Quinohaemoprotein alcohol dehydrogenase apoenzyme from *Pseudomonas testosteroni*

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Cell-free extracts of *Pseudomonas testosteroni*, grown on alcohols, contain quinoprotein alcohol dehydrogenase apoenzyme, as was demonstrated by the detection of dye-linked alcohol dehydrogenase activity after the addition of PQQ (pyrroloquinoline quinone). The apoenzyme was purified to homogeneity, and the holoenzyme was characterized. Primary alcohols (except methanol), secondary alcohols and aldehydes were substrates, and a broad range of dyes functioned as artificial electron acceptor. Optimal activity was observed at pH 7.7, and the presence of Ca^{2+} in the assay appeared to be essential for activity. The apoenzyme was found to be a monomer (M_r 67000 $\pm$ 5000), with an absorption spectrum similar to that of oxidized cytochrome *c*. After reconstitution to the holoenzyme by the addition of PQQ, addition of substrate changed the absorption spectrum to that of reduced cytochrome *c*, indicating that the haem *c* group participated in the enzymic mechanism. The enzyme contained one haem *c* group, and full reconstitution was achieved with 1 mol of PQQ/mol. In view of the aberrant properties, it is proposed to distinguish the enzyme from the common quinoprotein alcohol dehydrogenases by using the name 'quinohaemoprotein alcohol dehydrogenase'. Incorporation of PQQ into the growth medium resulted in a significant shortening of lag time and increase in growth rate. Therefore PQQ appears to be a vitamin for this organism during growth on alcohols, reconstituting the apoenzyme to a functional holoenzyme.

INTRODUCTION

During the past few years, several quinoprotein (PQQ-containing) oxidoreductases have been discovered in bacteria (Duine & Frank, 1981*b*). Obviously, these organisms are able to synthesize this prosthetic group, and some of them even excrete PQQ into their growth medium (Ameyama *et al.*, 1984; Duine *et al.*, 1984, 1985). On the other hand, it has been reported that synthesis of apoprotein and PQQ are not always synchronized (Van Schie *et al.*, 1984). Even an absence of PQQ synthesis has been observed, since *Acinetobacter lwoffii* (Van Schie *et al.*, 1984) and *Escherichia coli* (Hommes *et al.*, 1984) produce quinoprotein glucose dehydrogenase apoenzyme. In the latter case, by using an *E. coli* C (pts⁻) mutant in which the phosphorylative route of glucose dissimilation was blocked, it could be demonstrated that quinoprotein glucose dehydrogenase is functional in this organism, since growth on glucose could be restored on addition of PQQ to the growth medium (Hommes *et al.*, 1984).

Apart from providing support for the view that PQQ is a vitamin and that there exist unexpected metabolic routes in some cases, research on quinoprotein apoenzymes is important for the elucidation of the enzymic mechanism of quinoproteins, as it enables recombination studies with PQQ derivatives. The present paper describes the first isolation of a quinoprotein alcohol dehydrogenase apoenzyme and the properties of the reconstituted holoenzyme.

MATERIALS AND METHODS

Organisms and growth conditions

Pseudomonas testosteroni strains A.T.C.C. 15667 and A.T.C.C. 15666 were cultured with good aeration at 30 °C on a mineral medium, supplemented with 0.3% (v/v) ethanol or butanol. Cells were harvested at the end of the exponential growth phase, washed with 20 mM-potassium phosphate buffer, pH 7.2, and stored frozen. The mineral medium contained (per litre): K_2HPO_4 , 3H₂O, 15.4 g; KH_2PO_4 , 4.52 g; $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 0.5 g; $(\text{NH}_4)_2\text{SO}_4$, 3 g; CaCl_2 , 15 mg; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 15 mg; trace-element solution (Duine *et al.*, 1978), 1 ml.

