Biochimica et Biophysica Acta, 613 (1980) 266—274

BBA 69002

COMPARISON OF D-MALATE AND β,β-DIMETHYLMALATE DEHYDROGENASES FROM *PSEUDOMONAS FLUORESCENS* UK-1

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(Received November 6th, 1979)

Key words: D-Malate dehydrogenase; Dimethylate dehydrogenase; (P. fluorescens)

Summary

D-Malate dehydrogenase (D-malate:NAD^+ oxidoreductase (decarboxylating), EC 1.1.1.83) was purified to homogeneity from *Pseudomonas fluorescens* UK-1 grown on D-malate and some properties of the purified enzyme were compared with those of β,β-dimethylmalate dehydrogenase (3,3-dimethyl-D-malate:NAD^+ oxidoreductase (decarboxylating), EC 1.1.1.84). D-Malate dehydrogenase has the molecular weight and subunit size of 140 000 and 34 000, respectively (the same as those of β,β-dimethylmalate dehydrogenase). The amino acid compositions of the two enzymes are similar as well. D-Malate dehydrogenase cross-reacted with anti-β,β-dimethylmalate dehydrogenase and β,β-dimethylmalate dehydrogenase cross-reacted with anti-D-malate dehydrogenase. The two dehydrogenases have similar catalytical properties. Both of the dehydrogenases were unaffected by sulphydryl reagents but were inactivated by 1,2-butanedione. NAD provided better protection against inactivation than D-malate or β,β-dimethyl-DL-malate.

Introduction

Escherichia coli cells grown in the presence of D-malate contain inducible D-malate dehydrogenase (D-malate:NAD^+ oxidoreductase (decarboxylating, EC 1.1.1.83). The enzyme is activated by Mg^{2+} or Mn^{2+} [1]. In addition to D-malate dehydrogenase, β,β-dimethylmalate dehydrogenase (3,3-dimethyl-D-malate:NAD^+ oxidoreductase (decarboxylating), EC 1.1.1.84) and D-(+)-β-isopropylmalate dehydrogenase (EC 1.1.1.85) catalyze the analogous oxidative decarboxylation in the degradation of pantothenate [2,3] and biosynthesis of leucine [4], respectively. Stern and O'Brien [4] have compared the abilities of some *Salmonella typhimurium*, *Aerobacter aerogenes* and *E. coli* strains to

Abbreviations: Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid; SDS, sodium dodecyl sulphate.

oxidize D-malate and β-alkylmalates. In *S. typhimurium* grown on D-malate or citrate the oxidation of β-alkylmalates correlates closely the oxidation of β-isopropylmalate, whereas in *E. coli* and *A. aerogenes* the oxidation of β-alkylmalates correlates with the oxidation of D-malate. Based on inhibition experiments they concluded that in *S. typhimurium* β-alkylmalates are oxidized by the β-isopropylmalic enzyme rather than by D-malate dehydrogenase. β,β-Dimethylmalate dehydrogenase purified from *Pseudomonas* P-2 catalyzes the oxidative decarboxylation of D-malate. The relative *V* of the decarboxylation of D-malate is 93% of the relative *V* of the decarboxylation of β,β-dimethylmalate [3]. Because of some similar catalytic properties it appears probable that these dehydrogenases may employ similar mechanisms for oxidative decarboxylation. Similarities in mechanism might accrue from similarities in structure. In this paper, we show that D-malate and β,β-dimethylmalate dehydrogenase have the same molecular weight, subunit size and amino acid composition but have small differences in catalytic and immunological properties.

Experimental

Materials. Ultrogel AcA 34 and AcA 44 were purchased from Industrie Biologique Francaise. DEAE-Sephadex A-50 was from Pharmacia, Uppsala, Sweden. D-Malate and 1,2-butanedione were from Sigma, St. Louis, MO, U.S.A. β,β-Dimethylmalate was prepared as described [2,5] and bromopyruvate as described [6,7]. $Na^{125}I$ was obtained from Amersham, U.K. β,β-Dimethylmalate dehydrogenase was purified from *Pseudomonas fluorescence* UK-1 as previously described [8].

