA-containing buffers (examples are found in *A. calcoaceticus* and *Gluconobacter oxydans*).

INTRODUCTION

Bacteria converting aldose sugar(s) into the corresponding lactones frequently contain an NAD(P)-independent dye-linked glucose (aldose) dehydrogenase. The enzyme from *Acinetobacter calcoaceticus* (formerly known as *Bacterium anitratum*) has been purified to homogeneity and partially characterized (Hauge *et al.*, 1964). Although at that time from the scarce data no structural assignments could be made, it was already clear that the enzyme contained an unusual cofactor. Despite this important finding, no further work on this aspect was reported. After the discovery of PQQ as the cofactor in methanol dehydrogenase (EC 1.1.99.8), comparative studies revealed that the cofactor of glucose dehydrogenase from *A. calcoaceticus* was also PQQ (Duine *et al.*, 1979). This resulted in a reclassification and the enzyme is now indicated as 'quinoprotein (PQQ-containing) glucose dehydrogenase' (EC 1.1.99.17) (Nomenclature Committee of the International Union of Biochemistry, 1979). Since in the meantime several similar enzymes have been reported to occur in other bacteria, e.g. *Pseudomonas aeruginosa* (Duine & Frank, 1980), *Gluconobacter suboxydans* (Ameyama *et al.*, 1981), *Pseudomonas fluorescens* (Matsushita & Ameyama, 1982) and *Klebsiella aerogenes* (Neijssel *et al.*, 1983), it seems safe to conclude that all bacterial dye-linked glucose dehydrogenases are quinoproteins.

Recent work on non-acid-producing *Acinetobacter lwoffii* strains revealed that they contain quinoprotein glucose dehydrogenase apoenzyme (van Schie *et al.*, 1984). This phenomenon appears to be widespread, and

even the common *Escherichia coli* laboratory strains harbour it, the apoenzyme becoming functional only after addition of PQQ (Hommes *et al.*, 1984). From this, it might be concluded that the enzyme has been overlooked in the past, and that in the natural environment non-phosphorylative glucose dissimilation occurs more frequently than previously thought.

In view of the recent discoveries, more information on glucose dehydrogenase is required. Moreover, the enzyme is an attractive model system to study the enzymology of quinoproteins, since, in contrast with methanol dehydrogenase, its activity does not depend on the presence of an activator, and reconstitutable apoenzyme forms do exist, or can be prepared from holoenzyme (Duine *et al.*, 1979).

Unfortunately, the strain originally used by Hauge (1964) has been lost, and even some doubt has been expressed about its identity (Beardmore-Gray & Anthony, 1983). As a consequence, it was necessary to purify the enzyme from a genuine *A. calcoaceticus* strain, to compare the results with those obtained by Hauge (1964), and to characterize the enzyme more extensively.

EXPERIMENTAL

Materials

Wurster's Blue was prepared as described previously (Duine *et al.*, 1978). The PQQ used was prepared by both biological (Duine & Frank, 1980) and synthetic methods (Corey & Tramontano, 1981). 2-Mercaptoethanol was from Bio-Rad Laboratories. Tris was from Janssen

Abbreviations used: PQQ, pyrroloquinoline quinone (2,7,9-tricarboxy-1H-pyrrolo[2,3-f]quinoline-4,5-dione); Wurster's Blue is the free radical of *NNN'*-tetramethyl-*p*-phenylenediamine.

Chimica. Alcohol dehydrogenase (product no. 102709) was from Boehringer. Fractogel TSK HW-50(S), KH_2PO_4 , Na_2HPO_4 , D-glucose, L-arabinose, D-xylose, lactose, 2,6-dichlorophenol-indophenol, conc. HCl, SDS, guanidinium chloride and NaCl were from Merck. CM-Sephadex, Sephadex G-100, Sepharose CL-6B, gradient gels PAA 4/30 and molecular-mass calibration (low- M_r and high- M_r) kits were from Pharmacia. Polyacrylamide, bisacrylamide, *NNN'*-tetramethylethylenediamine, Coomassie Brilliant Blue G-250, bovine serum albumin (product no. 11920), carbonic anhydrase (product no. 15880), chymotrypsin (product no. 17160) and conalbumin (product no. 17465) were from Serva. Cytochrome *c* (product no. C-7752), ribonuclease (product no. R-4875), ovalbumin (product no. A-7641) and phosphorylase *b* (product no. P-6635) were from Sigma Chemical Co. N_2 was from Hoekloos and the O_2 filter (model 7970) was from Chrompack.