Apoenzyme isolation

Frozen cells (25 g) of strain A.T.C.C. 15667, grown on butanol, were suspended in 20 ml of 50 mM-potassium phosphate buffer, pH 7.0, and the cells were disrupted in a French pressure cell at 110 MPa. The suspension (viscosity was lowered by adding deoxyribonuclease) was centrifuged at 48000 *g* for 30 min at 4 °C. The supernatant (38 ml) was dialysed against 10 mM-potassium phosphate buffer, pH 7.0, containing 0.01% (v/v) 2-mercaptoethanol. The dialysis residue (40 ml) was applied to a DEAE-Sephacel column (15 cm $\times$ 2 cm) in 10 mM-potassium phosphate buffer, pH 7.0. As the enzyme passes through under these conditions, the column was washed with 10 mM-Tris/HCl buffer,

Abbreviation used: PQQ, pyrroloquinoline quinone (2,7,9-tricarboxy-1*H*-pyrrolo[2,3-*f*]quinoline-4,5-dione).

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pH 7.0. Active fractions were pooled and applied to a hydroxyapatite (Bio-Rad HPT) column (4 cm × 2.2 cm) in the same buffer. After the column had been washed with 50 mM-potassium phosphate buffer, pH 7.0, the apoenzyme was eluted with 0.5 M-potassium phosphate buffer, pH 7.0 (the flow rate during adsorption and washing should not exceed 15 ml/h). The pooled fractions were dialysed against 5 mM-potassium phosphate buffer, pH 7.0, and the dialysis residue was applied to a CM-Sepharose column (4 cm × 0.5 cm) in the same buffer. After the column had been washed with 20 ml of 40 mM-potassium phosphate buffer, pH 7.0, followed by 40 ml of 70 mM-potassium phosphate buffer, pH 7.0, the apoenzyme was eluted with 0.15 M-potassium phosphate buffer, pH 7.0. The active fractions were pooled and further purified by h.p.l.c. gel filtration on a Serva Si 200 polyol column (50 cm × 0.95 cm) with 0.1 M-sodium phosphate buffer, pH 6.6, and a flow rate of 0.5 ml/min. The apoenzyme peak was analysed for homogeneity by a Hewlett-Packard HP 1040 A photodiode-array detector, by scanning the peak up-slope, at the top and down-slope.

Enzyme assay

Apoenzyme (20 µl), usually in 0.15 M-potassium phosphate buffer, pH 7.0, was mixed with 200 µl of 30 µM-PQQ in water. After incubation for 5 min at 20 °C in a 1 ml cuvette, 0.8 ml of 20 mM-Tris/HCl buffer, pH 7.7, containing 3 mM-CaCl₂, 0.15 mM-Wurster's Blue and 0.1 mM-butan-1-ol was added and the rate was determined by measuring the reduction of Wurster's Blue spectrophotometrically at 612 nm, by using a molar absorption coefficient of $1 \times 10^4 \text{ M}^{-1} \cdot \text{cm}^{-1}$. The latter value was determined from an absorption spectrum of this electron acceptor, by using a molar absorption coefficient of $9 \times 10^3 \text{ M}^{-1} \cdot \text{cm}^{-1}$ at 600 nm (Duine & Frank, 1980). In the case of cell-free extract, the assay mixture also contained 1 mM-KCN in order to prevent the reoxidation of reduced Wurster's Blue via components of the respiratory chain.

Protein determinations

Protein in cell-free extracts was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard. For the pure apoenzyme samples, concentrations were calculated from the absorbance values at 205 and 280 nm by using the following formula:

$$A_{280}^{0.1\%} = 34.14 \times A_{205}/A_{280} - 0.02$$

M_r determinations

The M_r of the native apoenzyme was determined by gel filtration on a Sephadex G-100 column (55 cm × 1 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: cytochrome *c* (horse heart), ribonuclease, chymotrypsin, carbonic anhydrase, ovalbumin, bovine serum albumin, conalbumin and alcohol dehydrogenase (baker's yeast). The M_r of the denatured apoenzyme was determined by gel filtration on a Sepharose C1-6B column (55 cm × 1 cm) in 6 M-guanidinium chloride with or without the addition of 2-mercaptoethanol (0.3 M) to the sample. Phosphorylase *b*, bovine serum albumin, carbonic anhydrase, ribonuclease and cytochrome *c* were used as standards for calibration of the column.