Enzyme source. P. fluorescens UK-1 was grown at 30°C to late logarithmic phase (40 h) in 5-l flasks containing 3 l of salts medium [9] and 10 mM D-malate as the inducer and carbon source. In some experiments 5 mM glucose was used as an additional carbon source.

Enzyme assay. D-Malate dehydrogenase was assayed at 25°C in a reaction mixture of 1 ml containing 1 mM NAD, 10 mM D-malate, 0.1 mM $MnSO_4$ and 0.1 M Tris-HCl buffer (pH 8.5). One unit is the amount of enzyme which catalyzes the reduction of 1 μmol NAD per min. β,β-Dimethylmalate dehydrogenase was assayed as described earlier [3].

Electrophoresis. SDS-polyacrylamide gel electrophoresis was performed on a 7.5% (w/v) gel as described earlier [10,11].

Inactivation experiments. 45 μg D-malate dehydrogenase and 54 μg β,β-dimethylmalate dehydrogenase were incubated in 0.2 ml 26 mM borate buffer (pH 7.6) in the presence of 5 mM 1,2-butanedione. 0.02 ml samples were withdrawn as indicated and assayed for D-malate and β,β-dimethylmalate dehydrogenase. Effects of 100 μM *p*-chloromercuribenzoate, 1 mM *N*-ethylmaleimide, 1 mM iodoacetic acid and 1 mM bromopyruvate were determined in the same way.

Amino acid analysis. Analyses were performed with a Perkin Elmer KLA automatic amino acid analyzer. Samples of protein were hydrolyzed in 6 N HCl for 24 and 48 h at 110°C.

Preparation of antibodies. Antibodies to D-malate and β,β-dimethylmalate

dehydrogenase from *P. fluorescens* UK-1 were raised in New Zealand rabbits. The IgG fraction of immune and preimmune sera was purified by $(NH_4)_2SO_4$ precipitation [12]. The purified IgG fractions were exhaustively dialyzed against 30 mM potassium phosphate (pH 7.4)/0.85% NaCl and stored at −20°C.

Iodination. Antigens were iodinated using the chloramine T procedure of Greenwood et al. [13]. Usually, 2.3 mCi ^{125}I was used per 100 μg protein. After gel filtration (Sephadex G-25) antigen was diluted 50-fold with 2 mg/ml bovine serum albumin in 30 mM potassium phosphate (pH 7.4).

Radioimmunoassay. Reaction mixtures of 0.44 ml contained antibody (0.9 μg anti-D-malate dehydrogenase and 0.7 μg anti-β,β-dimethylmalate dehydrogenase) sufficient to precipitate 80% of iodinated antigen. The amount of competing antigen was as indicated. After incubation for 24 h at 4°C, precipitation was achieved by adding an excess of goat anti-rabbit γ-globulin. After another 24 h incubation at 4°C the precipitates were sedimented by centrifugation and washed with ice-cold 30 mM potassium phosphate (pH 7.4)/0.85% NaCl.

Immunodiffusion. Ouchterlony double diffusion [14] was performed on 0.8% agarose plates.

Titration of enzymatic activity. Titration of enzymatic activity was performed with both of the antisera against D-malate and β,β-dimethylmalate dehydrogenase. The enzymes to be titrated were diluted so that 1 ml contained about 0.01 unit of activity. Amounts of antiserum ranging from 0.7 ng to 7 μg were used. The samples were incubated at 4°C overnight. Control serum caused no inactivation during incubation.

Results

Purification of D-malate dehydrogenase from P. fluorescens UK-1

The purification procedure for D-malate dehydrogenase was modified from that used for β,β-dimethylmalate dehydrogenase [8].

Crude extract. Cells (26 g wet weight) were suspended in 1.4 ml of buffer A (0.1 M potassium phosphate, pH 7.2/10 mM 2-mercaptoethanol/1 mM EDTA) per g of the cells and disrupted as described earlier [8].

Streptomycin and $(NH_4)_2SO_4$ precipitations. Nucleic acids were removed as described [8]. D-Malate dehydrogenase was precipitated between 33 and 44% $(NH_4)_2SO_4$ saturation. The pellet was dissolved in 15 ml of buffer B (buffer A containing 0.1 M KCl).

Heat treatment (1). The heat treatment was at 55°C for 5 min. After cooling the precipitate was removed by centrifugation at 23 500 × *g* for 30 min.

