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D-Malate Dehydrogenase from *Pseudomonas fluorescens* UK-1

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An enzyme catalyzing oxidative decarboxylation of D-malate was induced by D-malate and β,β-dimethylmalate in *Pseudomonas fluorescens* UK-1. D-Malate dehydrogenase was purified to homogeneity from the cells grown on D-malate by using ammonium sulfate precipitation, heat treatment, DEAE-Sephadex chromatography, Ultrogel AcA 34 gel filtration and 5'-AMP-Sepharose affinity chromatography. D-Malate dehydrogenase has a molecular weight of 140 000 and contains 4 identical subunits of 34 000. The enzyme is very stable against sulfhydryl group reagents or alkylating agents and rather stable against heat or protein denaturants like urea or sodium dodecyl sulfate. NAD protects against heat inactivation and trypsinization but not against protein denaturants. D-Malate dehydrogenase from *Escherichia coli* does not cross-react with anti-D-malate dehydrogenase to D-malate dehydrogenase from *P. fluorescens* UK-1.

Formation of D-malate dehydrogenase (EC 1.1.1.83) in *Escherichia coli*, *Aerobacter aerogenes* and *Salmonella typhimurium* SL 1636 is an inducible process.[1] The enzyme produced in the presence of D-malate utilizes many β-alkylmalates as a substrate.[1,2] Two other analogous enzymes β,β-dimethylmalate (EC 1.1.1.84)[3] and β-isopropylmalate (EC 1.1.1.85)[4] dehydrogenase have been detected in bacterial cells, but so far no comparisons of structural properties of these enzymes have been done. In this paper we describe purification and some properties of D-malate dehydrogenase from *Pseudomonas fluorescens* UK-1.

MATERIALS AND METHODS

Materials. Ultrogel AcA 34 was obtained from Industrie Biologique Francaise. DEAE-Sephadex and N^6-(6-aminohexyl)5'-AMP-Sepharose 4B were from Pharmacia, Uppsala, Sweden. D-Malate was purchased from Sigma, St. Louis, Mo., U.S.A. Trypsin was from Merck AG, Darmstadt, Germany. β,β-Dimethylmalate was prepared as described[3,5] and 1,8-dimethoxy-octane-1,8-diimine (dimethyl suberimidate) as described.[6]

Growth conditions. P. fluorescens UK-1 was grown as described.[7]

Enzyme assay. D-Malate dehydrogenase was assayed as described.[1,2] One unit is the amount of the enzyme which catalyzes the formation of 1 μmol NADH per min.

Purification of D-malate dehydrogenase. The purification procedure described previously[2] was followed except the steps "heat treatment (1)" and "Ultrogel AcA 44" were replaced by 5'-AMP-Sepharose affinity chromatography. After DEAE-Sephadex chromatography a sample (1.8 mg protein) was dialyzed against 10 mM citrate buffer (pH 5.8) −2 mM 2-mercaptoethanol − 1 mM EDTA and applied to a 5'-AMP-Sepharose column (1.2 × 5 cm) equilibrated with the same buffer (pH 5.8). The column was washed with the same citrate buffer and then with the same buffer containing a linear NAD (0 to 2 mM) plus D-malate (0 to 10 mM) gradient. The most active fractions were pooled, dialyzed against 20 mM potassium phosphate (pH 7.2) − 1 mM EDTA and stored at −70 °C.

Electrophoresis. Polyacrylamide gel electrophoresis was performed as described[8,9] and in the presence of SDS as described.[10,11]

Molecular weight determination. Molecular weight estimation of D-malate dehydrogenase was performed in an Ultrogel AcA 34 column (1.5 × 124 cm). Standard proteins used are; hemoglobin (64 000), L-malate dehydrogenase (67 000), alkaline phosphatase (86 000) and alcohol dehydrogenase (140 000).

Amidination of D-malate dehydrogenase. Amidination of D-malate dehydrogenase was carried out in 0.2 M triethanolamine.HCl (pH 8.5) as described.[12] 1,8-Dimethoxy-octane-1,8-diimine and protein solutions were mixed to give 3.0 mg/ml of the cross-

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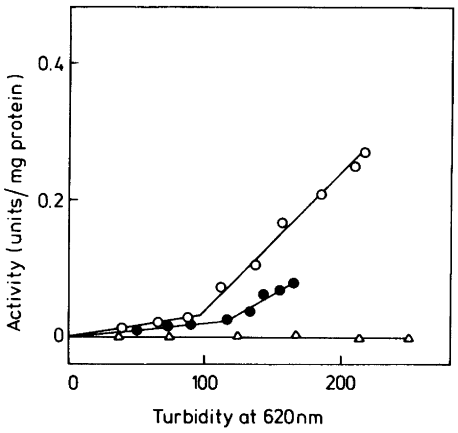