Culture conditions

Acetate-limited chemostat cultures of *A. calcoaceticus* L.M.D. 79.41 (identified with the API 20 NE system) were provided by B. J. van Schie (van Schie *et al.*, 1984). The cells were harvested and stored frozen at -20°C .

Enzyme purification

Cell-free extract (400 ml) was prepared from cells (200 g wet wt.) as described previously (Duine *et al.*, 1979) and applied to a CM-Sephadex column (4.4 cm $\times$ 5 cm) equilibrated with 20 mM-potassium phosphate buffer, pH 7.0. The column was washed with the same buffer (5 column volumes). The enzyme was eluted with 0.2 M-potassium phosphate buffer, pH 7.0. After concentration by pressure filtration on a type PTGC 047.10 Pellicon membrane (Millipore), gel-filtration chromatography was performed on a Fractogel TSK HW-50(S) column (2 cm $\times$ 60 cm) in 50 mM-potassium phosphate buffer, pH 7.0. The pooled fractions were applied to a CM-Sephadex column (1 cm $\times$ 5 cm) and eluted with a linear gradient of 0–0.2 M-NaCl in 20 mM-potassium phosphate buffer, pH 7.0. Finally, h.p.l.c. gel filtration was performed on a Serva Si-300-polyol column (0.5 cm $\times$ 9.1 cm) in 0.1 M-sodium phosphate buffer, pH 6.5, at a flow rate of 0.5 ml/min.

Enzyme assay

Activities were determined at room temperature by measuring the rate of discoloration of Wurster's Blue at 610 nm in a mixture containing 80 μM -Wurster's Blue, 1 mM-KCN, enzyme or cell-free extract, 0.1 M-Tris/HCl buffer, pH 7.0, and 20 mM substrate in a final volume of 2 ml. The reaction was started by adding the substrate. One enzyme unit refers to 1 μmol of aldose sugar converted/min under these conditions. The calculations were based on a molar absorption coefficient (calculated from absorbance measurements) for Wurster's Blue of $12400\text{ M}^{-1}\cdot\text{cm}^{-1}$ at 610 nm. Activities were also determined with 2,6-dichlorophenol-indophenol as electron acceptor, by the assay described by Hauge (1960).

Protein determinations

During purification, protein concentrations were determined by the method of Bradford (1976), with bovine serum albumin as a standard. For the purified enzyme the *a* value was obtained from absorbance measurements at 205 and 280 nm by using the

chromatographic procedure described by van Iersel *et al.* (1985). Protein concentrations of pure enzyme solutions were calculated from the absorbance values at 280 nm.

Polyacrylamide-gel electrophoresis

Glucose dehydrogenase was electrophoresed in gel slabs of 7.7% polyacrylamide cross-linked with 0.2% bisacrylamide, in 0.02 M-potassium phosphate buffer, pH 7.0, with the anode above the application side of the gel. For enzyme activity staining, the gels were immersed in a solution containing 0.1 M-Tris/HCl buffer, pH 7.0, 20 mM-glucose and 600 μM -Wurster's Blue. Enzyme activity was visible as a white band on a dark-blue background. Protein staining was done with Coomassie Brilliant Blue G-250 (Pharmacia, 1984). Electrophoresis under denaturing conditions was performed on polyacrylamide gradient gels (PAA 4/30) in the presence of SDS, with the use of high- M_r and low- M_r electrophoresis calibration kits as a reference (method described by Pharmacia, 1984).

Kinetic measurements

Substrate specificity. Activity of cell-free extract and of partially purified enzyme towards aldose sugars (20 mM) was determined spectrophotometrically as described above.

Kinetic parameters. Initial reaction rates were determined with various concentrations of glucose and Wurster's Blue at room temperature under the conditions used in the enzyme assay. An enzyme concentration of 1 nM was used. The measured values and concentrations were plotted according to the method of Lineweaver & Burk (1934). Kinetic parameters were determined from secondary plots.