Polyacrylamide-gel electrophoresis

Gel slabs of 5.3% (w/v) polyacrylamide, cross-linked with 0.19% (w/v) bisacrylamide, were electrophoresed in 36 mM-Tris/21 mM-H₃PO₄ buffer, pH 6.5, for 60 min at 175 V. Application of the enzyme was at the anode side. After reconstitution, alcohol dehydrogenase and aldehyde dehydrogenase activities were detected in the gel by using butan-1-ol and acetaldehyde respectively in the assay mixture and an appropriate amount of Wurster's Blue to give a sufficiently blue background to the gel.

Haem and PQQ analysis

Characterization and determination of haem were as described by Bartsch (1971). Cytochrome *c* (horse heart) was used as reference material. It gave a molar absorption coefficient of $23\,200 \text{ M}^{-1} \cdot \text{cm}^{-1}$ at 550 nm in the alkaline pyridine solution. For PQQ determinations in the solution used for reconstitution of the enzyme, a molar absorption coefficient of $19\,400 \text{ M}^{-1} \cdot \text{cm}^{-1}$ at 249 nm was used (Duine *et al.*, 1980).

Materials

All chemicals were from commercial suppliers, except for Wurster's Blue, which was prepared as described by Duine *et al.* (1978), and PQQ, prepared as described by Corey & Tramontano (1981).

RESULTS

Apoenzyme induction

Both *Ps. testosteroni* strains grew (albeit poorly) on ethanol, propan-1-ol, butan-1-ol and pentan-1-ol (not on methanol or higher alcohols than pentan-1-ol). In contrast with the observation for many other *Pseudomonas* species (Duine *et al.*, 1985), PQQ was not found in the culture medium after growth on ethanol. Cell-free extracts of ethanol-grown organisms showed NAD-dependent ethanol dehydrogenase activity and a very low dye-linked activity (0.03 µmol of Wurster's Blue reduced/min per mg of protein). Addition of PQQ to the cell-free extracts resulted, however, in a high dye-linked ethanol dehydrogenase activity (1.8 µmol of Wurster's Blue reduced/min per mg of protein). Similar results were obtained after growth on the other alcohols. When gluconate was used as a carbon source for growth, cell-free extracts were devoid of any dye-linked ethanol dehydrogenase activity. Incorporation of PQQ in the growth medium resulted in a large decrease of the lag time and an increase in growth rate (Fig. 1). Cell-free extracts of these cells contained substantial amounts of dye-linked alcohol dehydrogenase activity, and the enzyme was identical with the reconstituted apoenzyme isolated from cells grown without PQQ. The positive effect of PQQ on growth characteristics was observed only with alcohols, not with gluconate as a carbon source.

Isolation

Results of the purification are presented in Table 1. The enzyme was eluted from the h.p.l.c. column as a distorted peak at 47.2 min. However, it appeared to be homogeneous, since the spectra taken up-slope, at the top and down-slope of the peak were identical, so that the distortion may arise from adsorption effects. Homogeneity was further confirmed by electrophoresis, since a single

Fig. 1. Effect of PQQ on growth of *Ps. testosteroni*

Strain A.T.C.C. 15667 was grown on butan-1-ol as described in the Materials and methods section. A culture, pre-grown on butan-1-ol, was used as the inoculum (3 ml). The culturing was performed in a 100 ml flask (containing 25 ml of medium) on a reciprocal shaker. Turbidity was monitored with a Klett-Summerson colorimeter (red filter). ●, No PQQ added; ○, PQQ in water, sterilized in an autoclave, was added to the medium before inoculation.

Fig. 2. Recombination stoichiometry of apoenzyme with PQQ

Samples (100 μ l) containing 82 pmol (●) or 190 pmol (○) of apoenzyme were incubated with various amounts of PQQ (100 μ l) and assayed according to the normal procedure.

Table 1. Purification of quinohaemoprotein alcohol dehydrogenase

For experimental details see the text.

