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PURIFICATION OF PANTOATE AND DIMETHYLMALATE DEHYDROGENASE FROM *PSEUDOMONAS FLUORESCENS* UK-1

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Summary

Pantoate dehydrogenase and dimethylmalate dehydrogenase were purified 69- and 112-fold, respectively, from *Pseudomonas fluorescens* UK-1 by ammonium sulphate precipitation. Ultrogel AcA 34 gel filtration, hydroxyapatite column chromatography, heat treatment and Ultrogel AcA 44 gel filtration.

The enzymes were evaluated for homogeneity (pantoate dehydrogenase was estimated to be about 95% pure) by disc and sodium dodecyl sulphate gel electrophoresis and by immunodiffusion.

Pantoate and dimethylmalate dehydrogenases have molecular weights of 83 000 and 138 000, respectively, and are dissociable into four identical subunits with molecular weights of 24 000 and 34 000.

Introduction

Some species of *Pseudomonas* are able to grow with pantothenate as the only source of carbon and nitrogen [1]. The first step in the degradation of pantothenate is cleavage of the amide bond by pantothenase (D-pantoate:NAD^+ 4-oxidoreductase, EC 1.1.1.106) [2]. Pantoate is oxidized to 2-oxoisovalerate by three separate dehydrogenases, pantoate, aldopantoate and dimethylmalate dehydrogenases [3,4]. These enzymes have been partially purified from *Pseudomonas* P-2. They are specific for NAD and rather heat stable. Magee and Snell [4] have reported D-malate to be a good substrate for dimethylmalate dehydrogenase.

One purpose of the present work was to prepare homogenous enzymes for comparative studies of pantoate and dimethylmalate dehydrogenases and for immunological studies of dimethylmalate and D-malate dehydrogenases, and the "malic enzyme".

Experimental

Materials

Ultrogel AcA 34 and 44 were the products of Industrie Biologique Francaise. Hydroxyapatite and deoxyribonuclease II were from Sigma, St. Louis, Mo., U.S.A. 1,6-Dicyanohexane was purchased from Aldrich, Belgium. Dimethyl suberimidate was prepared according to the method of McElvain and Schroeder [5]. The other chemicals were of the highest grade commercially available and were used without further purification.

Growth conditions

Pseudomonas fluorescens UK-1 was grown in a pantothenate medium as described by Airas [6]. The cells were collected when their fresh weight reached 1.7 mg/ml and stored at −20°C.

Enzyme assays

Pantoate and dimethylmalate dehydrogenase activities were determined as described earlier [3,4]. In each case a unit of activity corresponds to the amount of enzyme catalyzing the utilization of 1 μmol NAD per min at room temperature. The standard enzymes were assayed by standard techniques as described by Boehringer Mannheim GmbH [7]. Haemoglobin and cytochrome *c* were measured at 405 and 550 nm, respectively.

Protein determination

Modified Biuret [8] and Lowry et al. [9] methods were used to determine the protein concentrations with bovine serum albumin as standard protein.

Electrophoresis

Polyacrylamide gel electrophoresis was performed on a 7.5% (w/v) polyacrylamide gel [10,11], and SDS polyacrylamide gel electrophoresis on a 10% (w/v) gel [12,13].

Molecular weight determination

Two methods were used to determine the molecular weights of pantoate and dimethylmalate dehydrogenases. In the first, migration of the enzymes was studied in a sucrose density gradient [14]. Sucrose gradients from 5 to 20% were used. Samples in 0.17 ml of buffer B (0.1 M potassium phosphate, pH 7.2, 10 mM 2-mercaptoethanol, 1 mM EDTA and 0.1 M KCl) contained 28 μg pantoate dehydrogenase, 23 μg dimethylmalate dehydrogenase and the standard enzymes and proteins. In the second, an Ultrogel AcA 44 column (1.5 × 120 cm) was used. The sample (0.270 ml) containing 56 μg pantoate dehydrogenase, 46 μg dimethylmalate dehydrogenase and standard enzymes and proteins was eluted with buffer A (0.1 M potassium phosphate buffer, pH 7.2, 10 mM 2-mercaptoethanol and 1 mM EDTA).