Fig. 1. Formation of D-malate oxidizing activity in *Pseudomonas fluorescens* UK-1. Samples of 10 ml were taken and the cells were sedimented, washed and disrupted in an ultrasonic disintegrator. The extracts were assayed for D-malate dehydrogenase as described.[1,2] (○), Growth on D-malate (10 mM) + glucose (5 mM); (●), growth on β,β-dimethyl-DL-malate (20 mM) + glucose (5 mM); (△), growth on L-malate (10 mM) + glucose (5 mM).

linking reagent and 0.8 mg/ml of protein in a volume of 0.2 ml. After 2 h at room temperature the samples were denatured and the products analyzed by electrophoresis in the presence of 0.1 % SDS.

Immunochemical procedures. Anti-D-malate dehydrogenase was raised in New Zealand white rabbit and prepared as described.[13] D-Malate dehydrogenase was iodinated and radioimmunoassay performed as described.[2]

RESULTS

Formation of D-malate dehydrogenase. P. fluorescens UK-1 grew under aerobic conditions at 30 °C on a defined basal salt medium[7] containing 10 mM D-malate, 10 mM L-malate or 20 mM β,β-dimethyl-DL-malate as a sole source of carbon or 5 mM glucose as an additional source of carbon. An enzyme(s) catalyzing oxidative decarboxylation of D-malate was induced in the presence of D-malate or β,β-dimethyl-DL-malate, but not in the presence of L-malate (Fig. 1). Stern and Hegre[14] have shown that D-malate dehydrogenase from *E. coli* cells which are grown in the presence of D-malate is stereospecific for D-malate, although the reaction catalyzed by L-malic enzyme is exactly analogous.

Purification of D-malate dehydrogenase. D-Malate dehydrogenase was purified from the *P. fluorescens* UK-1 cells grown on D-malate. The purified enzyme had specific activity 11.6 units/mg protein which is close to that reported for β,β-dimethylmalate dehydrogenase, 12.5 units/mg.[15] The purification procedure was modified from that used to purify β,β-dimethylmalate dehydrogenase.[15] Heat treatment at 50 °C and gel filtration in an Ultragel AcA 44 column were replaced by a 5'-AMP-Sepharose 4B column. The affinity chromatography step was carried out at pH 5.8. At pH 7 the enzyme was very loosely bound to the 5'-AMP-Sepharose column and was eluted from the column with washing buffer. The enzyme was judged to be homogeneous by the criteria of polyacrylamide gel electrophoresis in the presence and absence of SDS (Fig. 2).

Molecular structure of D-malate dehydrogenase. The subunit size was determined by SDS gel electrophoresis. The determination gave a subunit weight of 34 000. When incubated with dimethyl suberimidate four bands were detected in the gel after SDS electrophoresis (Fig. 3). When the

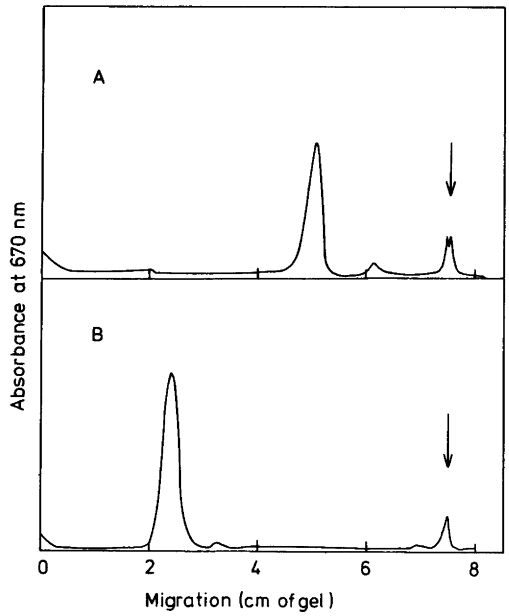

Fig. 2. Densitometer tracings of SDS gel electrophoresis (A) and disc electrophoresis (B) of D-malate dehydrogenase (15 μg). The direction of electrophoresis is from left to right. The arrows show the position of the tracking dye.

