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Reversible thermal inactivation of the quinoprotein glucose dehydrogenase from *Acinetobacter calcoaceticus*

Ca^{2+} ions are necessary for re-activation*

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The soluble form of the homogeneous quinoprotein glucose dehydrogenase from *Acinetobacter calcoaceticus* is reversibly inactivated at temperatures above 35 °C. An equilibrium is established between active and denatured enzyme, this depending on the protein concentration and the inactivation temperature used. Upon thermal inactivation the enzyme dissociates into the prosthetic group pyrroloquinoline quinone and the apo form of glucose dehydrogenase. After inactivation at 50 °C active enzyme is re-formed again at 25 °C. Ca^{2+} ions are necessary for the re-activation process. The velocity of re-activation depends on the protein concentration, the concentration of the prosthetic group pyrroloquinoline quinone and the Ca^{2+} concentration. The apo form of glucose dehydrogenase can be isolated, and in the presence of pyrroloquinoline quinone and Ca^{2+} active holoenzyme is formed. Even though native glucose dehydrogenase is not inactivated in the presence of EDTA or *trans*-1,2-diaminocyclohexane-*NNN'N'*-tetra-acetic acid, Ca^{2+} stabilizes the enzyme against thermal inactivation. Two Ca^{2+} ions are found per subunit of glucose dehydrogenase. The data suggest that pyrroloquinoline quinone is bound at the active site via a Ca^{2+} bridge. Mn^{2+} and Cd^{2+} can replace Ca^{2+} in the re-activation mixture.

INTRODUCTION

Quinoproteins are a novel class of oxidoreductases, which depend on pyrroloquinoline quinone (PQQ). The prosthetic group reversibly accepts the reduction equivalents (Fig. 1). Besides these oxidoreductases another enzyme, nitrile hydratase, is reported to possess PQQ as prosthetic group (Nagasawa & Yamada, 1987). In some of the oxidoreductases the prosthetic group is bound covalently to the protein, as in methylamine dehydrogenase from bacterium W3A1 (McIntire & Stults, 1986) or bovine plasma amine oxidase (Lobenstein-Verbeek *et al.*, 1984). Other quinoproteins contain PQQ bound non-covalently. At low pH and in the presence of 3 M-KBr the prosthetic group is removed from methanol dehydrogenase from bacterium W3A1 (Davidson *et al.*, 1985).

With the quinohaemoprotein alcohol dehydrogenase from *Pseudomonas testosteroni* the presence of Ca^{2+} is essential for activity (Groen *et al.*, 1986), and PQQ is readily removed from the quinoprotein glucose dehydrogenase from *Pseudomonas aeruginosa* by dialysis against EDTA-containing buffer (Duine *et al.*, 1983). In a number of Gram-negative bacteria is found an apo form of glucose dehydrogenase, which can be converted into the active holoenzyme in the presence of Mg^{2+} or Ca^{2+} (Ameyama *et al.*, 1985; Geiger & Görisch, 1987).

The quinoprotein glucose dehydrogenase from *Acinetobacter calcoaceticus* is not inactivated by EDTA or by *o*-phenanthroline (Hauge, 1964). The prosthetic group PQQ is removed from the enzyme at low pH or high salt concentrations (Hauge, 1964; Duine *et al.*, 1979). In the present paper we demonstrate that the prosthetic group is also removed upon thermal inactivation of *A. calcoaceticus* glucose dehydrogenase. *A. calcoaceticus* glucose dehydrogenase is a dimeric enzyme containing one molecule of PQQ per subunit of M_r 54000 (Geiger & Görisch, 1986). We find two Ca^{2+} ions per subunit of the enzyme, and Ca^{2+} is essential for the formation of active holoenzyme of the quinoprotein glucose dehydrogenase from *A. calcoaceticus*.

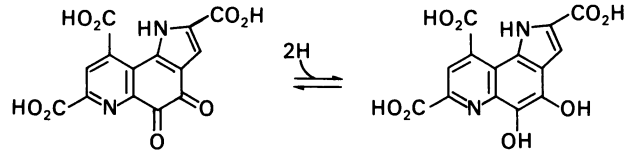

Fig. 1. Pyrroloquinoline quinone (PQQ) or methoxatin

MATERIALS AND METHODS

Chemicals

The inorganic chemicals used were of analytical-reagent grade.

DEAE-Sephadex A-25 was obtained from Pharmacia (Uppsala, Sweden), and the Centricon-10 micro-concentrator was from Amicon (Witten, Germany).