Ultrogel AcA 34 filtration. Gel filtration was performed as described [8].

Heat treatment (2). The heat treatment was at 60°C for 5 min.

Ultrogel AcA 44 filtration. Gel filtration was as described [8].

DEAE-Sephadex. A DEAE-Sephadex column (1 × 18 cm) was equilibrated with 5 mM potassium phosphate (pH 7.6), 1 mM 2-mercaptoethanol, 1 mM EDTA. The pooled fractions after Ultrogel AcA 44 filtration were dialyzed overnight against the same buffer and applied to the column. The proteins were eluted with a 0—0.6 M linear KCl gradient in the same 5 mM potassium phosphate buffer (pH 7.6). The most active fractions were pooled, concentrated by

ultrafiltration (Amicon PM-30 membrane), dialyzed against 20 mM potassium phosphate (pH 7.2), 1 mM EDTA and stored at −70°C. Table I summarizes the purification of D-malate dehydrogenase.

Structure comparison

Molecular weight of D-malate dehydrogenase. As reported earlier [8] β,β-dimethylmalate dehydrogenase has a molecular weight of 140 000 and contains four identical subunits. Purified D-malate dehydrogenase and β,β-dimethylmalate dehydrogenase were indistinguishable by SDS gel electrophoresis and by Ultrogel AcA 44 gel filtration. These results indicate that the two enzymes are of similar size and contain four identical subunits.

Amino acid composition. Table II presents a comparison of the amino acid composition of D-malate dehydrogenase with that of β,β-dimethylmalate dehydrogenase. Numbers of residues are calculated for the subunits of molecular weight 34 000 which is obtained from SDS gel electrophoresis and from the amino acid composition of β,β-dimethylmalate dehydrogenase [8]. Linear extrapolation to zero time was used to obtain values for serine, threonine and tyrosine. The values obtained on the 48 h sample were used for valine and isoleucine. The half-cysteine content was determined by spectrophotometric titration with 5,5'-dithiobis(2-nitrobenzoic acid) in 0.1% SDS. These data emphasize the overall similarity in the amino acid composition of the two enzymes.

Immunological comparison

Although D-malate and β,β-dimethylmalate dehydrogenase are of similar molecular weight and subunit size, further evidence is needed to evaluate structural and evolutionary similarity. As shown in an Ouchterlony test (Fig. 1) the dehydrogenases cross-reacted with the antibodies prepared against the purified enzymes. Precipitation lines were identical when either anti-D-malate or anti-β,β-dimethylmalate dehydrogenase was used as the antibody. No spurs were detected between precipitation lines of D-malate and β,β-dimethylmalate dehydrogenase.

TABLE I

PURIFICATION OF D-MALATE DEHYDROGENASE FROM *P. FLUORESCENS* UK-1

Step	Protein (mg)	Activity (units)	Specific activity (units/mg)	Yield (%)
1. Crude extract	2522	803	0.32	100
2. Streptomycin sulphate	2187	521	0.24	65
3. $(NH_4)_2SO_4$ (0.33—0.44% saturation)	674	405	0.60	50
4. Heat 55°C	172	311	1.72	39
5. Ultrogel AcA 34	147	189	1.27	23
6. Heat 60°C	12.8	139	10.90	17
7. Ultrogel AcA 44	10.5	127	12.07	16
8. DEAE-Sephadex	5.5	71	13.02	9

* A unit of activity corresponds to the amount of enzyme catalyzing the utilization of 1 μmol NAD/min at 25°C.

TABLE II

AMINO ACID COMPOSITION OF D-MALATE AND β,β-DIMETHYLMALATE DEHYDROGENASE FROM *P. FLUORESCENS* UK-1

Amino acid	mol/mol of subunit	
	D-Malate dehydrogenase	β,β-Dimethylmalate dehydrogenase *
Tyr	5.8	4.9
Phe	9.2	8.4
Lys	10.1	9.3
His	8.9	8.8
Arg	10.1	9.8
Asp	28.1	28.0
Glu	29.9	32.4
Thr	16.3	16.3
Ser	15.9	17.4
Pro	16.3	18.3
Ala	40.1	38.1
Gly	37.0	34.0
Val	15.7	16.2
Met	4.9	6.4
Ile	13.4	13.5
Leu	20.3	19.6
Cys	2.4	2.8

* See Ref. 8.