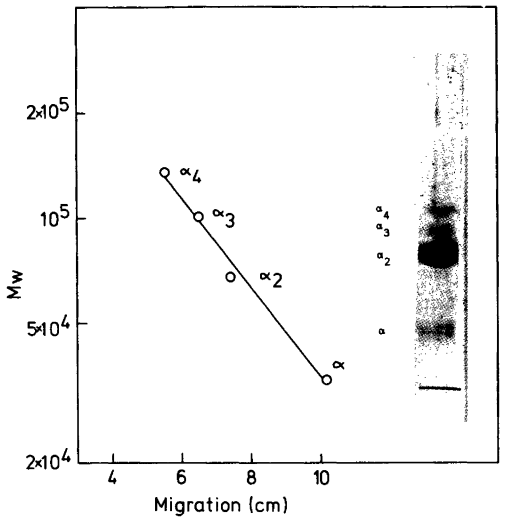

Fig. 3. Molecular weights of the multiple forms of D-malate dehydrogenase subunits. The cross-linking reagent and protein were mixed to give 3.0 mg/ml and 0.8 mg/ml in a volume of 0.2 ml, respectively. After 2 h incubation at room temperature the samples were denatured and an electrophoresis run in the presence of 0.1 % SDS. The inset shows a photograph of the sodium dodecyl sulfate gel.

migration of each band with respect to Coomassie dye was plotted, the molecular weights of the covalent species were multiples of the protomer molecular weight corresponding α,α_2, α_3 and α_4 structure of D-malate dehydrogenase subunits. As seen after 2 h incubation at room temperature the most abundant multiple was the dimer. The tetrameric structure of D-malate dehydrogenase was about the same molecular weight as alcohol dehydrogenase.

Radioimmunoassay. An antibody dilution of 1:300 (0.4 μg anti-D-malate dehydrogenase) was able to precipitate 50 % of the radioactivity. Homologous D-malate dehydrogenase and crude extracts from *P. fluorescens* UK-1 inhibited precipitation of radioactivity by about 80 and 75 %, respectively. D-Malate dehydrogenase from *E.coli* did not cross-react with anti-D-malate dehydrogenase to the dehydrogenase from *P. fluorescens* UK-1 (Fig. 5).

Inactivation of D-malate dehydrogenase. D-Malate dehydrogenase was stable against sulfhydryl group reagents or alkylating agents. Rather high concentrations of *p*-chloromercuribenzoate (0.5 mM) and iodoacetate (5 mM) inactivated the enzyme by 45 % and 24 % within 30 min. The enzyme is also rather stable against heat and protein denaturants like urea or SDS. NAD provided a substantial protection against heat inactivation and trypsinization but not against urea (Fig. 6).

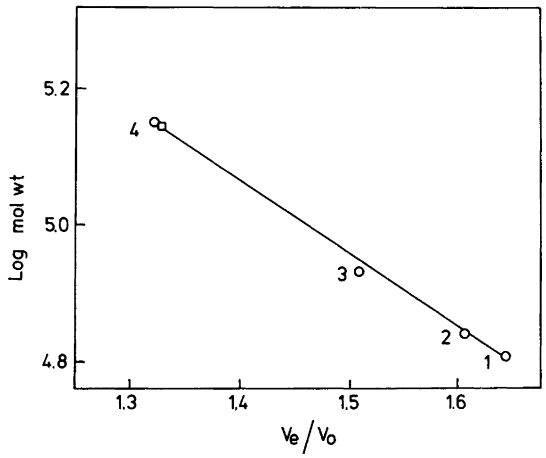

Fig. 4. Molecular weight estimation of D-malate dehydrogenase by an Ultrogel AcA 44 column (1.5 × 124 cm). Standard proteins are: (1) hemoglobin, (2) L-malate dehydrogenase, (3) alkaline phosphatase and (4) alcohol dehydrogenase. (□) is D-malate dehydrogenase.

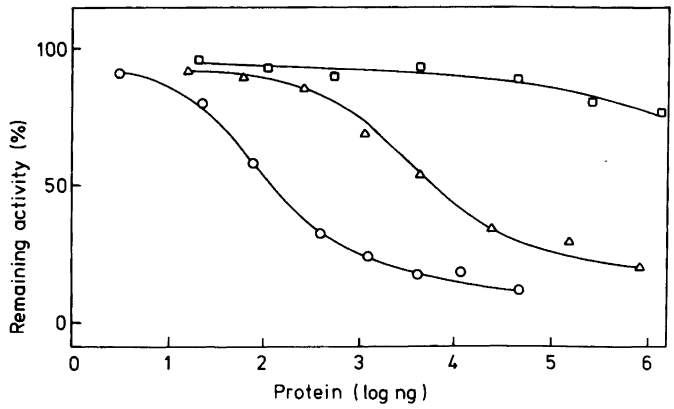

Fig. 5. Double antibody precipitation. The reaction mixtures contained 3 kBq ^{125}I-D-malate dehydrogenase, 0.8 μg anti-D-malate dehydrogenase, excess of goat antirabbit immunoglobulin, and purified D-malate dehydrogenase from *P. fluorescens* UK-1 (○); crude extract from *P. fluorescens* UK-1 (△); crude extract from *E. coli* (□). The data are corrected for nonspecific precipitation.