NNN'N'-Tetramethyl-*p*-phenylenediamine dihydro-

Abbreviations used: PQQ, pyrroloquinoline quinone or methoxatin; DCTA, *trans*-1,2-diaminocyclohexane-*NNN'N'*-tetra-acetic acid.

* Dedicated to Professor Dr. L. Jaenicke on the occasion of his 65th birthday.

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chloride was purchased from Fluka (Buchs, Switzerland), and EDTA from Riedel de Haen (Seelze, Germany).

Wurster's Blue was synthesized by the method of Michaelis & Granick (1943). PQQ was isolated as described previously (Geiger & Görisch, 1987).

Organism and growth conditions

The strain of *A. calcoaceticus*, formerly described as *Bacterium anitratum* (Hauge, 1960), was provided by Dr. J. Hauge, Veterinary College of Norway, Oslo, Norway. The organism was grown as described by Geiger & Görisch (1986).

Purification of quinoprotein glucose dehydrogenase

Homogeneous glucose dehydrogenase was prepared as described previously (Geiger & Görisch, 1986).

Assay of enzyme activity

The activity of glucose dehydrogenase was determined with Wurster's Blue as electron acceptor. The assay method and definition of the unit of glucose dehydrogenase activity have been described elsewhere (Geiger & Görisch, 1986).

Protein determination

Protein was determined by the procedure of Sedmak & Grossberg (1977), with bovine serum albumin as the standard.

Amino acid analysis

Amino acid analysis was performed in accordance with Spackman (1967). Three 0.1 mg samples of the protein were hydrolysed in 6 M-HCl for 24, 48 and 72 h at 110 °C in sealed tubes. The amino acids were analysed on a Biotronic LC 6000 analyser. The threonine and serine contents were corrected for losses sustained during hydrolysis. Valine, leucine and isoleucine were extrapolated to infinite time of hydrolysis. Cysteine was determined after oxidation of a sample with performic acid as described by Moore (1963). Tryptophan was determined after hydrolysis of a sample with toluene-*p*-sulphonic acid as described by Liu (1972). In addition, cysteine was determined by titration with Ellman's reagent in accordance with Riddles *et al.* (1983). Tryptophan was also determined by the spectroscopic method of Edelhoch (1967). The difference spectrum was used, obtained by measuring a solution 6.7 μM in enzyme subunits against a 6.7 μM solution of the prosthetic group.

X-ray fluorescence spectrometry

The metal determination was carried out with an energy-dispersive X-ray fluorescence apparatus (system 77; Finnigan International, Sunnyvale, CA, U.S.A.). The statistical error of the individual measurements was below 2%. The samples to be analysed contained 0, 0.2, 0.4, 0.8 or 2 nmol of glucose dehydrogenase subunits and 100 nmol of potassium phosphate, pH 7. With the highest amount of protein used the self-absorption will decrease the measured signal by about 3%.

Thermal inactivation and re-activation of glucose dehydrogenase

The inactivation and re-activation experiments were performed in 50 mM-potassium phosphate buffer, pH 6, in small plastic reaction vessels containing 0.5 ml of enzyme solution. The various temperatures used were maintained by means of different water baths.

Thermal stability of glucose dehydrogenase

Samples of glucose dehydrogenase were incubated at temperatures between 20 and 70 °C for 10 min, in the presence and in the absence of 1 mM-$CaCl_2$. After rapid cooling on ice residual enzyme activities were determined.

Kinetics of thermal inactivation

Samples of glucose dehydrogenase were incubated at 35, 40, 45 and 50 °C. At different times small samples were withdrawn and residual activities determined.

Re-activation of glucose dehydrogenase

For studying the re-activation process of glucose dehydrogenase samples of the enzyme were first thermally inactivated at 50 °C for 20 min. Re-activation was initiated by transferring the reaction tubes to a water bath at 25 °C. Alternatively, re-activation studies were performed with the apo form of glucose dehydrogenase.