M_r determinations

The M_r of the native enzyme was determined by the method of Andrews (1965) by gel filtration on a Sephadex G-100 column (1 cm $\times$ 55 cm) in 50 mM-potassium phosphate buffer, pH 7.0, containing 0.1 M-NaCl at a flow rate of 7.8 ml/h. Proteins used for calibration were: horse heart cytochrome *c* (M_r 12500), ribonuclease (M_r 13700), chymotrypsin (M_r 25000), carbonic anhydrase (M_r 30000), ovalbumin (M_r 43000), bovine serum albumin (M_r 67000), conalbumin (M_r 86000) and baker's-yeast alcohol dehydrogenase (M_r 141000). M_r determinations of the denatured enzymes were performed by gel filtration on a Sepharose CL-6B column (1 cm $\times$ 55 cm) in 6 M-guanidinium chloride with or without the addition of 2-mercaptoethanol (0.3 M) to the sample. Phosphorylase *b* (M_r 92500), bovine serum albumin (M_r 67000), carbonic anhydrase (M_r 30000), ribonuclease (M_r 13700) and cytochrome *c* (M_r 12500) were used as standards for calibration of the column (Ansari & Mage, 1977).

PQQ analysis

A 150 μl sample of the enzyme in 0.1 M-sodium phosphate buffer, pH 6.5, containing 0.2% SDS was kept at 100°C for 10 min. The solution was subsequently injected on a Serva Si-300 (4.5 mm $\times$ 250 mm) h.p.l.c. gel-filtration column eluted with 0.1 M-sodium phosphate buffer, pH 6.5, containing 0.1% SDS at a flow rate of 0.3 ml/min. The eluate was monitored at 280, 249 and 330 nm with a photodiode array detector (Hewlett–

Table 1. Purification of glucose dehydrogenase from *A. calcoaceticus* L.M.D. 79.41

For experimental details see the text.

Step	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Yield (%)
Cell-free extract	12500	8800	0.7	100
CM-Sepharose (0.2 M eluate)	400	7900	20	90
Gel filtration (Fractogel)	42	4600	110	50
CM-Sepharose (gradient)	8.7	2700	340	30
H.p.l.c. gel filtration	1.5	960	640	10

Packard model 1040A). The PQQ content of the enzyme sample was calculated from the peak height from the low- M_r factor monitored at 330 nm (identified as PQQ, by comparing the retention time and absorption spectrum with those of authentic PQQ). The system was calibrated by injecting 150 μ l samples of six different PQQ concentrations in the range 2–20 μ M.

Aromatic amino acids

The aromatic amino acid composition of the enzyme was determined by multicomponent analysis of the absorption spectrum of the denatured enzyme in 6 M-guanidinium chloride, measured with a Hewlett-Packard model HP 8450A spectrophotometer. Standards used for the calculations were those described by Levine & Frederici (1982). The absorption spectrum above 300 nm appeared to be identical with that of PQQ under these conditions. This was used to correct the spectrum below 300 nm for the presence of PQQ before performance of the multicomponent analysis. It was also used as a method to measure the amount of PQQ in the enzyme.

Isoelectric point

Isoelectric focusing was performed on a horizontal gel slab [2 mm thick, 5% acrylamide, 0.15% bisacrylamide and 2% (v/v) Ampholine buffer, pH 3–10 (LKB-Produkter)] by following the method described by LKB-Produkter (1977).

RESULTS

Purification

Results of the purification procedure are presented in Table 1. Glucose dehydrogenase was eluted from the CM-Sepharose column at a concentration of 80 mM-NaCl. It had a retention time of 45 min on the h.p.l.c. column. The final preparation gave one single band on polyacrylamide-gel electrophoresis, by protein staining as well as by enzyme-activity staining. The normalized absorption spectra taken from the enzyme peak eluted from the h.p.l.c. column (upslope, at the top and downslope) were identical.

Substrate specificity

Other substrates found to be active with the purified enzyme were D-xylose (20%), L-arabinose (35%), lactose (65%), galactose (30%), D-melibiose (10%), cellobiose (70%) and maltose (90%) (activities compared with D-glucose given in parentheses). Similar ratios were obtained with 2,6-dichlorophenol-indophenol as electron acceptor. The enzyme has a pH optimum of 9.0 (in

Fig. 1. Initial reaction rates of quinoprotein glucose dehydrogenase (1 nM) with glucose as the variable substrate

The Wurster's Blue concentrations were 63 μ M ($\blacktriangle$), 82 μ M ($\triangle$), 155 μ M ($\blacksquare$) and 286 μ M ($\square$).

Tris/HCl buffers) with Wurster's Blue and 6.0 (in potassium phosphate buffers) with 2,6-dichlorophenol-indophenol as electron acceptor. $K_3Fe(CN)_6$ was not an electron acceptor in the assay, either at pH 9.0 or at pH 6.0.

