A novel dye-linked formaldehyde dehydrogenase with some properties indicating the presence of a protein-bound redox-active quinone cofactor

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Dye-linked formaldehyde dehydrogenase from methylamine-grown *Hyphomicrobium zavarzinii* ZV 580, a tetramer of M_r 210000 with subunits of M_r 54000, was purified to homogeneity in five steps with 10% yield. The enzyme shows optimal affinity for, and activity with, formaldehyde (K_m 67 μ M) compared with other aldehydes. Pyridoxal phosphate, pyrroloquinoline quinone and other cofactors that would give the enzyme a distinctive absorption spectrum are absent. Slight changes are observed in the spectrum at 300–550 nm on oxidation of the enzyme with Wurster's Blue (WB) and reduction with formaldehyde. Titration of the native reduced enzyme with WB accounts for 2 mol of electrons per mol of tetrameric enzyme. The circumstantial evidence supporting the presence of a redox-active quinone cofactor bound to the polypeptide chain comprises a signal at

$g = 2.0049$ in the X-band e.p.r. spectrum of the enzyme oxidized with WB, which disappears on reduction with formaldehyde, and a positive reaction of the native as well as the denatured and dialysed enzyme in the redox-cycling assay with glycinate and NitroBlue Tetrazolium (quinone staining). The oxidized enzyme is inhibited by equimolar amounts of phenylhydrazine, which is also a reductant. Hydrazone formation was absent with completely inhibited enzyme, according to photometric evidence. Likewise, the glycinate-dependent reduction of NitroBlue Tetrazolium was not affected by the inhibitor. It is concluded that an oxidation product of the hydrazine is the actual inhibitor which reacts with an amino acid residue of the active site rather than with the prospective quinone cofactor.

INTRODUCTION

Biochemical problems presented by dye-linked aldehyde dehydrogenases (dAldDHs) (EC 1.2.99.3) include the properties of their cofactors as well as their role in the oxidative pathways for hydrocarbons, alcohols and certain amines. Identified cofactors of enzymes from this group are FAD plus molybdopterin and iron–sulphur groups [1,2] or tungsten and iron–sulphur groups [3]. For other dAldDHs there is evidence for the presence of haem *c* [4,5] but see also [6] or pyrroloquinoline quinone (PQQ) [7,8]. The enzyme studied here seems to contain yet another cofactor, a covalently bound quinone or redox-active modified amino acid residue. Cofactors of this type range, in the order of discovery, from the tyrosine radical of ribonucleotide reductase [9] to 6-hydroxydopaquinone (topaquinone) of bovine serum amine oxidase [10], tryptophan tryptophylquinone of methylamine dehydrogenase [11] and the tyrosine radical thioether linked to a cysteine residue in galactose oxidase [12].

Although dAldDHs were first discovered in methylotrophic bacteria [13] and occur in several species of this group [2,4,5,14–18] as well as other bacteria [1,3,7,8,19], interest in their possible role in (form)aldehyde oxidation was lessened by the existence of other prominent enzymes and pathways for this purpose, among them the NAD(P)⁺-dependent (form)aldehyde dehydrogenases (for overviews see [6,20]). In addition, dAldDHs showed little substrate specificity and affinity and often low activities, as exemplified by the enzyme from methanol-grown *Hyphomicrobium* X [16]. Only a few dAldDHs with sole, or at least marked, specificity for formaldehyde have been reported; they include the partially purified enzyme from *Pseudomonas* sp.

RJ1, which exclusively oxidizes formaldehyde [14], and the formaldehyde/formate ester dehydrogenase from *Comamonas testosteroni* with a high affinity towards formaldehyde [2]. The enzyme in the present paper, termed dye-linked formaldehyde dehydrogenase because of its optimal affinity towards formaldehyde [21], is different from the dAldDHs of methanol-grown *Hyphomicrobium* X [16] and of methanol-grown *H. zavarzinii* ZV 580 [22,23], and is induced or derepressed and repressed depending on the carbon source used for growth (F. P. Kessler and A. C. Schwartz, unpublished work).