Preparation of the apo form of glucose dehydrogenase

The enzyme must be inactivated in very dilute solution, otherwise the protein will be precipitated irreversibly. A 95 ml volume of 50 mM-potassium phosphate buffer, pH 6, containing DEAE-Sephadex A-25 gel (5 ml of settled gel) was heated to 50 °C. Then 0.5 mg of homogeneous glucose dehydrogenase in 5 ml of 50 mM-potassium phosphate buffer, pH 6, was added. The suspension was slowly agitated and incubated for 20 min at 50 °C. After a further incubation at 25 °C for 20 min the anion-exchange resin was removed by filtration. The resin was washed with 10 ml of 50 mM-potassium phosphate buffer, pH 6. To concentrate the very dilute protein solution, the combined filtrates were applied on to a column (1 cm × 2 cm) containing 1.5 ml of hydroxyapatite. A flow rate of 20 ml/h was maintained. The apoenzyme was eluted with 0.5 M-potassium phosphate buffer, pH 7, at a flow rate of 4 ml/h. Fractions containing protein with a total volume of 5 ml were concentrated to 800 μl by ultrafiltration in a Centricon-10 microconcentrator. The preparation was dialysed for 14 h against 50 mM-potassium phosphate buffer, pH 6.

RESULTS

Metal ion content

X-ray fluorescence spectrometry was used to determine the metal ion content of the quinoprotein glucose dehydrogenase from *A. calcoaceticus*. By this means 1.95 ± 0.08 mol of Ca^{2+}/mol of enzyme subunit was detected. For Sr, Ba, Pb, Mn, Fe, Ni, Cu and Zn less than 0.06 mol/mol of subunit was found. Mg^{2+} will not be detected by this method.

Stability towards EDTA treatment

The activity of glucose dehydrogenase was determined in the presence and in the absence of 10 mM-EDTA. No effect of the chelating agent was observed. The enzyme was also dialysed at 4 °C for 24 h against 5000 vol. of 0.1 M-Tris/HCl buffer, pH 7.5, containing 10 mM-EDTA. Subsequently glucose dehydrogenase was dialysed again for 24 h against 0.1 M-Tris/HCl buffer, pH 7.5, without EDTA. Within experimental error, the EDTA treatment did not influence the activity of glucose dehydrogenase

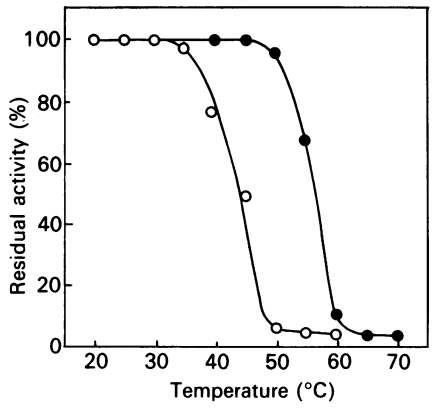

Fig. 2. Thermal stability of glucose dehydrogenase

Samples of homogeneous enzyme [7.7 μg/ml (= 20 units/ml)] were incubated for 10 min in 50 mM-potassium phosphate buffer, pH 6, at the temperatures indicated. Residual activities were determined under standard conditions. Heat treatment was in the presence of 1 mM-$CaCl_2$ (●) and in the absence of $CaCl_2$ (○).

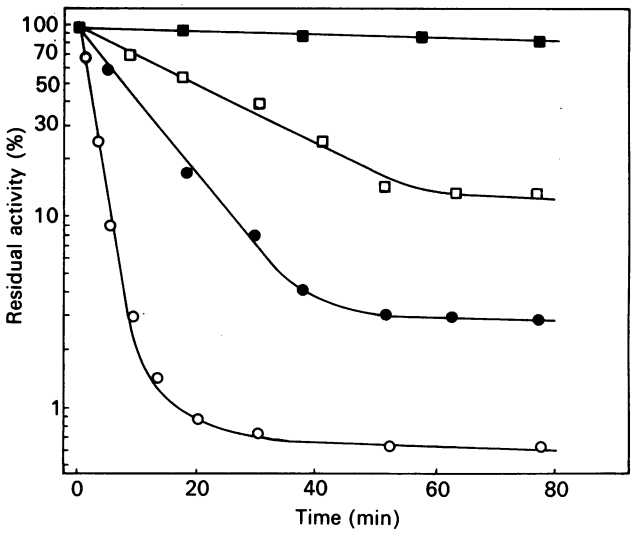

Fig. 3. Time course of the thermal inactivation of glucose dehydrogenase

Samples of homogeneous glucose dehydrogenase [3.8 μg/ml (= 10 units/ml)] in 50 mM-potassium phosphate buffer, pH 6, were incubated at 35 °C (■), 40 °C (□), 45 °C (●) and 50 °C (○). Residual activities were determined under standard conditions at the times indicated.

when compared with a control sample. DCTA, a chelator with higher affinity for Ca^{2+}, also does not inactivate the enzyme under these conditions.

