

Quinoprotein alcohol dehydrogenase from ethanol-grown *Pseudomonas aeruginosa*

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Cell-free extracts of *Pseudomonas aeruginosa* strains, grown on ethanol, showed dye-linked alcohol dehydrogenase activities. The enzyme responsible for this activity was purified to homogeneity. It appeared to contain two molecules of pyrroloquinoline quinone per enzyme molecule. In many respects, it resembled other quinoprotein alcohol dehydrogenases (EC 1.1.99.8), having a substrate specificity intermediate between that of methanol dehydrogenases and ethanol dehydrogenases in this group. On the other hand, it also showed dissimilarities: the enzyme was found to be a monomer (M_r 101 000), to need only one molecule of the suicide substrate cyclopropanol to become fully inactivated, and to have a different aromatic amino acid composition.

Quinoprotein ethanol dehydrogenase from a non-methylotrophic bacterium was first reported to occur in an *Acinetobacter calcoaceticus* strain (Duine & Frank, 1981). In that report it was postulated that other dye-linked alcohol dehydrogenases, frequently observed in a large variety of bacteria, also could be quinoproteins.

Since then it was found (B. Groen & J. A. Duine, unpublished work) and reported by Beardmore-Gray & Anthony (1983) that genuine *Acinetobacter calcoaceticus* strains do not possess such an enzyme. Since the original strain has been lost by ourselves and by others, it was considered necessary to substantiate our postulate by investigating one of the organisms suspected to contain such an enzyme. As *Pseudomonas aeruginosa* strains show dye-linked alcohol dehydrogenase activity (Van der Linden & Huybregtse, 1969; Tassin *et al.*, 1973), it was decided to purify such an enzyme and to characterize it.

Materials and methods

Organisms and growth conditions

Pseudomonas aeruginosa strains LMD 80.53 and PAO₁ were cultured at 35°C on a mineral medium (Duine *et al.*, 1978), supplemented with 0.5% (v/v) ethanol. Cells were harvested at the end of the exponential growth phase, washed with

Abbreviations used: PQQ, pyrroloquinoline quinone (2,7,9-tricarboxy-1*H*-pyrrolo[2,3*f*]quinoline-4,5-dione); h.p.l.c., high-performance liquid chromatography.

0.02M-potassium phosphate buffer, pH 7.2, and frozen.

Enzyme isolation

Frozen cells of strain LMD 80.53 were suspended in an equal volume of 36 mM-Tris/39 mM-glycine buffer, pH 9.0, and the cells were disrupted in a French pressure cell at 110 MPa (three times). The suspension (viscosity was lowered by adding deoxyribonuclease) was centrifuged at 48 000*g* for 30 min at 4°C. The supernatant was dialysed against 18 mM-Tris/19.5 mM-glycine buffer, pH 9.0, containing 0.05% (v/v) 2-mercaptoethanol. The dialysis residue (30 ml) was applied to a DEAE-Sephacel column (20 cm × 1.5 cm) in 18 mM-Tris/19.5 mM-glycine buffer, pH 9.0. As the enzyme passes through under these conditions, the column was washed with this buffer. Active fractions were pooled and applied to a silica-gel column (10 cm × 1 cm) in the same buffer to remove cytochrome *c* (Duine *et al.*, 1978). The eluate was concentrated by filtration, by using an immersible CX 30 ultrafilter (Millipore). The concentrated enzyme solution was centrifuged and further purified by h.p.l.c. gel filtration (Serva Si 200 polyol; 50 cm × 0.95 cm column) at a flow rate of 0.5 ml/min with 0.1 M-sodium phosphate buffer, pH 6.6, as the eluting buffer. The eluted enzyme peak (39.8 min) was analysed for homogeneity by a Hewlett-Packard HP 1040 A photodiode-array detector, by scanning the peak upslope, at the top, and downslope.

Enzyme assay

Samples (20 μ l) were added to a cuvette with 1 ml of 0.16M-sodium pyrophosphate buffer, pH9.0, containing 10mM-ethylamine, 1mM-KCN and 0.2mM-ethanol. The reaction was started by adding 1ml of 0.2mM Wurster's Blue solution (Duine & Frank, 1980). Rates were determined by monitoring the absorbance at 600nm and using a molar absorption coefficient at this wavelength of $9 \times 10^3 \text{ M}^{-1} \cdot \text{cm}^{-1}$.