MATERIALS AND METHODS

Materials

DNAase (Benzonase) was obtained from Merck (Darmstadt, Germany) and other enzymes were from Boehringer (Mannheim, Germany). Media for chromatography were from Pharmacia (Freiburg, Germany) and IBF (Serva, Heidelberg, Germany). Wurster's Blue (WB) was prepared chemically [24]. Other chemicals were of analytical or biochemical grade.

Organisms and growth

H. zavarzinii strain ZV 580 from the collection of the Institut für Allgemeine Mikrobiologie, Universität Kiel (D-24118 Kiel, Germany) was maintained and grown on methylamine as described [22] in batches of 16 litres, which were continuously stirred and aerated with 12 l/h sterile air per bottle. Cells were harvested at the end of the exponential phase after 5 days with a yield of about 2.6 g/l (wet weight).

Abbreviations used: DCIP, dichlorophenol–indophenol; dAldDH, dye-linked aldehyde dehydrogenase; NBT, NitroBlue Tetrazolium; PMS, phenazine methosulphate, *N*-methylphenazinium sulphate; PQQ, pyrroloquinoline quinone; WB, Wurster's Blue, the radical cation of *NNN'*-tetramethyl-*p*-phenylenediamine.

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Purification of the enzyme and determination of size

All steps were performed at 4 °C. Fresh or frozen cells (200 g) were suspended in 2 vol. of 50 mM Tris/HCl buffer, pH 7.4, containing 0.2 mM phenylmethanesulphonyl fluoride as proteinase inhibitor, 2.5 units/ml DNAase and 3 mM MgCl₂, and were passed twice through an Aminco–French pressure-cell at about 80 MPa, with cooling and pH adjustment between the passages. Cell debris was removed by centrifuging at 48 000 *g* for 30 min. The enzyme was then precipitated between 60 and 75 % (NH₄)₂SO₄ saturation. The dissolved sediment was dialysed against 50 mM Tris/HCl buffer, pH 8.5, applied to a column (4.5 cm × 20 cm) of DEAE-Sephacel (same buffer) and eluted in fractions of volume 3 ml (same fraction size in all steps) with the same buffer without using a gradient. Fractions containing more than 60 % of the highest activity were pooled, concentrated with an Amicon Diaflo PM30 membrane, transferred to 50 mM potassium phosphate buffer, pH 7.2, and chromatographed on a column (2.5 cm × 70 cm) of Sephacryl S-300 in the same buffer. Active fractions pooled as above were made 30 % saturated with (NH₄)₂SO₄ and applied to a column (4.5 cm × 10 cm) of phenyl-Sephacel equilibrated under the same conditions. The enzyme was eluted with a gradient of 30–0 % (NH₄)₂SO₄ in this buffer, and washed with the same buffer. The last step was again chromatography on a column (4.5 cm × 10 cm) of DEAE-Trisacryl equilibrated in 10 mM potassium phosphate buffer, pH 8.5, to which the enzyme bound slightly more tightly than in the previous DEAE-Sephacel step. It was eluted with the same buffer, or a gradient of 10–100 mM NaCl in this buffer.

The *M_r* was determined for both the activity in the (NH₄)₂SO₄ fraction and the purified enzyme by chromatography on a column (2.6 × 90 cm) of Sephacryl S-300, which was calibrated with the marker proteins ferritin (*M_r* 450 000), catalase (*M_r* 240 000), aldolase (*M_r* 158 000), BSA (*M_r* 68 000) and cytochrome *c* (*M_r* 12 500) in a separate run.

Chromatographic analysis of purified and denatured enzyme was performed by size-exclusion h.p.l.c. on a column (1 cm × 30 cm) of Superose-12 connected to a Hewlett–Packard 1040 diode-array detector. The enzyme was denatured by heating 37 pmol of enzyme protein in 200 µl of 50 mM sodium phosphate buffer, pH 6.5, with 0.2 % (w/v) SDS for 2 min at 100 °C. After cooling in an ice bath the sample was chromatographed in the same buffer with 0.1 % SDS and 100 mM NaCl at a flow rate of 0.5 ml/min.