Thermal stability

Glucose dehydrogenase was incubated in 50 mM-potassium phosphate buffer, pH 6, at different temperatures for 10 min. Residual activity was determined in the standard test. The enzyme is inactivated at temperatures above 35 °C. In the presence of 1 mM-$CaCl_2$ glucose dehydrogenase is more stable and inactivation is observed only at temperatures of 50 °C and above (Fig. 2).

Time course of inactivation

The time course of heat inactivation of homogeneous glucose dehydrogenase at different temperatures is shown in Fig. 3. The enzyme is not completely inactivated. The amount of active enzyme depends on the inactivation temperature and on the concentration of enzyme used. In addition, the inactivation is a reversible process. When glucose dehydrogenase is inactivated at 50 °C for 20 min and afterwards the temperature is shifted to 40 °C the activity of the sample increases again. Within experimental error, the final residual activity reached is the same as that of an identical control sample that was simply inactivated at 40 °C. The main part of the initial inactivation process follows apparent first-order kinetics. First-order rate constants for the inactivation were determined from the initial part of the inactivation reaction. The first-order rate constants were found to be k (40 °C) = 5.8×10^{-4} s^{-1}, k (45 °C) = 1.37×10^{-3} s^{-1} and k (50 °C) = 5.06×10^{-3} s^{-1}. Within the experimental error of 10% the values of the rate constants fall on a straight line in the Arrhenius plot. An activation energy of E_a = 195 kJ/mol is obtained. According to transition-state theory, the temperature-dependence of the velocity constant of the inactivation process is given by eqn. (1):

$$k = \frac{RT}{Nh} \cdot \exp\left[-\left(\frac{\Delta H^{\ddagger}}{RT} - \frac{\Delta S^{\ddagger}}{R}\right)\right] \qquad (1)$$

where $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ are the enthalpy and entropy of activation, N is Avogadro's number, h is Planck's constant, and R and T are the gas constant and the absolute temperature. The activation enthalpy and activation entropy were found to be $\Delta H^{\ddagger} = 187$ kJ·mol^{-1} and $\Delta S^{\ddagger} = 290$ J·mol^{-1}·K^{-1}.

Re-activation of heat-inactivated enzyme

Different concentrations of glucose dehydrogenase were inactivated for 20 min at 50 °C. Afterwards the temperature was shifted to 25 °C and the re-activation process was recorded by determining the activity of samples removed after different time intervals (Fig. 4). The reaction order cannot be determined unambiguously from the time course of the re-activation, since the data do not follow a straight line exactly, whether plotted as a first-order or as a second-order process. The initial velocity of the re-activation reactions is a function of the square of the protein concentration. When treated as a second-order process, similar rate constants are obtained for the first 50% of the re-activation reaction with all enzyme concentrations used. The experimental error associated with the data, however, does not allow a detailed kinetic analysis of the process.

Influence of Ca^{2+} on the inactivation/re-activation of glucose dehydrogenase

Inactivation and re-activation experiments were performed in the presence of Ca^{2+} and/or EDTA. In the presence of 1 mM-$CaCl_2$ almost no inactivation of glucose dehydrogenase occurs at 50 °C. In contrast, 2 mM-EDTA prevents completely the re-activation of an inactivated enzyme sample at 25 °C. $CaCl_2$ (4 mM) in the presence of 2 mM-EDTA again stabilizes the enzyme against thermal inactivation. Addition of $CaCl_2$ to a solution of inactivated glucose dehydrogenase increases the rate of re-activation at 25 °C.

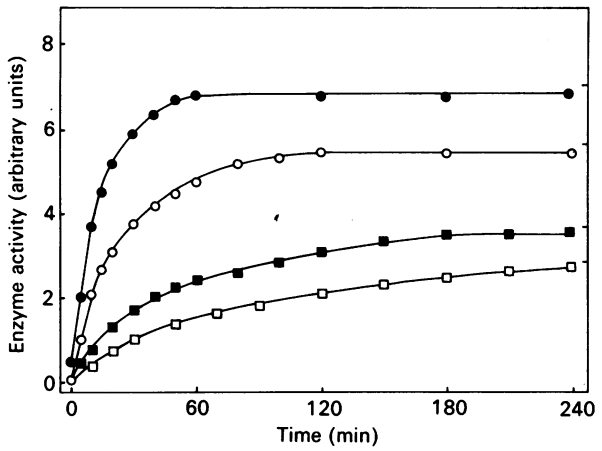

Fig. 4. Re-activation of heat-inactivated glucose dehydrogenase

Samples containing various concentrations of glucose dehydrogenase in 50 mM-potassium phosphate buffer, pH 6, were incubated at 50 °C for 20 min and subsequently incubated at 25 °C. The time course of re-activation was monitored by withdrawing 10 μl samples at the times indicated and measuring glucose dehydrogenase activity. The activities were determined under standard conditions. The following concentrations of glucose dehydrogenase were used: 21.2 μg/ml (●), 16.5 μg/ml (○), 10.5 μg/ml (■) and 8.3 μg/ml (□). Enzyme activity is expressed in arbitrary units. The activities of the samples before heat treatment are indicated by bars on the right-hand ordinate.