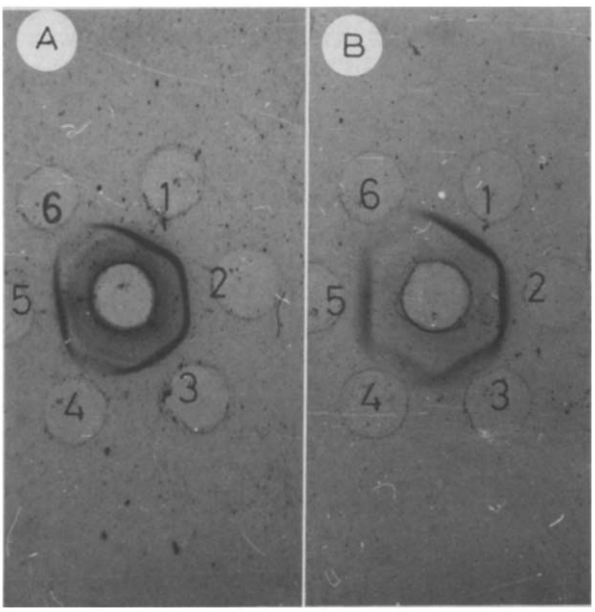

Fig. 1. Immunodiffusion of D-malate dehydrogenase and β,β-dimethylmalate dehydrogenase with anti-D-malate and anti-β,β-dimethylmalate dehydrogenase. Center wells: (A) 10.6 μg anti-β,β-dimethylmalate dehydrogenase; (B) 13.3 μg anti-D-malate dehydrogenase. Side wells: (1) 14.5 μg β,β-dimethylmalate dehydrogenase; (2) 8 μg D-malate dehydrogenase; (3) 7.2 μg β,β-dimethylmalate dehydrogenase; (4) 2 μg D-malate dehydrogenase; (5) 4 μg β,β-dimethylmalate dehydrogenase plus 2 μg D-malate dehydrogenase; (6) 62 μg *P. fluorescens* UK-1 crude extract.

Cross-reactivity of the enzymes was detected in radioimmunoassay, as well (Fig. 2). The values for maximal inhibition of precipitation of the ^{125}I-labeled dehydrogenase reflects the number of the cross-reacting determinants [15]. Addition of high concentrations of β,β-dimethylmalate or D-malate dehydrogenase to ^{125}I-labeled β,β-dimethylmalate dehydrogenase-antibody or ^{125}I-labeled D-malate dehydrogenase-antibody mixture results in almost equal dilution of radioactivity. Maximal competition was 96—98% for precipitation anti-β,β-dimethylmalate dehydrogenase and using anti-D-malate dehydrogenase maximal competition by β,β-dimethylmalate dehydrogenase was 92—94%. The amount of protein required for half-maximal inhibition, on the other hand, is an estimate of binding affinity [15]. At low antigen concentrations (lower than 2 mg/ml) the homologous antigen decreased radioactivity more than the heterologous dehydrogenase. The amounts of D-malate and β,β-dimethylmalate dehydrogenase to inhibit precipitation of ^{125}I-labeled β,β-dimethylmalate dehydrogenase by anti-β,β-dimethylmalate dehydrogenase by 50% were 0.250 and 0.125 μg, respectively. The amounts of D-malate and β,β-dimethylmalate dehydrogenase to inhibit precipitation of ^{125}I-labeled D-malate dehydrogenase by anti-D-malate dehydrogenase were 0.250 and 0.380 μg, respectively.

Enzymatic activity was titrated more effectively with the homologous than with the heterologous antibody. The amounts of antibodies required for 50% inhibition of D-malate dehydrogenase were 140 ng anti-D-malate dehydrogenase and 190 ng anti-β,β-dimethylmalate dehydrogenase. The amounts of antibodies to inhibit β,β-dimethylmalate dehydrogenase by 50% were 85 ng anti-D-malate dehydrogenase and 45 ng anti-β,β-dimethylmalate dehydrogenase (results not shown). Addition of non-immuno serum did not inhibit the dehydrogenases.