Enzyme assays

The enzyme was assayed with a Clark oxygen electrode [16,22] in 50 mM potassium phosphate buffer, pH 8.0, with 2.8 mM phenazine methosulphate (PMS) and 1 mM formaldehyde at 30 °C or in a photometer with 125 µM dichlorophenol–indophenol (DCIP) (ϵ_{578} 13 000 M⁻¹·cm⁻¹) and 2.5 mM PMS in the same buffer at 30 °C (modified from [16]). Corrections for the pH-dependent change in DCIP absorbance were applied when necessary. Activities in the two assays were similar. Formaldehyde was freshly prepared by hydrolysis of paraformaldehyde. PMS was freshly dissolved every day and kept on ice in the dark.

Reduction of Methylene Blue and WB was recorded at 578 nm and 30 °C (ϵ_{578} 12 000 M⁻¹·cm⁻¹ for Methylene Blue B and 10 980 M⁻¹·cm⁻¹ for WB). The reduction of Methylene Blue was tested in an anaerobic cuvette flushed with nitrogen. Reduction of cytochrome *c* from horse heart was tested as described in [25].

K_m and *V_{max}* values were determined by the direct graph method [26].

In order to establish the stoichiometry of the catalysed reaction, residual formaldehyde was determined with alcohol dehydro-

genase from yeast in a method originally developed for glycolaldehyde [27]. Production of formate was assayed with NAD⁺-dependent formate dehydrogenase from *Candida boidinii* [28], after PMS and DCIP had been removed from the samples with charcoal.

U.v./visible and e.p.r. spectrometry

Absorption spectra of the purified enzyme were recorded with a Hewlett–Packard 8452 diode-array spectrophotometer. Oxidized enzyme was obtained by adding an excess of WB, which was then removed by chromatography on a column (1 cm × 30 cm) of Superose-12 in 100 mM sodium phosphate buffer, pH 6.5, at a flow rate of 0.5 ml/min. For titration, 160 µl of enzyme in 50 mM potassium phosphate buffer, pH 8, in a Hellma quartz microcuvette was oxidized with 0.1 µl portions of WB (2.2 mM in water), added with a 1 µl syringe.

E.p.r. spectra of the enzyme were recorded with a Varian E9 spectrometer in a standard flat cell at room temperature.

PAGE and isoelectric focusing

Disc or slab-gel PAGE was performed in gels with various acrylamide concentrations or gradients and with Tris/glycinate buffer, pH 8.9 [29]. For denaturing electrophoresis, 0.2 % (w/v) SDS was added to the gels and 0.1 % SDS to the electrode buffer. Protein samples were boiled for 10 min with 0.2 % SDS and 0.4 % 2-mercaptoethanol when applicable. Gels were stained for protein with Coomassie Blue R250, for enzyme activity with NBT and substrate [22] and for the presence of a quinone cofactor (quinone staining) with 1.5 M sodium glycinate at pH 9.0 and 0.25 mM NBT in an adaptation of the method of Paz et al. [30].

Isoelectric focusing was carried out in agarose gels with Pharmacia ampholyte pH 4.0–6.5 as described in *Pharmacia Information* (1982). The enzyme was stained with the activity stain above or with Coomassie Blue after fixation with sulphosalicylic acid plus trichloroacetic acid.

Miscellaneous methods

The presence of PQQ in the enzyme was tested for by reconstituting the apoenzyme of glucose dehydrogenase with acidic methanol extracts of purified enzyme [31]. The presence of an alternative quinone cofactor was tested for redox cycling with glycinate and NBT at pH 10, as described by Paz et al. [32] except that serum albumin was omitted. The presence of pyridoxal phosphate in the enzyme was assayed for by its reaction with phenylhydrazine after extraction with perchloric acid [33].

Analysis of heavy metals was performed by Mikroanalytisches Labor Pascher (An der Pulvermühle 3, D-53424 Remagen, Germany) by using emission spectrometry and a sample of 2 mg of lyophilized enzyme. Protein concentrations during purification were determined by the Biuret method [34] and, from the chromatography steps onwards, by the u.v. method [35]. For the purified enzyme, which was dialysed against distilled water, lyophilized and then redissolved, the *A*₂₈₀ of a solution of 1 mg/ml was 1.25, as determined by a spectrophotometric method [36].