Protein determinations

Protein concentrations during the purification were determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard, but for pure enzyme samples concentrations were calculated from the absorbance values at 205 and 280nm by the procedure of Scopes (1974).

M_r and PQQ determinations

The M_r of the native enzyme was determined by gel filtration on a Sephadex G-200 column (50cm \times 1cm) in 0.1M-sodium phosphate buffer, pH6.6 (Andrews, 1965), and by gel filtration on the h.p.l.c. column described above under 'Enzyme isolation', with the same marker proteins and eluting buffer as recommended for the Sephadex-column method.

The M_r of the denatured enzyme was estimated on a Serva Si 200 polyol h.p.l.c. gel-filtration column (25cm \times 0.46cm) in 0.1M-sodium phosphate buffer, pH6.6, containing 0.1% (v/v) sodium dodecyl sulphate. The eluted protein and co-enzyme peaks were monitored by the photodiode-array detector, as described above. Proteins for calibration (Pharmacia; 'low-molecular-weight' electrophoresis calibration kit) were chromatographed under the same conditions. The enzyme (50 μ l) was denatured by adding 150 μ l of 0.1M-sodium phosphate buffer, pH6.6, containing 1% (v/v) sodium dodecyl sulphate and 1% (v/v) 2-mercaptoethanol. The mixture was incubated for 60min at 95°C, centrifuged and injected on the column. To determine the amount of PQQ in the enzyme, the same procedure was followed except that 2-mercaptoethanol was omitted (Dijkstra *et al.*, 1984).

Aromatic amino acids

Aromatic amino acids were determined by multicomponent analysis of the absorbance spectrum of the enzyme, denatured by guanidinium chloride, by using a Hewlett-Packard HP 8450 A spectrophotometer, as described by Levine & Federici (1982). A similar procedure was developed to determine PQQ, with the use of a reference PQQ absorption spectrum taken in the same buffer. The region between 245 and 265 nm

was used to calculate the PQQ and phenylalanine concentrations, and that between 280 and 300 nm to calculate tryptophan and tyrosine concentrations.

Inactivation by cyclopropanol

Enzyme samples (4.4 μ M) in 0.1M-sodium pyrophosphate buffer, pH9.0, containing 10mM-ethylamine, 10mM-KCN and various concentrations of cyclopropanol were oxidized by adding Wurster's Blue (10 times the enzyme concentration) and titrating with 0.1M- $\text{K}_3\text{Fe}(\text{CN})_6$ until the blue colour persisted (Dijkstra *et al.*, 1984). The inactivation process was monitored by measuring the activities of samples of the mixture with the enzyme assay.

Materials

All chemicals were from Merck B.V., Amsterdam, The Netherlands, except guanidinium chloride, which was from BDH Chemicals (AristaR grade), cyclopropanol, which was prepared as described by Dijkstra *et al.* (1984), and Wurster's Blue, prepared as described by Duine *et al.* (1978).

Results

Induction of the enzyme

Cell-free extracts of *Ps. aeruginosa* LMD 80.53 grown on succinate, acetate or nutrient broth did not show dye-linked ethanol dehydrogenase activity. The activity was, however, detected in cells grown on ethanol. The strains *Ps. aeruginosa* PAO₁ and *Pseudomonas putida* LMD 72.6 behaved similarly.

Isolation

Purification of the dye-linked alcohol dehydrogenase activity was performed, from ethanol-grown *Ps. aeruginosa* LMD 80.53 cells. As the activity was not adsorbed on the DEAE-Sephacel column at high pH, this provided an easy way to purify the apparently basic protein. The enzyme, eluted from the h.p.l.c. column as a peak at 39.8min, appeared to be homogeneous, since the spectra taken upslope, at the top, and downslope, were identical. The absorption spectrum (Fig. 1) looks similar to those of other quinoprotein alcohol dehydrogenases (Anthony, 1982). On close examination it appears, however, that there is a slight difference in absorption maximum (340nm compared with 345nm) and A_{280}/A_{340} absorbance ratio (6 compared with 10 respectively). Most probably, the enzyme isolated is for a large part in the fully reduced form (MDH_{red}) instead of the half-reduced (semiquinone) form (MDH_{ox}) usually observed for these isolated enzymes (De Beer *et al.*, 1983).