SDS gel electrophoresis was used to determine subunit molecular weights.

Amidination of the enzymes

Dimethyl suberimidate and the enzymes were mixed to give 0.84 mg per ml

of pantoate dehydrogenase or 0.69 mg per ml of dimethylmalate dehydrogenase and 4.2 mg per ml of suberimidate in a volume of 0.2 ml according to the method of Davies and Stark [15]. Samples (40 μl) were taken at 30-min intervals, denatured and subjected to polyacrylamide gel electrophoresis in the presence of 0.1% SDS.

Preparation of antisera

New Zealand white rabbits were immunized [16] with 1 nmol of homogeneous enzyme (in 0.5 ml of 0.9% NaCl) suspended in an equal volume of Freund's complete adjuvant. Injections were repeated at 1-week intervals and blood was collected 4 weeks after the first injection. Antiserum was partially purified as described by Reiners and Zalkin [16]. Non-immune serum was prepared from blood obtained before the first immunization.

Immunodiffusion

Immunodiffusion experiments were conducted as described by Ouchterlony [17]. The microscope slides were coated with 0.8% Colab ion agar 2, 0.85% NaCl and 0.05 M potassium phosphate, pH 7.4. Diffusion was for 24 h at 4°C. Slides were washed in 0.85% NaCl overnight and stained for 7 min using 1% Aniline Blue-Black in 0.5 M acetate buffer (pH 3.8).

Results

Purification of pantoate and dimethylmalate dehydrogenases

Step 1. Crude extract. Cells (170 g) were suspended in 3 ml of buffer solution A/g cell paste and disrupted in a French press (10 000 kg/cm^2). $MgCl_2$ (5 mM) and 20 μg of deoxyribonuclease/ml were added to the cell extract, which was slowly stirred at room temperature for 30 min. Cell debris was removed by centrifugation at 27 000 $\times g$ for 1 h.

Step 2. Streptomycin precipitation. The remaining nucleic acids were removed from the supernatant solution by a slow addition of 1% streptomycin sulphate (10 ml 10% streptomycin sulphate/100 ml crude extract). After centrifugation the precipitate was discarded.

Step 3. Ammonium sulfate precipitation. Saturated ammonium sulphate solution (pH 7.0) was added to the supernatant from Step 2 to give 30% saturation. Stirring was continued for 30 min. After centrifugation the precipitate was discarded and ammonium sulphate was added to 48% saturation. The protein isolated after centrifugation was dissolved in 80 ml of buffer B.

Step 4. Heat treatment. The heat treatment was at 50°C for 5 min [3]. After centrifugation the supernatant was concentrated by ultrafiltration using an Amicon PM-30 membrane.

Step 5. Ultrogel AcA filtration. The concentrate (18 ml) obtained after the heat treatment was applied to two tandem columns (2.5 × 100 cm) containing Ultrogel AcA 34. Protein was eluted with buffer B. Fractions of 8 ml were collected. Fig. 1 shows the elution profiles of pantoate and dimethylmalate dehydrogenases. Fractions which contained most of the enzyme activity were pooled.