Table 1. Re-activation of heat-inactivated glucose dehydrogenase in the presence of bivalent metal ions

Before heat treatment the samples showed an activity of ΔA_{610}/min = 2.8. Effective radii of metal ions in octahedral co-ordination are taken from Bell (1977).

Salt added (1 mM)	Ionic radius of the cation (nm)	Activity after 5 min of re-activation (ΔA_{610}/min)
–	–	0.42
NaCl	0.102	0.38
$MgCl_2$	0.072	0.4
$Co(NO_3)_2$	0.073*	0.26
$MnCl_2$	0.082*	1.8
$CdCl_2$	0.095	1.6
$CaCl_2$	0.100	2.68
$Ca(NO_3)_2$		2.4
$HgCl_2$	0.102	0
$AlCl_3$	0.053	0.26

* High spin.

Influence of bivalent metal ions upon the rate of re-activation of heat-inactivated glucose dehydrogenase

Samples of glucose dehydrogenase [8.1 μg of enzyme/ml)] were inactivated at 50 °C for 20 min. The temperature was shifted to 25 °C for re-activation and solutions of different metal salts were added to obtain final concentrations of 1 mM. The activity of the samples was determined after 5 min at 25 °C. NaCl does not influence the normal re-activation process of the enzyme, and Co^{2+} and Al^{3+} are slightly inhibitory. In the presence of 1 mM-$HgCl_2$ no re-activation occurs. Ca^{2+}, Mn^{2+} and Cd^{2+} accelerate the re-activation rate appreciably (Table 1).

Influence of PQQ on the rate of re-activation of glucose dehydrogenase

Glucose dehydrogenase was inactivated at 50 °C. Addition of the prosthetic group PQQ resulting in final concentrations of 1 μM or 10 μM increases the rate of re-activation at 25 °C, by factors of 5-fold or 18-fold respectively.

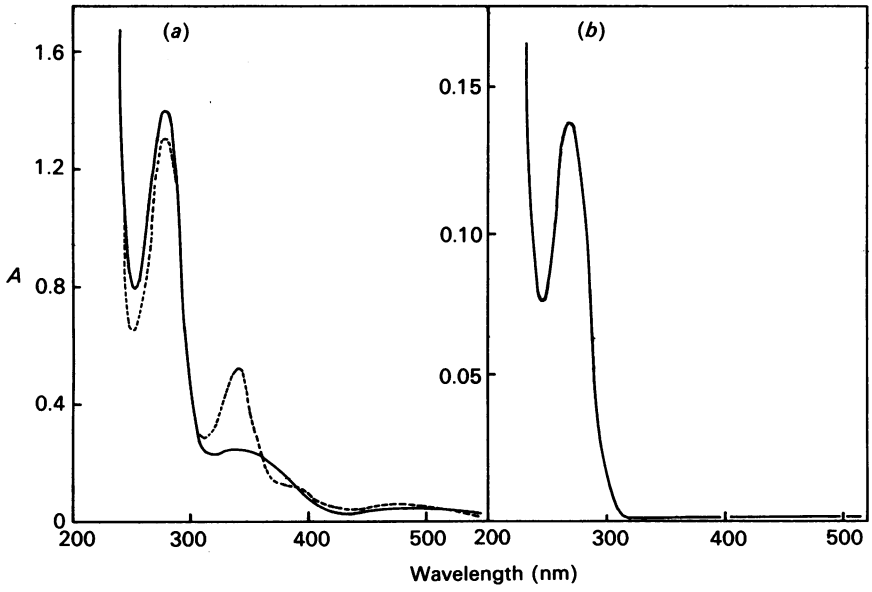

Fig. 5. Absorption spectrum of glucose dehydrogenase from *A. calcoaceticus*

(*a*) Glucose dehydrogenase (1 mg/ml) in 20 mM-potassium phosphate buffer, pH 7: ——, oxidized form; ------, reduced form.
(*b*) Apo form of glucose dehydrogenase (0.1 mg/ml) in 50 mM-potassium phosphate buffer, pH 6.