Comparison of catalytical properties

β,β-Dimethylmalate dehydrogenase from *Pseudomonas* P-2 utilizes D-malate

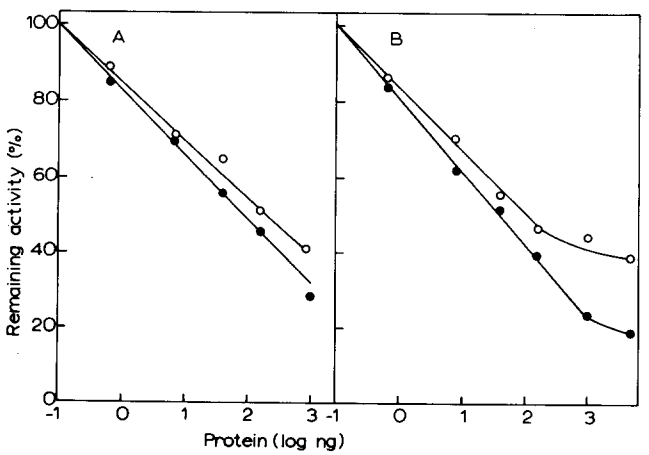

Fig. 2. Cross-inhibition of D-malate and β,β-dimethylmalate dehydrogenase. (A) Inhibition of β,β-dimethylmalate dehydrogenase (●) and D-malate dehydrogenase (○) by anti-β,β-dimethylmalate dehydrogenase. (B) Inhibition of β,β-dimethylmalate dehydrogenase (○) and D-malate dehydrogenase (●) by anti-D-malate dehydrogenase.

as the substrate, as well. Although the K_m for D-malate is increased about 70-fold compared with the K_m for β,β-dimethylmalate the maximal activity is 93% of the maximal activity when β,β-dimethylmalate is used as the substrate [3]. β,β-Dimethylmalate dehydrogenase from *P. fluorescens* UK-1 utilizes D-malate as the substrate, and D-malate dehydrogenase utilizes β,β-dimethylmalate as well. Some of the main properties of D-malate and β,β-dimethylmalate dehydrogenases are summarized in Table III. As shown the pH optima and temperature stability of the enzymes are the same, but K_m values for D-malate and β,β-dimethylmalate are different. At high substrate concentration relative V for β,β-dimethylmalate was 68%.

Effect of group specific reagents. Both β,β-dimethylmalate and D-malate dehydrogenase were fairly stable against SH-group reagents. 0.2 mM *p*-chloromercuribenzoate and 1 mM *N*-ethylmaleimide inactivated the enzymes only by 10 and 18%, respectively. Alkylating agents, 1 mM iodoacetic acid and 1 mM bromopyruvate were almost without effect as well. 5 mM 1,2-butanedione in 26 mM borate buffer inactivated both D-malate and β,β-dimethylmalate dehydrogenase (Fig. 3). D-Malate dehydrogenase was inactivated by 50% of the control within 7—8 min and only 5% activity remained after 2 h incubation. β,β-Dimethylmalate dehydrogenase was inactivated by 50% within 15 min. The rate of inactivation was significantly reduced if borate was omitted. Under conditions where the activity is reduced to 5% in the presence of 26 mM borate buffer (pH 7.6) 72% of D-malate dehydrogenase and 76% of β,β-dimethylmalate dehydrogenase activity remained if borate was replaced by Hepes buffer. In both cases, inactivation was reversible. If the in borate buffer modified enzymes were eluted from a Sephadex G-10 column with 20 mM Hepes buffer (pH 7.6) almost 80% of the original activity was restored.

1 mM NAD provided better protection against inactivation than either 10 mM D-malate or 20 mM β,β-dimethylmalate. D-Malate and β,β-dimethylmalate, on the other hand, provided equal protection against inactivation of the both dehydrogenases (results not shown). These data strongly suggest that in both cases inactivation is due to the modification of essential arginyl residues.

TABLE III

CATALYTIC PROPERTIES OF D-MALATE AND β,β-DIMETHYLMALATE DEHYDROGENASE

V^R is the relative V.