Finally, the purified enzyme showed a specific

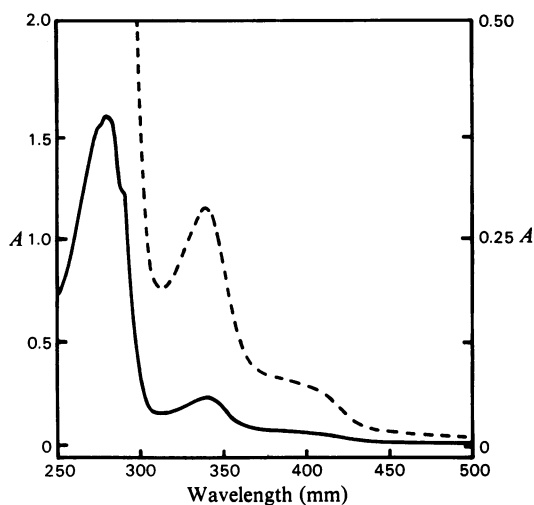


Fig. 1. Absorption spectrum of quinoprotein alcohol dehydrogenase from *Ps. aeruginosa*

Enzyme (3.9 mg/ml) was in 0.1 M-sodium pyrophosphate buffer, pH 9.0. The continuous line refers to the left-hand scale and the broken line refers to the right-hand scale.

activity of 35 μ mol of Wurster's Blue reduced/min per mg of protein, compared with a value of 0.23 for the cell-free extract.

Kinetic properties

As is to be expected for a quinoprotein alcohol dehydrogenase, enzyme activity was dependent on the presence of an activator and high pH (a pH optimum of 9.5 was found, with 0.2 mM-ethanol in the assay medium). Besides ammonium salts, amines were found to behave as activators [K'_m values and V' values relative to ethylamine (%) given in parentheses]: NH_4Cl (100 mM and 91), methylamine (7 mM and 100), ethylamine (2 mM and 100) and octylamine (0.05 mM and 86). The following compounds were tested as substrates [K'_m values and V' values relative to ethanol (%) are given in parentheses]: methanol (8 mM and 43), ethanol (0.013 mM and 100), propan-1-ol (0.010 mM and 95), propan-2-ol (0.64 mM and 88), octanol (0.0035 mM and 80), decanol (0.0020 mM and 74), formaldehyde (4 mM and 90), acetaldehyde (0.12 mM and 60) and octanal (0.038 mM and 60).

M_r

Both methods used to estimate the M_r of the native enzyme gave a value of 101 000. After denaturation, a value of 100 000 was found with gel filtration in the sodium dodecyl sulphate system.

Nature and quantity of coenzyme

Gel filtration of the denatured enzyme on the h.p.l.c. column revealed that the protein peak did

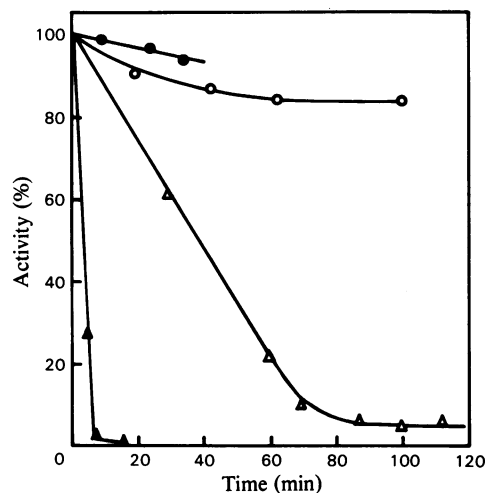


Fig. 2. Inactivation of quinoprotein alcohol dehydrogenases by cyclopropanol

Quinoprotein alcohol dehydrogenase from *Ps. aeruginosa* (4.4 μM) was oxidized as described in the text, and activities were measured during incubation without (\bullet) and with (\blacktriangle) 4.4 μM -cyclopropanol. For comparison, the results of quinoprotein alcohol dehydrogenase from *Hyphomicrobium X* (3.0 μM) are also indicated for incubation without (\circ) and with (\triangle) 6.0 μM -cyclopropanol. Activities of the latter enzyme were measured as described (Dijkstra *et al.*, 1984).

not contain substances absorbing above 300 nm. The peak of the low- M_r material showed an absorption spectrum identical with that of PQQ under these conditions. By using a value of 101 000 for the M_r of the enzyme, it was calculated that 2 ± 0.2 molecules of PQQ were extracted per enzyme molecule.