Step 6. Hydroxyapatite chromatography. The pooled fractions of pantoate

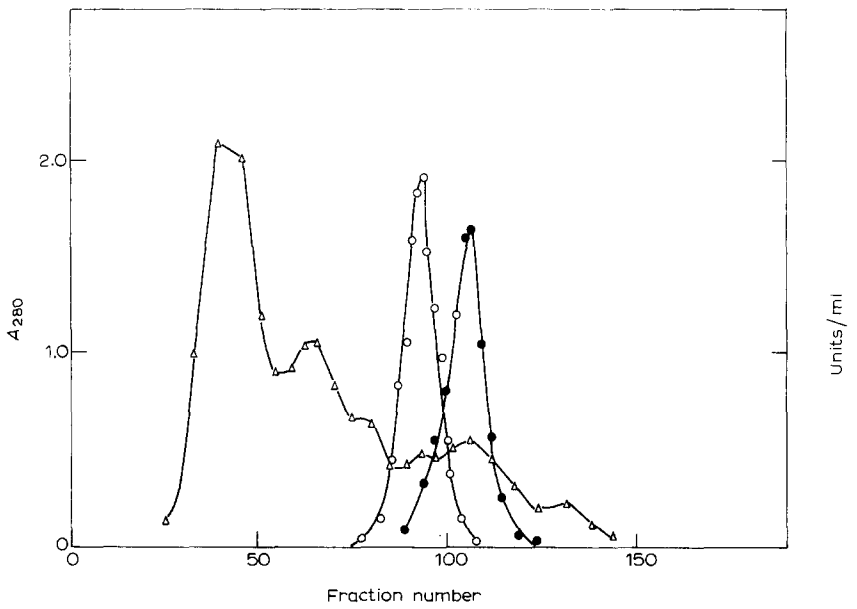

Fig. 1. Separation of pantoate and dimethylmalate dehydrogenases from *Pseudomonas fluorescens* UK-1 on 2 tandem columns (2.5 × 100 cm) of Ultrogel AcA 34. Fractions of 8 ml were collected at a flow rate of 38 ml per h. △———△, absorbance at 280 nm; ○———○, dimethylmalate dehydrogenase; ●———●, pantoate dehydrogenase.

dehydrogenase (104 ml) and dimethylmalate dehydrogenase (120 ml) were concentrated separately by ultrafiltration (Amicon PM-30 membrane) and then dialyzed for 4 h against 5 mM potassium phosphate buffer, pH 7.2, containing 10 mM 2-mercaptoethanol and 1 mM EDTA. The dialyzed enzymes were applied separately to an equilibrated hydroxyapatite column (2 × 2.4 cm) and eluted with the same dialyzing buffer. Fractions of 2 ml were collected (the total volumes were about 150 ml). Fractions 2—10 (18 ml) contained pantoate dehydrogenase activity and fractions 5—15 (22 ml) contained dimethylmalate dehydrogenase activity.

Step 7. The second heat treatment. A concentrated buffer solution was added to the pooled fractions to produce conditions identical to buffer B. The fractions were then concentrated as above. The concentrated solution of dimethylmalate dehydrogenase was kept at 60°C for 10 min.

Step 8. Ultrogel AcA 44 filtration. The concentrated fractions of pantoate dehydrogenase from Step 6 (4.5 ml) and dimethylmalate dehydrogenase from Step 7 (6.2 ml) were applied separately to a column (1.5 × 120 cm) of Ultrogel AcA 44. The protein was eluted with buffer B. Fractions of 2.1 ml were collected. The most active fractions were pooled and concentrated as above. The enzymes were dialyzed for 4 h against 50 mM potassium phosphate buffer, pH 7.2 containing 1 mM EDTA and stored at −70°C. Table I summarizes the purification procedure of pantoate and dimethylmalate dehydrogenases.

Purity of the enzymes

The purified preparation of dimethylmalate dehydrogenase was judged to

TABLE I

SUMMARY OF PURIFICATION OF PANTOATE AND DIMETHYLMALATE DEHYDROGENASES FROM *P. FLUORESCENS* UK-1

Step	Total protein (mg)		Total enzyme (units)		Specific activity (units/mg)		Yield (%)	
	PDH *	DMDH **	PDH *	DMDH **	PDH *	DMDH **	PDH *	DMDH **
1. Crude extract	11 880		3326	1306	0.28	0.11	100	100
2. Streptomycin sulphate	10 930		3148	1260	0.29	0.12	94.6	96.5
3. $(NH_4)_2SO_4$ precipitate 0.30—0.48 saturation	1 370		2710	945	1.98	0.69	81.6	72.4
4. Heat 50°C	535		2364	877	4.42	1.64	71.1	67.2
5. Ultrogel AcA 34	119	103	1649	706	13.86	6.85	49.6	54.1
6. Hydroxyapatite	94	81	1470	674	15.64	8.32	44.2	51.6
7. Heat 60°C		46		468		10.18		35.8
8. Ultrogel AcA 44	36	27	695	338	19.34	12.54	20.9	25.8

* PDH = Pantotate dehydrogenase.