Fraction	Protein (mg)	Sp. activity (μ mol of Wurster's Blue/min per mg)	Yield (%)	Purification (fold)
Cell-free extract	950	1.8	100	1
DEAE-Sephacel	293	4	68	2.2
Hydroxyapatite	203	5	59	2.8
CM-Sephadex	30	8	14	4.4
H.p.l.c.	10	15	9	8.3

band was observed on activity staining with ethanol or aldehyde as a substrate, and on protein staining with Coomassie Brilliant Blue G-250. The purified enzyme showed a specific activity of 15 μ mol of Wurster's Blue reduced/min per mg of protein. For routine purposes the apoenzyme was stored in 0.15 M-potassium phosphate buffer, pH 7.0, at 4 $^{\circ}$ C. For prolonged periods, it could be stored at -70° C, without loss of activity.

Kinetic parameters

Before the performance of kinetic experiments with the holoenzyme, optimal reconstitution conditions were determined. As follows from the points of intersection of the tangents that can be drawn for the curves of Fig. 2, 82 pmol of apoenzyme combines with 80 pmol of PQQ,

and 190 pmol of apoenzyme with 190 pmol of PQQ. This indicates that one molecule of PQQ is bound per enzyme molecule. With the use of Mes/NaOH, Mops/NaOH, Tes/NaOH and Tris/HCl buffers (20 mM) in the appropriate region of buffering, a pH optimum of 7.7 was found for the enzyme. Ammonium, primary alkylammonium or benzylammonium salts were not activators, either at pH 7.0 or pH 9.0. On the other hand, calcium salts had a strong stimulating effect (Fig. 3), and magnesium salts were active but far less so. The low activity observed in the absence of calcium salts completely disappeared on addition of EDTA, so that it may originate from traces of calcium salts in the test system.

The apparent kinetic parameters of a number of alcohols and aldehydes are shown in Table 2. It should be noted that methanol is not a substrate and that octanol and benzyl alcohol were substrates only at very low concentrations (strong substrate inhibition was observed with the latter two compounds). As appears from Table 3, one-electron and two-electron acceptors (in anionic as well as cationic form) were active in the assay system. The enzyme behaves as a dye-linked dehydrogenase, since no O_2 consumption was observed in the absence of artificial electron acceptors, and NAD^+ and $NADP^+$ were not active.

Properties

The apoenzyme is a basic protein, as follows from its chromatographic behaviour. Gel filtration on a calibrated column revealed that its M_r is 70000. A value of 65000 was found under denaturing conditions, while the eluted protein still contained the haem *c* group. The apoenzyme showed an absorption spectrum (Fig. 4) reminiscent of that of oxidized cytochrome *c*. Only after reconstitution with PQQ did the spectrum change into that of reduced

Fig. 3. Effect of Ca^{2+} ions in the assay

Activities were measured according to the normal procedure with various concentrations of CaCl_2 .

Table 2. Substrate specificity of quinohaemoprotein alcohol dehydrogenase

Apoenzyme (40 pmol) was mixed with PQQ (100 pmol) and assayed with various amounts of substrate, as indicated in the Materials and methods section. The apparent kinetic parameters K'_m and V' were determined by the plotting method of Lineweaver & Burk (1934). The V' values are given as percentages of that obtained with pentan-1-ol.

Substrate	K'_m (mM)	V' (%)
Ethanol	5	70
Propan-1-ol	0.06	80
Butan-1-ol	0.005	85
Pentan-1-ol	0.005	100
Octan-1-ol	0.005	75
Benzyl alcohol	0.001	60
Butane-1,3-diol	0.28	40
6-Aminohexan-1-ol	0.20	90
Formaldehyde	3	20
Acetaldehyde	0.9	80
Butyraldehyde	0.1	60
Octanal	0.04	15

cytochrome *c* on addition of butanol (Fig. 4). Since the haem was not extractable with butan-2-one, it is concluded that the apoenzyme contains haem *c*. This is in accordance with the absorption maxima found in alkaline pyridine, which were identical with those of cytochrome *c* (412 and 550 nm). By using the value of the molar absorption coefficient of reduced cytochrome *c* at 550 nm ($23\,200\text{ M}^{-1}\text{ cm}^{-1}$) in the alkaline pyridine solution, it was calculated that 1.05 mol of haem *c* is present/mol of apoenzyme.