RESULTS AND DISCUSSION

Purification of the enzyme, *M_r* and isoelectric point

The enzyme was obtained in an apparently homogenous form with specific activity 10–40 µmol of formaldehyde oxidized/min

Table 1 Purification of dye-linked formaldehyde dehydrogenase from *H. zavarzinii* ZV 580Units of enzyme activity are μmol of formaldehyde oxidized/min.

Purification step	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Purification (fold)	Yield (%)
Cell-free extract	3960	1740	0.44	1	100
$(\text{NH}_4)_2\text{SO}_4$ fraction, dialysed	1900	1320	0.69	1.6	76
DEAE-Sephacel eluate	99.3	1170	11.8	27	67
Sephacryl S-300 eluate	31.1	539	17.3	39	31
Phenyl-Sepharose eluate	14.4	284	19.7	45	16
DEAE-Trisacryl eluate	5.5	139	25.3	58	8

per mg of protein, and with a yield of 8–12 % after about 60-fold enrichment (Table 1). The first chromatographic step (DEAE-Sephacel) separated the enzyme from the constitutive methanol dehydrogenase and a trace of the other dAldDH, which is typical of methanol-grown cells [16,22]. Both unwanted activities bind more strongly to DEAE-Sephacel than the required enzyme. PAGE in denaturing SDS gradient gels with 5–15 % acrylamide and in non-denaturing 7.5 % gels produced one band for the purified enzyme, which occurred at identical positions in non-denaturing gels stained for protein and activity as well as redox cycling with glycine and NBT (Figure 1).

The M_r of the purified enzyme as well as the activity in the $(\text{NH}_4)_2\text{SO}_4$ fraction, determined by chromatography on Sephacryl S-300, was 210000, and that of the four apparently identical subunits, determined by SDS/PAGE, was 54000 in both the absence and presence of thioethanol.

The isoelectric point of the enzyme (5.25) was determined in a gel with a pH gradient between 4.0 and 6.5. The position of the enzyme was verified by both protein and activity staining.

pH optimum of the enzyme

With potassium phosphate or Tricine/KOH buffer (50 mM), a plateau of activity between pH 7.8 and 9.0 was obtained with both assays, and with 50 mM sodium borate a plateau at pH 9–9.7 was obtained. For other buffers, a narrower range of pH optima was observed for enzyme activity. Tris/HCl gave an optimum at pH 8, and 2-amino-2-methyl-propan-1-ol (Amprop/HCl) and 3-cyclohexylamino-2-hydroxy-1-propane-sulphonic acid (Capso/KOH) both yielded optima at pH 9.0–9.2. For activities above pH 8.5, noticeable reactions in the absence of enzyme, which occurred in both tests and increased with pH, had to be subtracted for all buffers. For comparison, activities with Tricine/KOH, Hepes/KOH, Mops/KOH, sodium pyrophosphate and Tris/HCl (50 mM each) were determined at pH 8.5, and were observed to decrease from Tricine/KOH (100 %) to the least-suitable buffer Tris/HCl (57 % residual activity). For routine assay, potassium phosphate, pH 8, was used because of the absence of any spontaneous reaction.

Specificity for aldehydes and electron acceptors

The enzyme showed highest affinity for formaldehyde, and decreasing affinities and V_{max} values for aldehydes with higher chain length and substitutions on the molecule (Table 2). Benzaldehyde was less well oxidized by this enzyme than by the dAldDH from methanol-grown cells [21,22]. The enzyme did not oxidize methanol, ethanol, propanol or methylformate.