Table 2. Comparison of glucose dehydrogenase with its apoenzyme

Preparation	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	
			−PQQ	+PQQ (1 μM)
Glucose dehydrogenase holoenzyme	0.5	1300	2600	2600
Glucose dehydrogenase apoenzyme	0.08	1.76	22	540
	20% re-activated			2640*

* As determined from the titration experiment 20% of the total apoenzyme can be re-activated; this results in an enzyme with a specific activity of 2640 units/mg.

Thermal inactivation of glucose dehydrogenase is accompanied by dissociation into apoenzyme and prosthetic group

A concentrated sample (1 ml) of homogeneous glucose dehydrogenase with 11 μM subunits was inactivated at 50 °C for 20 min in 50 mM-potassium phosphate buffer, pH 6. Protein was removed from the solution in a Centricon-10 microconcentrator. A concentration of 2.5 μM free PQQ was found in the ultrafiltration solution by using an enzymic determination procedure for the prosthetic group as described elsewhere (Geiger & Görisch, 1987).

Absorption spectra of the holo form and apo form of glucose dehydrogenase

The absorption spectrum of the holo form of glucose dehydrogenase is depicted in Fig. 5(*a*). It shows an absorption band at 350 nm and a less intense but broad band around 515 nm, in addition to the absorption of the aromatic amino acid residues at 276 nm. Addition of glucose under anaerobic conditions results in the reduction of the prosthetic group, and a sharp absorption band is formed at 337 nm and the maximum of the broad band at 515 nm is shifted to 470 nm (Fig. 5*a*).

The apo form of *A. calcoaceticus* glucose dehydrogenase was prepared by thermal inactivation in the presence of an anion-exchange resin. The prosthetic group bound to the resin was removed as described in the Materials and methods section. Considerable loss of protein occurs during each step. The final preparation of the apo form of glucose dehydrogenase shows a residual specific activity of 22 units/mg compared with 2600 units/mg of the native enzyme. The apo form shows a simple protein spectrum with an absorption band at 276 nm (Fig. 5*b*). No absorption is found above 310 nm. From the specific activity of the preparation it is estimated that only 0.8% of the enzyme is present in the holo form. After re-activation of apoenzyme with its prosthetic group in the presence of 2.5 mM-Ca^{2+} the specific activity was increased to 520 units/mg (Table 2).

Apo form of glucose dehydrogenase is re-activated with PQQ in the presence of Ca^{2+}

The formation of enzymically active glucose dehydrogenase from apoenzyme and PQQ in the presence of Ca^{2+} does not occur instantaneously. The rate of re-activation depends on the Ca^{2+} concentration. At 25 °C in the presence of 2.5 mM-$CaCl_2$ the re-activation of glucose dehydrogenase is accomplished within 5 min. This behaviour allows titration of the apo form of the enzyme with PQQ. Fig. 6 demonstrates that the specific activity of the re-activation mixture at higher concentrations of the prosthetic group is an almost linear function of the PQQ concentration. The re-activation mixture was 500 nM in enzyme subunits, and from Fig. 6 it is estimated that the enzyme is completely re-activated by 102 nM-PQQ. Thus only 20% of the apo form of glucose dehydrogenase is able to form active enzyme again. The specific activity of the re-activated enzyme is calculated to be 2640 units/mg, a value very close to the specific activity of 2600 units/mg found for the native enzyme. Apparently 80% of the apo form is inactivated irreversibly (Table 2). So far we have no evidence concerning the nature of the irreversibly inactivated protein.

Amino acid composition

Table 3 presents the amino acid composition of quinoprotein glucose dehydrogenase from *A. calcoaceticus*. The enzyme does not contain cysteine. On the

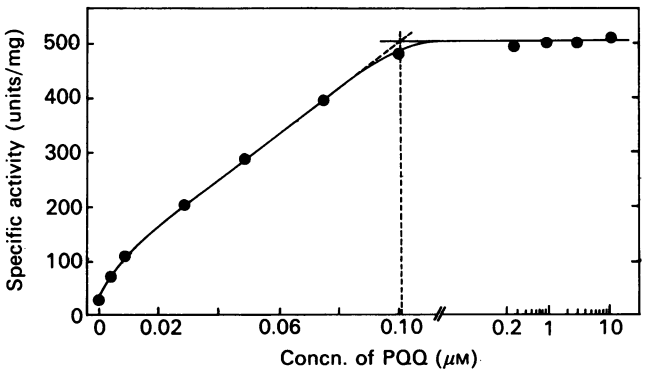

Fig. 6. Titration of apo form of glucose dehydrogenase with the prosthetic group PQQ

Active glucose dehydrogenase was obtained from the apoenzyme by re-activation at 25 °C in 50 mM-potassium phosphate buffer, pH 6, in the presence of 2.5 mM-$CaCl_2$. The samples contained 25 μg of the apoenzyme/ml and various concentrations of PQQ. After 5 min the enzyme activity was determined under standard conditions.