** DMDH = Dimethylmalate dehydrogenase.

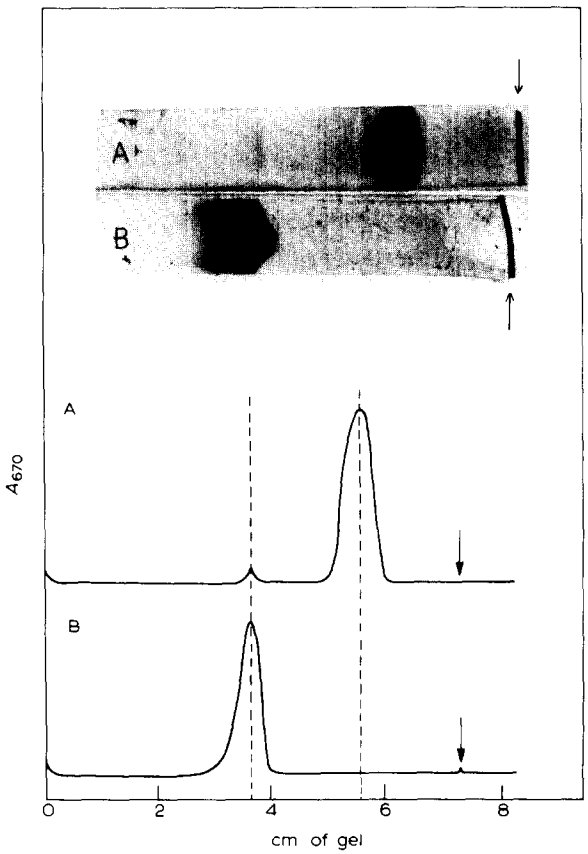

Fig. 2. Densitometer tracing of SDS polyacrylamide gel electrophoresis of pantoate and dimethylmalate dehydrogenases. (A) 18 μg pantoate dehydrogenase. (B) 16 μg dimethylmalate dehydrogenase. The arrows show the positions of the tracing dye. The inset shows a photograph of the SDS gels.

be homogenous by the criteria of gel electrophoresis (results not shown), SDS gel electrophoresis and immunodiffusion. Pantoate dehydrogenase was judged to be about 95% pure by the above mentioned criteria. The densitometer tracing of SDS gels of the enzymes are shown in Fig. 2. As can be seen the purified preparations exhibit single stained protein bands in the densitometer tracings. Only a faint impurity located at the dimethyl malate dehydrogenase position was found in the pantoate dehydrogenase preparations. The inset of Fig. 2 shows photographs of the SDS gels. Results of an immunodiffusion experiment are shown in Fig. 3. Single bands of precipitation were obtained for reaction of anti-pantoate dehydrogenase or anti-dimethylmalate dehydrogenase with crude or purified enzymes, respectively.

Molecular weights and the subunit structure

The molecular weights of pantoate and dimethylmalate dehydrogenases were determined by gel filtration on the columns of Ultrogel AcA 34 and AcA 44 (1.5 × 120 cm) and by ultracentrifugation. Fig. 4 shows the molecular weights of pantoate and dimethylmalate dehydrogenases when elution volumes were