Kinetic parameters

As determined experimentally, at certain ratios of glucose to Wurster's Blue concentrations the plots of the kinetic data (Fig. 1) gave parallel straight lines. Substrate inhibition was observed with glucose (Fig. 2) as well as with Wurster's Blue (the latter is not shown, but is clear from the downward curvature in Fig. 2). Assuming ping-pong kinetics, K_m values of 22 mM and 0.78 mM could be derived from secondary plots for glucose and Wurster's Blue respectively. The V_{max} was calculated to be 7.730 μ mol of glucose oxidized/min per mg of protein. The specific reaction rate with the assay described by Hauge (1960) was 490 units/mg of protein. The specific reaction rate at pH 9.0 and 25 $^{\circ}$ C in a mixture containing 80 μ M-Wurster's Blue, 1 mM-KCN, enzyme, 0.1 M-Tris/HCl buffer and 20 mM-glucose in a final volume of 2 ml was 1100 units/mg of protein.

M_r determinations

The M_r of the native enzyme, determined by gel filtration, was 94000 ($\pm$ 5000). The M_r of the denatured

Fig. 2. Substrate inhibition of quinoprotein glucose dehydrogenase (1 nM) by glucose

The Wurster's Blue concentrations were 16 μM (Δ), 102 μM ($\blacksquare$) and 336 μM ($\square$).

enzyme was 47 500 (± 5000) as determined by gel filtration and 48 000 (± 5000) as determined by electrophoresis on a polyacrylamide gradient gel in SDS.

PQQ content

From the h.p.l.c. gel-filtration chromatogram of enzyme denatured with SDS the PQQ concentration was calculated to be 18.4 μM (2.76 nmol) for an enzyme concentration of 10.2 μM (1.53 nmol) (by using $a = 14 \text{ litre} \cdot \text{g}^{-1} \cdot \text{cm}^{-1}$ and M_r 94 000). From the absorption spectrum of enzyme denatured with guanidinium chloride it was calculated that 1.88 μM (1.5 nmol) enzyme solution contains 4.04 μM (3.2 nmol) PQQ.

Aromatic amino acid content

All the common aromatic amino acids were present. The numbers of residues per enzyme molecule were found to be: phenylalanine, 21.8; tryptophan, 10.4; tyrosine, 41.6.

Isoelectric point

The isoelectric point was found to be 9.5, so that the enzyme is a basic protein, a property in accordance with its chromatographic behaviour.

Absorption spectrum

The absorption spectrum of the enzyme as it is isolated (Fig. 3) has a broad maximum at 350 nm, and the spectrum of the enzyme measured under anaerobic conditions in the presence of an excess of glucose has a sharp maximum at 338 nm.

DISCUSSION

Quinoprotein glucose dehydrogenase from *A. calcoaceticus* L.M.D. 79.41 was purified to homogeneity. The M_r of the native enzyme (94 000), the substrate specificity, the apparent K_m for glucose (3 mM) and the pH optimum (6.0) with 2,6-dichlorophenol-indophenol as electron acceptor are comparable with those of the enzyme from *Bacterium anitratum* (Hauge, 1964). Similarly, the ping-pong kinetic behaviour, as indicated by the parallel lines in Fig. 1 and the dual-substrate

Fig. 3. Absorption spectra of quinoprotein glucose dehydrogenase

The absorption spectrum was measured in 0.1 M-sodium phosphate buffer, pH 6.5, before (—) and after (----) the addition of glucose to the cuvette under anaerobic conditions (bubbling with N_2).

inhibition with glucose and Wurster's Blue, were also observed with glucose and 2,6-dichlorophenol-indophenol by Hauge (1960). Further support for the kinetic similarity is reflected by the comparable ratios of the activities (Hauge, 1960) obtained with different aldose sugars.

Just like the glucose dehydrogenases from *A. calcoaceticus* L.M.D. 70.9 and L.M.D. 79.39 (Duine *et al.*, 1979), the enzyme from *A. calcoaceticus* L.M.D. 79.41 is a quinoprotein. This, most probably, also applies to the enzyme from *B. anitratum* in view of the shape of the absorption spectra of the reduced and the oxidized enzyme forms (Hauge, 1964). The enzyme has an M_r of 94 000 and consists of two subunits with the same M_r (48 000), the subunits probably being identical as even numbers of aromatic amino acid residues and two molecules of PQQ per enzyme molecule were found. The enzyme from *B. anitratum* (Hauge, 1964) was reported to have a similar M_r (86 000) but only one cofactor per enzyme molecule (determined by titrating the oxidized enzyme form with glucose). The lower cofactor content of the enzyme from *B. anitratum* is also apparent from the absorption spectra (Hauge, 1964). Although the maxima of the reduced and the oxidized enzyme forms (338 and 350 nm) and the specific absorption coefficients ($a = 14 \text{ litre} \cdot \text{g}^{-1} \cdot \text{cm}^{-1}$) are identical, different ratios exist for the absorbancies in the spectra (data in parentheses are those estimated for the enzyme from *B. anitratum*): the oxidized enzyme form shows a broad maximum around 350 nm and ratios of A_{280}/A_{350} of 7.2 (11.3) and A_{280}/A_{280} of 1.56 (1.65). The reduced enzyme form has