Table 3. Amino acid composition of glucose dehydrogenase from *A. calcoaceticus*

Tryptophan was determined by the procedure of Liu (1972) ([a]) and that of Edelhoch (1967) ([b]). Cysteine was determined by the procedure of Moore (1963) ([c]) and that of Riddles *et al.* (1983) ([d]).

Amino acid	Amino acid composition (residues/subunit)	
Asx	67.2	(67)
Thr	28.2	(28)
Ser	27.1	(27)
Glx	54.9	(55)
Pro	38.8	(39)
Gly	36.8	(37)
Ala	30.8	(31)
Val	24.2	(24)
Met	3.8	(4)
Ile	22.7	(23)
Leu	34.1	(34)
Tyr	20.4	(20)
Phe	16.4	(16)
His	10.4	(10)
Lys	32.0	(32)
Arg	12.2	(12)
Trp	7.0[a] 8[b]	(7)
Cys	0.2[c] 0.06[d]	(0)

basis of an M_r of 54000 the subunit of glucose dehydrogenase contains 460–470 residues.

Absorption spectrum of PQQ in the presence of Ca^{2+}

The absorption spectrum of PQQ at pH 6 shows a strong absorption band at 249 nm and a band at 330 nm. In the presence of 5 mM-$CaCl_2$ the absorption band at 330 nm is shifted to 348 nm (Fig. 7). The heterocyclic tricarboxylic acid PQQ apparently forms a Ca^{2+} complex. A shift of the absorption band at 330 nm to longer wavelength is also observed in the presence of 5 mM-$MnCl_2$ or -$HgCl_2$.

DISCUSSION

Quinoprotein glucose dehydrogenase has been purified to homogeneity from several bacteria (Dokter *et al.*, 1986; Geiger & Görisch, 1986; Matsushita & Ameyama, 1982; Ameyama *et al.*, 1981). The homogeneous enzyme from *A. calcoaceticus* is inactivated at temperatures above 35 °C. The activation parameters determined for the thermal inactivation are typical for protein-denaturation processes (Stearn, 1949). It is estimated that about seven hydrogen bridges are broken in the activation process of the inactivation of glucose dehydrogenase. The inactivation reaction proceeds towards an equilibrium between active and inactive enzyme. The amount of active enzyme depends on the inactivation temperature and the enzyme concentration used.

The concentration-dependence could be understood assuming that glucose dehydrogenase dissociates into apoenzyme and the prosthetic group PQQ. We were able to demonstrate that, upon thermal inactivation, PQQ dissociates from the enzyme. In addition, the rate of re-activation of thermally inactivated enzyme is increased when exogenous PQQ is added.

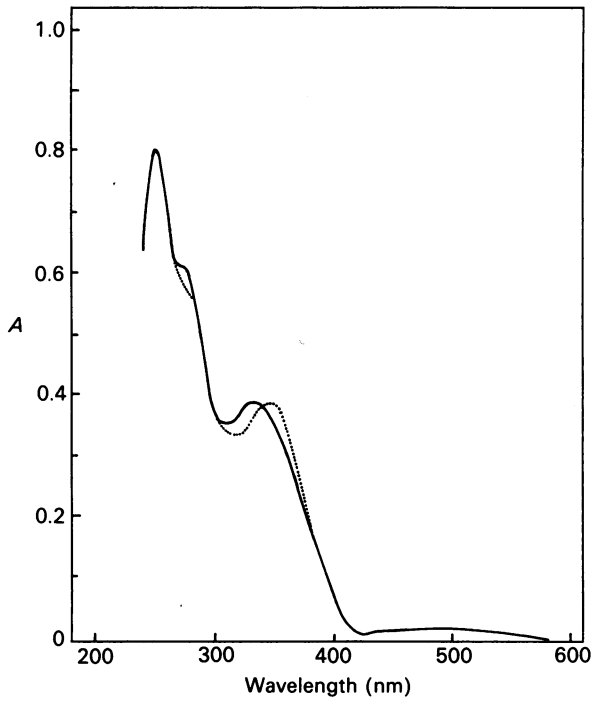

Fig. 7. Spectra of the prosthetic group PQQ

The spectra are of 44 μM-PQQ in 50 mM-sodium acetate buffer, pH 6 (——), and 50 mM-sodium acetate buffer, pH 6, containing 5 mM-$CaCl_2$ (·····).