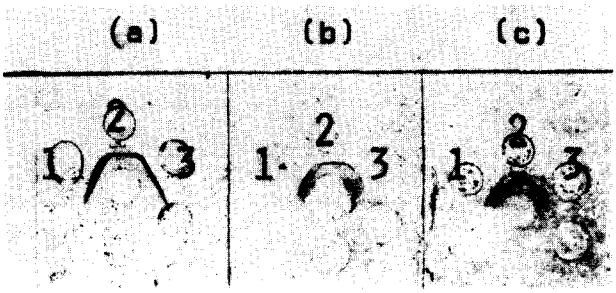

Fig. 3. Immunodiffusion of pantoate and dimethylmalate dehydrogenase. Center wells contain 0.01 ml antiserum. (A) Dimethylmalate dehydrogenase, (1) purified enzyme 22 μg, (2) crude extract, (3) purified enzyme 7 μg; (B) Pantoate dehydrogenase, (1) purified enzyme 6 μg, (2) crude extract, (3) purified enzyme 3 μg; (C) Pantoate dehydrogenase plus dimethylmalate dehydrogenase, (1) purified enzymes 6 and 3.5 μg, respectively, (2) crude extract, (3) purified enzymes 3 and 1.7 μg, respectively.

plotted against log molecular weight. According to gel filtration, molecular weights were 86 000 and 142 000, respectively. Ultracentrifugation gave the values 80 000 and 136 000.

The molecular weighst of the subunits were determined by SDS gel electrophoresis. Mean values of three determinations gave molecular weights of 24 000 for pantoate dehydrogenase and 34 000 for dimethylmalate dehydrogenase. These results suggest that both dehydrogenases consist of four identical

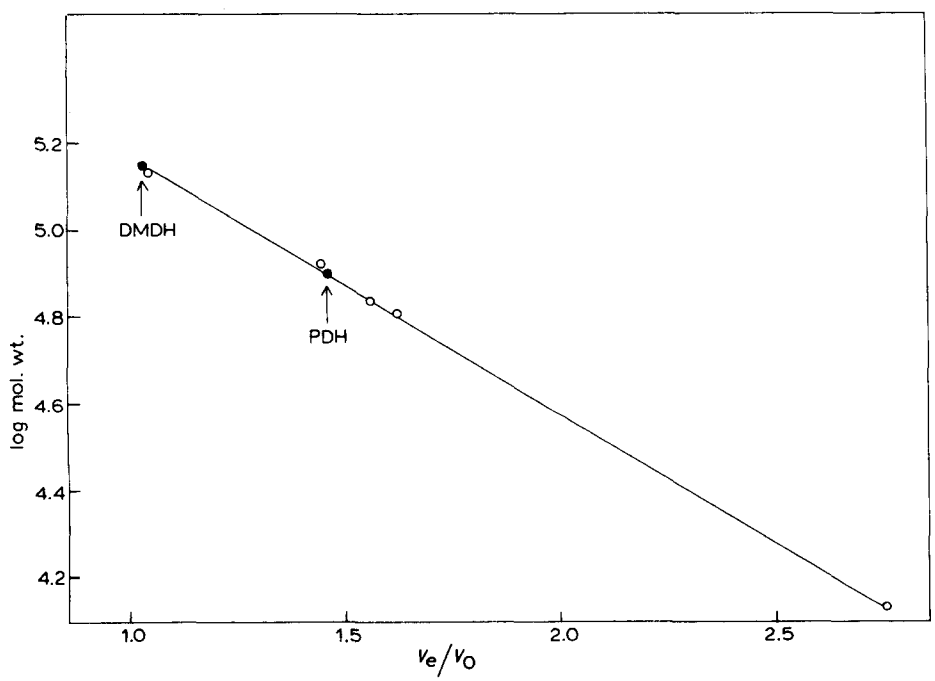

Fig. 4. Molecular weight estimation of native pantoate and dimethylmalate dehydrogenase by gel filtration on an Ultrogel AcA 44 column (1.5 X 120 cm). Standard mol. wt. proteins were alchohol dehydrogenase (141 000) alkaline phosphatase (86 000), L-malate dehydrogenase (67 000), haemoglobin (64 000), and cytochrome *c* (13 500). The column was eluted with buffer A. The fractions collected were 0.9 ml and were assayed for enzyme activities.