Of the electron acceptors tested, WB, which was available only

Figure 1 PAGE of purified formaldehyde dehydrogenase from methylamine-grown *H. zavarzinii* ZV 580

(a) Purification: lane 1, cell-free extract; lane 2, after $(\text{NH}_4)_2\text{SO}_4$ fractionation; lane 3, after the DEAE-Sephacel step; lane 4, after the Sephacryl S-300 step; lane 5, after the phenyl-Sepharose step; lane 6, after the DEAE-Trisacryl step. The protein stain was Coomassie Blue: lanes 1–4, 50 μg of protein each; lane 5, 25 μg ; lane 6, 10 μg . (b) Purified enzyme (20 μg per lane): lane 1, Coomassie Blue stain; lane 2, activity staining with formaldehyde, PMS and NBT; lane 3, redox cycling with glycinate and NBT at pH 9.

late during the investigation, was most active with the enzyme (Table 2). With DCIP in the absence of PMS only 2.3 μmol of formaldehyde was oxidized/min per mg of protein. Cytochrome *c* from horse heart, ferricyanide, Methylene Blue B and molecular oxygen itself were not reduced by the enzyme. These data provide further evidence that distinguishes this enzyme from the dAldDHs of methanol-grown strains [16,23].

The activity of the enzyme was also determined with [^3H]formaldehyde in both assays. Under conditions of initial-rate determination and saturating aldehyde concentration, the

Table 2 Apparent Michaelis constants and maximal velocities of dye-linked formaldehyde dehydrogenase for various aldehydes and electron acceptors

Kinetic parameters were determined by the direct graph method of Eisenthal and Cornish-Bowden [26].

Aldehyde or acceptor	K_m (μM)	V_{max} (μmol of aldehyde oxidized/min per mg of protein)
Formaldehyde*	67	40.6
Acetaldehyde*	168	37.6
Propionaldehyde*	180	23.3
Butyraldehyde*	205	21.7
Glycolaldehyde*	1000	4.5
Benzaldehyde†	1000	—
Vanillin†	9020	—
PMS‡	220	36.0
WB‡§	8.3	61.3

* PMS/DCIP assay, 3.2 μg of enzyme per determination.

† PMS/oxygen assay, different enzyme preparation.

‡ 1 mM formaldehyde as substrate in tests of electron acceptors.

§ Different enzyme preparation, 2.9 μg per determination.

Figure 2 Stoichiometry of the reaction catalysed by dye-linked formaldehyde dehydrogenase

PMS/DCIP assay: ●, formaldehyde consumed versus DCIP reduced, excess of DCIP (250 μM); ▲, same, but with an excess of formaldehyde (500 μM); ■, formaldehyde consumed versus formate produced (DCIP 250 μM).

activity of the batch of purified enzyme used was 9.95 (formaldehyde) compared with 5.0 ($[^2H]$ formaldehyde) $\mu mol/min$ per mg of protein, giving a ratio of V_{max} (formaldehyde)/ V_{max} ($[^2H]$ formaldehyde) of 2. This is not a significant isotope effect, and therefore hydrogen transfer is not the sole rate-limiting step of the reaction.

Stoichiometry of the enzyme-catalysed reaction

The stoichiometry was analysed by using the PMS/DCIP assay. With 250 μM DCIP present, added formaldehyde up to 160 μM was completely oxidized, and the electrons were quantitatively transferred to the electron acceptor (Figure 2). Likewise, added DCIP was completely reduced by equivalent amounts of for-

Figure 3 Kinetic determination of the reaction mechanism of dye-linked formaldehyde dehydrogenase with the PMS/DCIP assay

Eadie-Hofstee diagrams: (a) variation of formaldehyde concentration with fixed PMS concentrations: 0.05 mM (Δ), 0.25 mM ($\blacksquare$), 0.4 mM ($\square$), 1.5 mM ($\bullet$), 2.5 mM ($\circ$). (b) Variation of PMS concentration with fixed formaldehyde concentrations: 0.05 mM (Δ), 0.1 mM ($\blacksquare$), 0.2 mM ($\square$), 1.0 mM ($\bullet$), 1.3 mM ($\circ$). DCIP was always 125 μM .

maldehyde consumed in the presence of excess formaldehyde (Figure 2). Determination of formaldehyde consumed and formate produced also yielded a ratio of 1:1 over a similar range (Figure 2). The formaldehyde was freshly prepared from para-formaldehyde to avoid low readings caused by polymerization. Incubation of the enzyme in an assay mixture without an electron acceptor resulted in neither formaldehyde consumption nor formate production. The results permit formulation of the equation:

where A is the two-electron acceptor PMS [37] or DCIP.