Aromatic amino acid content

The multicomponent analysis method revealed the presence of all the aromatic amino acids. The following numbers of residues per enzyme molecule were calculated: 27 (41) Phe, 33 (34) Trp and 28 (30) Tyr. For comparison, the values of methanol dehydrogenase from *Hyphomicrobium X* are given in parentheses. In agreement with the results above, this method also showed that one enzyme molecule contains two molecules of the coenzyme PQQ.

Cyclopropanol inactivation

As shown in Fig. 2, this enzyme is rapidly inactivated as compared with the enzyme from *Hyphomicrobium X* (Dijkstra *et al.*, 1984). From the data presented in Fig. 3, a value of 1.0 ± 0.1 molecules of cyclopropanol per enzyme molecule was calculated for the complete inactivation of enzyme.

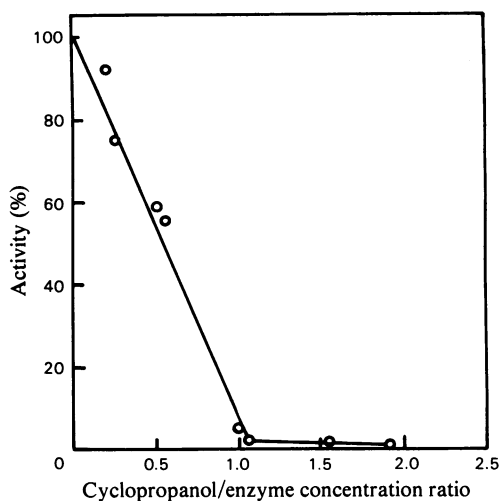


Fig. 3. Incubation of quinoprotein alcohol dehydrogenase from *Ps. aeruginosa* with different concentrations of cyclopropanol

Enzyme (4.4 μ M) was oxidized and incubated with various concentrations of cyclopropanol for 15 min. The procedure and measurement of enzyme activities were performed as described in the text.

Discussion

The purified enzyme appears to be a quinoprotein alcohol dehydrogenase, as it shows the typical absorption spectrum, contains PQQ, has a high pH optimum and needs an activator such as NH_4Cl . From its substrate and activator specificity, it appears to be an 'ethanol dehydrogenase', having, however, a somewhat better affinity for methanol than comparable enzymes (Bamforth & Quayle, 1978; Duine & Frank, 1981).

Most quinoprotein alcohol dehydrogenases are dimers with an M_r of about 120 000 (Anthony, 1982). The value of 101 000 found for this enzyme is substantially lower, but, more surprisingly, it is a monomeric enzyme. Since two coenzyme molecules were extracted, it could be envisaged that two catalytic centres exist in the enzyme. Since the application of the suicide substrate cyclopropanol clearly demonstrated that this was the case for methanol dehydrogenase from *Hyphomicrobium X* (Dijkstra *et al.*, 1984), similar experiments were performed with the enzyme. However, as the results indicated that addition of one molecule of cyclopropanol is sufficient to inactivate one molecule of enzyme, no conclusion can be drawn. Either this enzyme has two catalytic centres but modification of one PQQ eliminates the activity of the other, or this enzyme shows 'half-of-the-sites reactivity', so that in fact modification of

one site is sufficient to obtain complete inactivation. Another explanation could be that only half of the enzyme preparation is active. However, in view of the consistent ratio observed for different preparations, this seems very unlikely. Anyhow, the results show that the mechanism of action of this enzyme may be different from that of other quinoprotein alcohol dehydrogenases.

The existence of PQQ-containing dye-linked alcohol dehydrogenase in *Ps. aeruginosa* strains may imply that, in view of the widespread occurrence of dye-linked alcohol dehydrogenase activities, quinoprotein alcohol dehydrogenases are common enzymes in alcohol- or alkane-degrading bacteria. The results of the characterization studies on alcohol dehydrogenases from *Acetobacter* and *Gluconobacter* species support this view. Although these enzymes are different in several respects from the enzyme described here (they have a low pH optimum, they do not need an activator in the assay, negatively charged electron acceptors are active) and are isolated as a complex with, among other components, cytochrome *c*, they are probably quinoproteins, since PQQ has been detected after denaturation of the complex (Adachi *et al.*, 1982).

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