TABLE II

AMINO ACID COMPOSITION OF PANTOATE AND DIMETHYLMALATE DEHYDROGENASES FROM *P. FLUORESCENS* UK-1

Amino acid	mol/mol of subunit	
	PDH ††	DMDH ††
Tyr *	2.9	4.9
Phe	5.3	8.4
Lys	4.3	9.3
His	3.7	8.8
Arg	5.4	9.8
Asp	17.8	28.0
Glu	22.1	32.4
Thr *	12.3	16.3
Ser *	8.8	17.4
Pro	11.8	18.3
Ala	28.6	38.1
Gly	28.3	34.0
Val **	17.2	16.2
Met	2.7	6.4
Ile **	8.9	13.5
Leu **	14.7	19.6
Try ***	3.1	5.0
Cys †	1.9	2.8

* Values were extrapolated for zero time hydrolysis.
** Calculated for 96-h hydrolysates.
*** Determined by the method of Edelhoch [18].
† Measured by 5,5'-dithiobis (2-nitrobenzoic acid) titration of enzymes denatured in 0.1% SDS.
†† See footnote to Table I.

subunits. Amidination of the dehydrogenases with dimethylsuberimidate indicated that the enzymes contain 4 identical subunits.

Amino acid composition and ultraviolet absorption spectra

The amino acid composition of pantoate and dimethylmalate dehydrogenases is reported in Table II. Linear extrapolation to zero time was used to obtain values for serine, threonine and tyrosine.

According to Edelhoch [18], the amount of tryptophan residues was determined from the ultraviolet absorption spectrum measured in the presence of 6 M guanidine hydrochloride.

Discussion

Pantoate dehydrogenase, and especially dimethylmalate dehydrogenase, are rather heat stable. By using ammonium sulphate precipitations in addition to heat treatments, Goodhue and Snell [3] and Magee and Snell [4], succeeded in purifying the enzymes about 14- and 87-fold, respectively. By adding two gel filtrations and hydroxyapatite column chromatography to this procedure it was possible to produce almost homogeneous pantoate and completely homogeneous dimethylmalate dehydrogenases, as shown in Figs. 2 and 3.

The addition of 0.1 M KCl to buffer A increased stability; dimethylmalate dehydrogenase was stable at 60°C for at least 20 min. Treatment on an

Ultrogel AcA 34 column removed all of the high-molecular-weight proteins and partially separated pantoate (mol. wt. 83 000) and dimethylmalate (mol. wt. 138 000) dehydrogenases. Both pantoate and dimethylmalate dehydrogenase were very loosely bound to the hydroxyapatite column when 5 mM potassium phosphate buffer (pH 7.2) was used as an eluent. However, this step was successful, as can be seen in Table I. A "yellow" protein absorbing at 312 nm was trapped by the hydroxyapatite column.

The co-purification of pantoate and dimethylmalate dehydrogenases (Steps 1—5) did not completely separate the enzymes. Dimethylmalate dehydrogenase was purified from pantoate dehydrogenase by heat treatment at 60°C. This treatment denatured and precipitated pantoate dehydrogenase within one minute. Neither an addition of pantoate nor NAD stabilized the enzyme. Therefore, when this purification procedure was used it was not possible to remove tiny traces of dimethylmalate dehydrogenase from the pantoate dehydrogenase preparation. For this reason small errors in the amino acid composition of pantoate dehydrogenase are possible, as well.

Because highly purified antigens are essential for investigation of immunological cross-reactivity several criteria were used to determine the purity of dimethylmalate dehydrogenase. A single protein band was obtained in disc gel electrophoresis, SDS gel electrophoresis and immunodiffusion even using up to 90 μg of protein.

The specific activies of the purified materials were 19.34 (pantoate dehydrogenase) and 12.54 (dimethylmalate dehydrogenase) units per mg protein; about three times higher than those reported by Goodhue and Snell [3] and Magee and Snell [4]. However, the amounts of inactivated pantoate and dimethylmalate dehydrogenases in the final preparations are unknown. Although an addition of high concentration of 2-mercaptoethanol (up to 0.2 M) increased both activities somewhat, it remains uncertain how completely the inactivated forms are separated.

Immunological studies of dimethylmalate dehydrogenase and D-malate dehydrogenase are now in progress.

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