Kinetic investigation of the catalysed reaction

Formaldehyde and PMS concentrations were varied in the PMS/DCIP assay using a single batch of enzyme, which was selected for its size rather than its specific activity in order that the necessary number of determinations could be made. The results are plotted in Eadie-Hofstee diagrams (Figures 3a and 3b). In the simple treatment of the data, the lines for all concentrations of fixed substrate were calculated by linear regression. Then the means of all respective slopes from each set of data obtained for the two fixed substrates were determined. It was observed that the slope for 0.25 mM PMS and varying formaldehyde concentration deviated least from the means of all slopes obtained with fixed PMS concentrations. Therefore this slope was used to draw all the lines in Figure 3(a). Likewise, the

Figure 4 Absorption spectrum of dye-linked formaldehyde dehydrogenase and titration of the redox-active cofactor with WB

—, Native enzyme and oxidized enzyme after reduction with formaldehyde; ----, enzyme oxidized with WB (protein 1.1 mg/ml; path 1 cm). Insert: titration with WB (2.2 mM). Enzyme was 3.46 nmol (21.6 μ M) of subunits in potassium phosphate buffer, pH 8 (volume 160 μ l; specific activity 29 μ mol of formaldehyde oxidized/min per mg of protein). See the text for further details.

Figure 5 E.p.r. spectrum of dye-linked formaldehyde dehydrogenase

The spectrum is an average of two runs. The details were as follows: protein, 11.2 mg/ml; Varian E9 spectrometer; standard flat cell; microwave frequency 9514.3 GHz; modulation amplitude 0.1 mT; microwave power 0.5 mW; time constant 1 s; scan time 0.5 mT/min; 23 °C.

slope for 1 mM formaldehyde and varying PMS concentration was used for the lines in Figure 3(b). Both graphs indicate that the experimental points do not seriously deviate from the sets of parallel lines, and therefore permit the conclusion that the enzyme reacts with PMS by a Ping Pong mechanism. Although the kinetic analysis is preliminary because the second-best acceptor, PMS, was used instead of WB or the unknown natural

acceptor, it nevertheless provides an independent line of evidence that the enzyme must contain a redox cofactor which accepts electrons from the aldehyde before transferring them to PMS.

Oxidation and reduction of the enzyme

The absence of any distinctive structure in the u.v./visible absorption spectra of the slightly yellowish purified enzyme (Figure 4) suggests that the enzyme is devoid of the more common coenzymes and prosthetic groups. Addition of formaldehyde did not produce any spectral changes, indicating that the enzyme is apparently isolated in a fully reduced state. Oxidation with WB resulted in significant and characteristic alterations at 300–550 nm (Figure 4) which were reversed on addition of formaldehyde, proving that the chromophoric structure is involved in the redox reaction of the enzyme. The stepwise oxidation of the enzyme was recorded at 350 nm (Figure 4, insert). As the unreduced dye absorbs at 350 nm too, complete reduction of the portions of WB added was controlled at 612 nm. When the enzyme was completely oxidized, unreduced WB accumulated in the cuvette and, because of its own higher specific absorbance, led to a steeper increase in A_{350} (Figure 4, insert). Concomitantly, the spectrum of unreduced WB appeared in the region 500–700 nm after complete oxidation of the enzyme. The stoichiometry of the oxidation, 1.71 nmol of WB reduced per 3.46 nmol of enzyme subunits, as calculated from the intercept of the two lines in the inset to Figure 4, only accounts for the presence of 2 nmol of electrons in 1 nmol of the tetrameric enzyme. The photometric results do not permit determination of the nature of the cofactor of the enzyme. However, they are compatible with the presence of a redox-active quinone group, which is indicated by subsequent experiments.