DISCUSSION

Escherichia coli grown on, or in the presence of of D-malate possesses an inducible D-malate dehydrogenase.[14] An inducible β-isopropylmalate dehydrogenase was detected in *Salmonella typhimurium*.[4] This enzyme does not attack D- or L-malate. Extracts of *Pseudomonas* P-2 grown on pantothenate[3] or pantoate[2] as sole carbon source cause oxidative decarboxylation of β,β-dimethyl-DL-malate and D-malate. D-Malate and L-malate served as the sole source of carbon in *P. fluorescens* UK-1 but only D-malate induced the formation of D-malate dehydrogenase. Growth on D-pantothenate, D-pantoate or β,β-dimethyl-DL-malate produced a protein catalyzing decarboxylation of D-malate. This activity, however, may be due to the formation of β,β-dimethylmalate dehydrogenase, because Magee and Snell[3] have reported β,β-dimethylmalate dehydrogenase to decarboxylate D-malate, as well. Although the dehydrogenases may employ similar mechanisms for oxidative decarboxylation Stern and O'Brien[1] have shown at least D-malate and

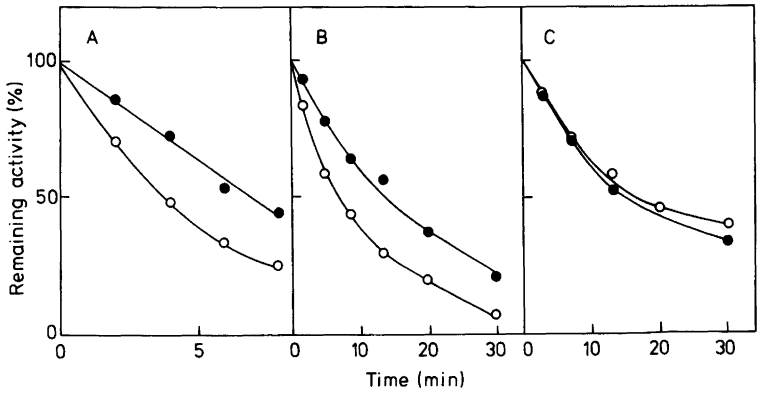

Fig. 6. Protection of D-malate dehydrogenase against heat, trypsin or urea by NAD. D-Malate (90 μg) was incubated at 60 °C (A) or at 30 °C with 0.1 % trypsin (B) or with 6 M urea (C). Samples (8 μg) were withdrawn at intervals indicated and assayed for D-malate dehydrogenase. (○), in the absence of NAD; (●), in the presence of 2 mM NAD.

β-isopropylmalate dehydrogenase to be distinct enzymes by selective inhibition and by other methods. Our preliminary results suggest that although very similar β,β-dimethylmalate and D-malate dehydrogenase from *P. fluorescens* are two separate enzymes catalyzing analogous oxidative decarboxylation reactions. The extent of similarity in the structure of D-malate dehydrogenase from different sources is presently unknown. This investigation has employed a highly sensitive radioimmunoassay to search for structural similarities of D-malate dehydrogenase from *P. fluorescens* UK-1 and *E. coli*. The data in Fig. 5 suggest that D-malate dehydrogenase from *E. coli* exhibits only small structural similarities to D-malate dehydrogenase from *P. fluorescens* UK-1. An extract from *E. coli* resulted in the same immunological effect when used about 2000-fold excess compared to the amount of the crude extract of *P. fluorescens* UK-1. The weak immunological effect of *E. coli* extract on the reaction with anti-D-malate dehydrogenase was not due to low D-malate dehydrogenase concentration in the extract. Specific activity of D-malate dehydrogenase in *E. coli* extract (0.48 units/mg)[1] is even higher than in *P. fluorescens* UK-1 (0.32 units/mg).[2] Reiners *et al.*[13] have reported that 800-fold excess of anthranilate synthetase from *P. putida* compared to the amount of the enzyme from *E. coli* was needed for half maximal competition for precipitation of the ^{125}I-labeled anthranilate synthetase from *E. coli*. Furthermore, these results suggest that the active sites play only a minor role in immunological precipitation reaction of D-malate dehydrogenase. Usually the active sites exhibit higher homology than the non-catalytic parts of the macromolecules because of the general catalytic mechanism. The catalytic site may not be exposed and this is one reason for poor immunological precipitation of heterologous enzymes.

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