Table 3. Activity with different electron acceptors

Activities were determined as indicated in the Materials and methods section by measuring the initial reduction rates of the electron acceptors at the appropriate wavelengths. The values are presented as percentages of that obtained with Wurster's Blue ($0.16\text{ }\mu\text{M}$). Abbreviations: DCIP, 2,6-dichlorophenol-indophenol; PMS, phenazine methosulphate.

Electron acceptor	Activity (%)
Wurster's Blue (0.16 mM)	100
DCIP (0.16 mM)	10
DCIP (0.16 mM) + PMS (0.12 mM)	75
Cytochrome <i>c</i> (0.7 mM)	5
Cytochrome <i>c</i> (0.7 mM) + PMS (0.1 mM)	60
$\text{K}_3\text{Fe}(\text{CN})_6$ (0.7 mM)	100

Fig. 4. Absorption spectra of quinohaemoprotein alcohol dehydrogenase

The spectrum of the apoenzyme (—) was measured in 20 mM-Tris/HCl buffer, pH 7.7, containing 2 mM- CaCl_2 . The spectrum of the holoenzyme (---) was measured after incubating apoenzyme with stoichiometric amounts of PQQ in the same buffer, followed by butan-1-ol (0.1 mM) addition. Both spectra correspond with an enzyme concentration of $5.9\text{ }\mu\text{M}$.

DISCUSSION

Cell-free extracts of *Ps. testosteroni* grown on alcohols contain PQQ-dependent dye-linked alcohol dehydrogenase activity. The purified apoenzyme appeared to be homogeneous. The holoenzyme had a very broad substrate specificity, since not only primary and secondary alcohols but also aldehydes were substrates. However, it is questionable whether this ability to oxidize a wide range of substrates is functional *in vivo*. Thus methanol dehydrogenase (EC 1.1.99.8) also has a broad

substrate specificity, but negative indications were obtained for whole cells with respect to the role of the enzyme in formaldehyde oxidation (Groeneveld *et al.*, 1984). Furthermore, *Ps. testosteroni* contains substantial amounts of a dye-linked flavoprotein aldehyde dehydrogenase (M. Dijkstra, P. Poels & J. A. Duine, unpublished work).

Although the enzyme from *Ps. testosteroni* can be regarded as a quinoprotein, it is quite different from already known quinoprotein alcohol dehydrogenases such as that from *Pseudomonas aeruginosa* (Groen *et al.*, 1984). Thus it has a low pH optimum of 7.7 instead of 9 or higher, ammonium, alkylammonium and benzylammonium salts are not activators, not only cationic one-electron acceptors but also two-electron acceptors and anionic electron acceptors are active in the assay, it contains one molecule of haem *c* per enzyme molecule, and the presence of calcium salts is essential in the assay. Moreover, it should be noted in this context that, despite several attempts (Patel *et al.*, 1978; Kilty *et al.*, 1982), reconstitutable apoenzyme of the common quinoprotein alcohol dehydrogenases has never been described so far. In view of these striking differences, it is proposed to distinguish the here-described enzyme from the other group by using the name 'quinohaemoprotein alcohol dehydrogenase'.

The haem *c* became reduced in the holoenzyme (not in the apoenzyme) on addition of substrate. Since no oxidase activity was found (dissociated PQQH₂ should become oxidized under these conditions), indirect reduction of the haem *c* group by dissociated PQQH₂ can be excluded. Therefore the haem *c* is involved in the enzymic mechanism. The two cofactors participating in the process might form two possibilities for electron transfer, explaining the broad electron acceptor specificity of the enzyme.

As was postulated previously from their PQQ production (Duine & Frank, 1981a), *Acetobacter* and *Gluconobacter* species have a quinoprotein alcohol dehydrogenase (Ameyama & Adachi, 1982). These enzymes have a low pH optimum and their activity is not dependent on the presence of ammonium, alkylammonium or benzylammonium salts. Although activity with aldehydes was not mentioned and the presence of calcium salts was apparently not necessary in their assay, the enzymes might nevertheless be related to the enzyme described in the present paper. Since the purified enzymes appeared to be large complexes consisting of several proteins (Ameyama & Adachi, 1982), characterization of the alcohol dehydrogenase component in these complexes is a prerequisite before conclusions on similarity can be drawn.