In the X-band e.p.r. spectrum, a signal at $g = 2.0049$ with a peak-to-peak linewidth of 0.53 mT was observed for the enzyme oxidized with an excess of WB (Figure 5). It disappeared on reduction of the enzyme with formaldehyde and was only slightly visible in the native enzyme, showing that again, in essence, fully reduced enzyme is obtained in these cases. The features of the signal are very similar to those of the PQQ radical and indicate the presence of a quinone free radical in the oxidized enzyme [38]. The amplitude of the e.p.r. signal is not stoichiometric with the concentration of the enzyme; the exact ratio was not determined. The hyperfine splittings, particularly on the low-field side of the spectrum (Figure 5), were not observed for the PQQ radical; they may be due to the interaction of the unpaired electron with hydrogen or nitrogen atoms in the enzyme.

Further assays of cofactors

The assay for the presence of free, i.e. non-covalently bound, PQQ, in the purified enzyme was negative. Addition of PQQ in concentrations of 0.1–20 μ M together with 1.3 mM CaCl_2 or 2.7 mM MgCl_2 did not increase the activity of the enzyme. Pyridoxal phosphate was also not found in the enzyme, and neither were heavy metals except for traces of impurities.

Redox cycling with glycinate and NBT, i.e. chemical catalysis of the oxidative deamination of glycine at pH 9–10 and transfer of the electrons to NBT, is a property of PQQ [32,39], other substances [40] and enzymes with covalently bound quinone residues (quinone staining [30]; see also Figure 1). As there were no spurious bands on gels of partially purified preparations subjected to quinone staining, false positive reactions of other enzymes in this test did not occur. In the liquid test at pH 10, formation of formazane was proportional to enzyme concentration (Figure 6). Quantitative and comparative data on the

Figure 6 Redox cycling of dye-linked formaldehyde dehydrogenase with glycinate and NBT at pH 10

Increasing concentrations of purified enzyme were incubated with 2 M glycinate (●) or 0.5 M valinate (○), each adjusted to pH 10 with NaOH, and 0.24 mM NBT for 30 min at 37 °C.

Table 3 Redox-cycling activity of denatured formaldehyde dehydrogenase

Enzyme samples from fractions obtained by DEAE-Trisacryl chromatography were denatured and subsequently dialysed in two different ways: treatment A was denaturation with 8 M urea for 5 min at 100 °C and dialysis against buffer at 4 °C; treatment B was denaturation with 6 M guanidinium chloride + 100 mM 2-thioethanol + 10 mM EDTA for 4 h at 50 °C, then 24 h at 22 °C and dialysis against distilled water at 4 °C. Different amounts of protein from each treatment were then subjected to redox cycling with glycinate and NBT at pH 10. Controls were treated in the same way except the denaturing agent was omitted from the buffer (50 mM potassium phosphate, pH 8). See the text for further details.

Mode of denaturation and dialysis	Protein (μg/assay)	Absorbance at 530 nm	
		Denatured sample	Control
Treatment A	70	0.270	0.285
	140	0.631	0.600
Treatment B	105	0.275	0.300
	210	0.751	0.700
	420	1.300	1.350

reaction of other quinoproteins in this test have not been reported. Valine was inactive as an electron donor in the test with our enzyme, as with PQQ [39].

Redox cycling was also used to investigate the mode of attachment of the quinone cofactor to the enzyme. When the enzyme was denatured with 8 M urea or 6 M guanidinium chloride and then exhaustively dialysed, redox-cycling activity was retained in all cases (Table 3), whereas diAldDH activity was completely abolished by these treatments as a result of denaturation of the protein moiety. This indicates that the quinone in question is covalently bound to, or integrated into, the polypeptide chain.

Evidence for the covalent attachment of the chromophore, which participates in catalysis according to the photometric experiments, was obtained by h.p.l.c. analysis of the denatured enzyme in SDS-containing buffer. The absorption spectrum of the eluted protein showed that the chromophore was co-eluted with the denatured enzyme (not shown). As a result of binding of

Figure 7 Inactivation of dye-linked formaldehyde dehydrogenase by phenylhydrazine

Purified enzyme (1 μM subunits) was incubated with phenylhydrazine in 50 mM potassium phosphate buffer, pH 8, at 30 °C: ●, 1 μM phenylhydrazine; ▲, 0.5 μM phenylhydrazine; ■, 1 μM phenylhydrazine + 2 μM DCIP. The PMS/oxygen assay was used. The activity of the control without phenylhydrazine decreased from 100 to 90% in 30 min.