a sharp maximum at 338 nm and ratios of A_{280}/A_{338} of 2.6 (3.6) and A_{280}/A_{260} of 1.76 (1.9). The lower cofactor content of the enzyme from *B. anitratum* might be due to the loss of one cofactor molecule during purification. However, this explanation is unlikely since its specific activity was comparable with that from the enzyme of *A. calcoaceticus* L.M.D. 79.41 in the assay with 2,6-dichlorophenol-indophenol. Otherwise it could be reasoned that the latter enzyme preparation contains inactive enzyme. However, this seems unlikely too in view of the fact that the oxidized enzyme could be fully converted into the reduced form by titration with glucose (Fig. 3). Therefore, despite the 2-fold higher PQQ content, it appears that the enzyme from *A. calcoaceticus* has a turnover number comparable with that of the enzyme from *B. anitratum*.

The enzyme as it is isolated from *A. calcoaceticus* L.M.D. 79.41 contains the two cofactors in their oxidized form since only PQQ was obtained under acidic denaturing conditions (PQQH₂ is stable against oxidation at low pH). Upon addition of glucose a characteristic change in the spectrum occurs, due to reduction of the cofactor. Interestingly, the absorption spectra of both redox forms of the enzyme are different from those of PQQ and PQQH₂ at pH 7 (Dekker *et al.*, 1982) but are similar to those of PQQ and PQQH₂ at high pH (Duine *et al.*, 1981).

Quinoprotein glucose dehydrogenases can be subdivided into two types. Type I enzymes have a low isoelectric point, PQQ can be removed by dialysis against EDTA-containing buffer and reconstitution has been achieved in the presence of Ca²⁺ salts (Imanaga *et al.*, 1979) or Mg²⁺ salts (Duine *et al.*, 1983). Representatives of this type have been isolated from *Pseudomonas aeruginosa* (Duine *et al.*, 1983, and unpublished work), *Klebsiella aerogenes* (Neijssel *et al.*, 1983, and unpublished work), *Escherichia coli* (Hommes *et al.*, 1984; Ameyama *et al.*, 1985) and *Pseudomonas fluorescens* (Imanaga *et al.*, 1979; Matsushita *et al.*, 1980). The last-mentioned enzyme has been subjected to several investigations. Although it has an M_r comparable with that of the enzyme from *A. calcoaceticus* L.M.D. 79.41, it is probably a monomer since the M_r observed under denaturing conditions was the same as that for the native enzyme (Matsushita *et al.*, 1980). It is only soluble in the presence of 1% Triton X-100 (Matsushita & Ameyama, 1982) and is inhibited by several nucleotides (Wurtz, 1982), whereas the enzyme from *A. calcoaceticus* L.M.D. 79.41 is soluble and is not inhibited by these compounds (results not shown).

Type II quinoprotein glucose dehydrogenases have high isoelectric points and PQQ is not removed by dialysis against EDTA-containing buffer. Most probably, all *A. calcoaceticus* glucose dehydrogenases belong to this type, since similar enzymes were present in several strains of this bacterium (P. Dokter, J. Frank & J. A. Duine, unpublished work). In addition, the enzyme from *Gluconobacter suboxydans* (Ameyama *et al.*, 1981) seems closely related, as it is a basic enzyme (isoelectric point pH 7.8), has a similar M_r and PQQ was not removed by dialysis against EDTA-containing buffer.

Since it was found that K₃Fe(CN)₆ is not active as electron acceptor, the difference in activities and the pH optimum of 6.0 with 2,6-dichlorophenol-indophenol are

probably due to the fact that 2,6-dichlorophenol-indophenol has a pK_a value around 6 and the genuine pH optimum of glucose dehydrogenase from *A. calcoaceticus* L.M.D. 79.41 is presumably at 9.0.

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