Quinoprotein apoenzymes have been found in the case of glucose dehydrogenase (EC 1.1.99.17). In *Escherichia coli* strains (Hommes *et al.*, 1984) this apoenzyme could form an additional dissimilative route under certain circumstances, provided that PQQ is present in the environment as a vitamin. A comparable situation exists

in *Ps. testosteroni*. The organism can grow on alcohols (albeit poorly), probably via an NAD⁺-dependent alcohol dehydrogenase. At the same time it synthesizes quinohaemoprotein alcohol dehydrogenase apoenzyme. This might be profitable under natural conditions where PQQ is provided by other organisms, since addition of PQQ to the growth medium improves growth enormously (Fig. 1). A similar finding has been described for a *Pseudomonas* species where growth on poly(vinyl alcohol) was only possible in the presence of PQQ (Shimao *et al.*, 1984). As poly(vinyl alcohol) oxidase is formed in a mixed culture, this might indicate that the bacterium produces poly(vinyl alcohol) oxidase apoenzyme, reconstitutable with PQQ. If correct, this could be another example of the wide diversity in the nature of quinoprotein alcohol-oxidizing enzymes.

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References

- Ameyama, M. & Adachi, O. (1982) *Methods Enzymol.* **89**, 450–457
- Ameyama, M., Hayashi, M., Matsushita, K., Shinagawa, E. & Adachi, O. (1984) *Agric. Biol. Chem.* **48**, 561–565
- Bartsch, R. G. (1971) *Methods Enzymol.* **23**, 344–363
- Corey, E. J. & Tramontano, A. (1981) *J. Am. Chem. Soc.* **103**, 5599–5600
- Duine, J. A. & Frank, J., Jzn. (1980) *Biochem. J.* **187**, 213–219
- Duine, J. A. & Frank, J., Jzn. (1981a) *J. Gen. Microbiol.* **122**, 201–209
- Duine, J. A. & Frank, J., Jzn. (1981b) *Trends Biochem. Sci.* **6**, 278–280
- Duine, J. A., Frank, J. & Westerling, J. (1978) *Biochim. Biophys. Acta* **524**, 277–287
- Duine, J. A., Frank, J., Jzn. & Verwiel, P. E. J. (1980) *Eur. J. Biochem.* **108**, 187–192
- Duine, J. A., Frank, J., Jzn. & Berkhout, M. P. J. (1984) *FEBS Lett.* **168**, 217–221
- Duine, J. A., Frank, J., Jzn. & Jongejan, J. A. (1985) *Proc. FEBS Congr. 16th, part A*, pp. 79–88
- Groen, B. W., Frank, J., Jzn. & Duine, J. A. (1984) *Biochem. J.* **223**, 921–924
- Groeneveld, A., Dijkstra, M. & Duine, J. A. (1984) *FEMS Microbiol. Lett.* **25**, 311–314
- Hommes, R. W. J., Postma, P. W., Neijssel, O. M., Tempest, D. W., Dokter, P. & Duine, J. A. (1984) *FEMS Microbiol. Lett.* **24**, 329–333
- Kilty, C. J., Maruyama, K. & Forrest, H. S. (1982) *Arch. Biochem. Biophys.* **218**, 623–625
- Lineweaver, H. & Burk, D. (1934) *J. Am. Chem. Soc.* **56**, 658–666
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Patel, R. M., Hou, C. T. & Felix, A. (1978) *J. Bacteriol.* **133**, 641–649
- Shimao, M., Yamamoto, H., Ninomiya, K., Kato, N., Adachi, O., Ameyama, M. & Sakazawa, C. (1984) *Agric. Biol. Chem.* **48**, 2873–2876
- Van Schie, B. J., van Dijken, J. P. & Kuenen, J. G. (1984) *FEMS Microbiol. Lett.* **24**, 133–138