Ca^{2+} stabilizes against thermal inactivation, and heat-inactivated enzyme can be re-activated only in the presence of Ca^{2+}. Other bivalent cations with similar ionic radii, such as Mn^{2+} and Cd^{2+}, can substitute for Ca^{2+}. Mg^{2+} ions do not support the formation of active enzyme. In this respect the behaviour of the quinoprotein glucose dehydrogenase apoenzyme from *A. calcoaceticus* differs from apoenzymes of other bacteria, which are re-activated in the presence of Ca^{2+} or Mg^{2+} (Ameyama *et al.*, 1985). Hg^{2+}, with an ionic radius very close to that of Ca^{2+}, prevents the slow formation of active enzyme that occurs in the absence of exogenous added metal ions (Table 1). This inhibition of the re-activation process, however, cannot be due to a reaction with cysteine residues, since quinoprotein glucose dehydrogenase does not contain cysteine (Table 3).

The prosthetic group PQQ forms a complex with Ca^{2+}, as demonstrated in Fig. 7. Upon complex-formation the absorption maximum of PQQ at 330 nm is shifted to 348 nm. This value is very close to the absorption maximum of the prosthetic group when bound to glucose dehydrogenase from *A. calcoaceticus* (Fig. 5*a*). When glucose dehydrogenase and free PQQ are reduced, the absorption maxima are shifted to shorter wavelength, and the absorption coefficients increase by a factor of 2.7-fold. However, in the reduced enzyme the absorption maximum of the prosthetic group is still shifted to longer wavelength when compared with the reduced form of free PQQ (Duine *et al.*, 1981). It appears that PQQ, whether oxidized or reduced, is bound as a Ca^{2+} complex to the enzyme. We found two Ca^{2+} ions/subunit of native *A. calcoaceticus* glucose dehydrogenase, but we do not know if both ions participate in the anchoring of the prosthetic group to the protein and thereby stabilize the

enzyme, or if one Ca^{2+} ion has a pure structural role for maintaining the native protein conformation.

The apoenzyme can be isolated from solutions of thermally inactivated glucose dehydrogenase. Enzyme activity is restored only in the presence of both Ca^{2+} and the prosthetic group PQQ. The apoenzyme can be titrated with PQQ, and from these titration data it appears that only about 20% of the preparation is in a state able to form enzymically active enzyme again. The specific activity calculated for the re-activated glucose dehydrogenase is 2640 units/mg, which is within experimental error the specific activity of the homogeneous native enzyme. Apparently the apoprotein is very unstable and 80% of the preparation is inactivated irreversibly during chromatography. Re-activation experiments performed directly after thermal inactivation result in restoration of about 95% of the original activity.

Bacterial quinoprotein glucose dehydrogenases have been divided into two groups (Dokter *et al.*, 1986). Enzymes of type I lose the prosthetic group when dialysed against buffer containing EDTA. Enzyme activity is reconstituted in the presence of Mg^{2+} or Ca^{2+} and the enzymes possess a low isoelectric point.

Quinoprotein glucose dehydrogenases of type II show high isoelectric points and the prosthetic group cannot be removed by EDTA treatment. The two enzymes known of this type have been purified from *Gluconobacter suboxidans* (Ameyama *et al.*, 1981) and from *A. calcoaceticus* (Dokter *et al.*, 1986; Geiger & Görisch, 1986). However, the prosthetic group is not bound in a completely different way from that in type I enzymes. In *A. calcoaceticus* quinoprotein glucose dehydrogenase a bivalent metal ion is necessary to form the active enzyme. *A. calcoaceticus* glucose dehydrogenase differs from the type I enzymes in that no re-activation occurs with Mg^{2+}. Enzyme activity is only restored by bivalent metal ions of ionic radius between 0.082 and 0.1 nm (0.82 and 1 Å).

Thus it appears that in both subdivisions of quinoprotein glucose dehydrogenases the prosthetic group is bound via a bivalent ion bridge. It remains to be seen whether the finding of high isoelectric points and stability against EDTA treatment in the enzymes from both *G. suboxidans* and *A. calcoaceticus* bears some structural and/or functional significance or is a fortuitous coincidence.

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