	D-Malate dehydrogenase	β,β-Dimethylmalate dehydrogenase
pH optimum	8.4	8.4
Temperature stability at 65°C	64% activity lost within 2 min	58% activity lost within 2 min
K_m^{app}		
NAD	0.49 mM	0.27 mM
β,β-Dimethylmalate *	1.9 mM	0.2 mM
D-Malate	2.2 mM	8.9 mM
V^R		
β,β-Dimethylmalate	90	100
D-Malate	100	68

* Calculated for D-isomer.

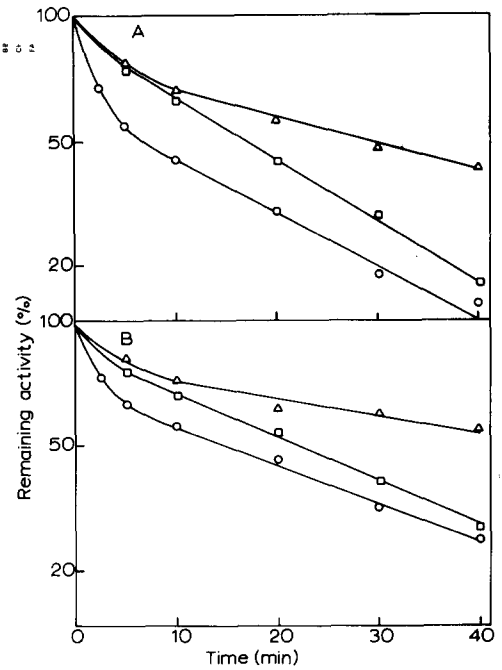

Fig. 3. Inactivation of D-malate (A) and β,β-dimethylmalate (B) dehydrogenase by 1,2-butanedione. Inactivation mixtures (0.2 ml) contained 45 μg D-malate dehydrogenase or 54 μg β,β-dimethylmalate dehydrogenase and 5 mM 1,2-butanedione in 26 mM borate buffer (pH 7.6). Samples (0.02 ml) were removed and assayed for activity. ○, 5 mM 1,2-butanedione; □, 5 mM 1,2-butanedione plus 10 mM D-malate (A) or 20 mM β,β-dimethyl-DL-malate (B); △, 5 mM 1,2-butanedione plus 1 mM NAD.

Discussion

Several lines of evidence suggest that D-malate and β,β-dimethylmalate dehydrogenase are very similar. (i) Purification procedures are almost the same; (ii) molecular weight and subunit size are equal; (iii) there are only a few differences in amino acid composition; (iv) immunological properties are not identical but close to each other; (v) the enzymes have similar pH optima and heat resistance; (vi) final specific activities are equal, and (vii) the enzymes behave similarly in the presence of group-specific reagents.

By using the same procedure as in the purification of β,β-dimethylmalate dehydrogenase homogeneous D-malate dehydrogenase was obtained. Most D-malate dehydrogenase precipitated between 0.33 and 0.44 $(NH_4)_2SO_4$ precipitation while 0.30—0.48 saturation was used to precipitate β,β-dimethylmalate dehydrogenase. This difference may be due to twice higher protein concentration of the D-malate dehydrogenase preparation. Because of weak binding of β′,β-dimethylmalate dehydrogenase to a hydroxyapatite column [8] DEAE-Sephadex was substituted for hydroxyapatide. Gel filtration and SDS gel electrophoresis gave the same molecular weights to D-malate and β,β-dimethylmalate dehydrogenase and the subunits, respectively. When the amount of tryptophan was not counted amino acid analysis (average of three determinations) gave a molecular weight of 34 500 to D-malate dehydrogenase and 33 200 to β,β-dimeth-

ylmalate dehydrogenase. This difference is so small that it may be due to variations in different determinations. However, the small immunological difference of the dehydrogenases does not support that idea that the enzymes are identical but two separate enzymes with a slight structural or conformation difference. Stern and O'Brien [4] concluded that in a mutant strain of *S. typhimurium* D-malate dehydrogenase and β-isopropylmalate dehydrogenase are two separate enzymes and that the genes are mapped in separate segments of the *Salmonella* chromosome. However, it is difficult to explain why, in this mutant, the levels of both D-malate and β-isopropylmalate dehydrogenase were increased. Our results strongly support the conclusion that D-malate and β,β-dimethylmalate dehydrogenase are separate enzymes which are very similar but have some differences in catalytical properties.

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