SDS, the denatured enzyme had a retention time of 20 min, whereas the native enzyme was eluted at 23.8 min.

Inhibition by phenylhydrazine

Inhibition by phenylhydrazine was investigated by incubating purified enzyme with inhibitor concentrations in the equimolar range and withdrawing samples for the PMS/oxygen assay. The enzyme was rapidly and completely inhibited, even more so with the oxidants DCIP or WB present (Figure 7). Some preparations were only inhibited when an oxidant was added. Photometric observation indicated that phenylhydrazine is also a reductant of the enzyme. Absorption spectra of completely inhibited enzyme did not show hydrazone formation with either phenylhydrazine or nitrophenylhydrazine. As the enzyme was not re-activated by molecular sieving with Sephadex G-25, some mode of binding of the hydrazine to the active centre must have occurred. In line with the absence of hydrazone formation, the redox-cycling assay with glycinate and NBT was not impaired at all in completely inhibited enzyme samples. Obviously, the hydrazines do not act on the enzyme as carbonyl reagents. It is assumed at present that they are oxidized by the enzyme and that an oxidation product is the actual inhibitor, which reacts with an essential amino acid residue of the active site without causing distinctive spectral changes.

Preincubation with *p*-hydroxymercuriphenylsulphonate, *N*-ethylmaleimide, *o*-phenanthroline or cyclopropanol (up to a 50-fold excess [40]) did not affect the activity of the enzyme.

Concluding remarks

The most intriguing question to arise from the investigation of this enzyme is the identity of its covalently bound redox cofactor. It is assumed at present that it is a single redox entity, which manifests itself in the Ping Pong mechanism as well as in the various activities and physical signals of the enzyme, and which may interact with other amino acid residues of the active site. The notion that the cofactor is a quinone residue is supported by the e.p.r. signal at $g = 2.0049$ of the oxidized enzyme, which is typical of a protein-bound quinone free radical [38], and the glycinate-dependent reduction of NBT, which is catalysed by quinone cofactors (quinone stain [30]). Inhibition of phenylhydrazine, which acts as a carbonyl reagent in many quinoproteins [10,41], does not lead to hydrazone formation, and therefore, although irreversible, cannot affect the proposed

quinone in our enzyme. This is corroborated by the absence of inhibitory effects of hydrazines on the glycinate-dependent reduction of NBT. As phenylhydrazine is oxidized by the enzyme, it is assumed that an oxidation product is the actual inhibitor which then interacts with an amino acid residue of the active site. Inhibition by phenylhydrazine that does not lead to hydrazone formation has been observed in lipoxygenase-1 [42] and dopamine β -hydroxylase [43]. The u.v./visible spectral changes observed on oxidation and reduction of the enzyme cannot be ascribed to any recognized structure, but are compatible with the presence of a quinone residue. A_{350} was used to titrate apparent cofactor sites in the native reduced enzyme through consumption of the oxidant WB. The results account for the presence of 0.5 mol of electrons per mol of subunit, i.e. 2 mol of electrons per mol of native reduced tetrameric enzyme from very active preparations, and indicate that, at most, one-half of the enzyme has reacted in the titration presented here. It is not known whether inactive enzyme was present, whether the native enzyme was indeed fully reduced in spite of the absence of physical signals on addition of formaldehyde or which other causes may have led to this result. Therefore the question of whether only two or all four subunits, which are apparently identical in size and electrophoretic mobility, contain one molecule of cofactor harbouring the necessary minimum of one electron cannot be decided at present. The transfer of one electron per active subunit or site is compatible with the redox change of the quinone residue between the reduced state and the radical or semiquinone state, which was observed by e.p.r. spectroscopy.

This diAldDH from *H. zavarzinii* ZV 580 exhibits sufficient activity and substrate specificity to play a major role in formaldehyde oxidation during growth on and catabolism of methylamine.

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