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FAD-DEPENDENT MALATE DEHYDROGENASE, A PHOSPHOLIPID-REQUIRING ENZYME FROM *MYCOBACTERIUM* SP. STRAIN TAKEO *

PURIFICATION AND SOME PROPERTIES

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Summary

FAD-dependent malate dehydrogenase, a phospholipid-requiring enzyme, was homogeneously purified from the particulate fraction of *Mycobacterium* sp. strain Takeo.

The isolated enzyme contains no FAD and few phospholipid, and has a specific activity of 300—360 units/mg of protein. In the assay system without addition of phospholipid (cardiolipin), the enzyme activity was only about 3% of maximum activity.

The molecular weight was estimated to be 51 000—55 000 by four methods. Titration by *p*-chloromercuribenzoate revealed the presence of one cysteine residue/mol of enzyme. The isoelectric point was found to be pH 6.9 by isoelectric focusing. From circular dichroism spectral data, the enzyme protein was found to contain α-helix structure of 24%.

Introduction

In a previous paper from our laboratory, it was reported that a malate dehydrogenase, located in the particular fraction of *Mycobacterium* sp. strain Takeo, was a flavoprotein with FAD as the coenzyme [1]. The enzyme was solubilized from acetone powder of the particulate fraction, and then the solubilized enzyme was acidified and fractionated with $(NH_4)_2SO_4$, it was not enzymatically active. One of the components necessary for the activity in the residual particulate fraction was shown to be phospholipid [2]. The particulate

* The name *Mycobacterium avium* (strain Takeo) has been used in the previous papers. Refer to the literature for details (Kusunose, M. et al. (1976) Arch. Microbiol. 108, 65—73).

fraction could reduce 2,6-dichlorophenolindophenol with concomitant oxidation of malate without addition of any electron acceptor, such as vitamin K-3 or vitamin K-1, but vitamin K-3 could be served as an artificial and efficient electron acceptor for the acetone-treated preparation [2]. A natural electron acceptor extracted into acetone from the particulate fraction was determined later to be a vitamin K-2 derivative [3] which had previously been identified as an electron acceptor in the electron transport system of *Mycobacterium phlei* [4].

Similar enzymes have been reported in many bacteria [5—10] and one enzyme was purified to 85% homogeneity from *M. phlei* and its properties studied [11, 12]; however, the enzyme was not shown to be electrophoretically homogeneous.

In the present report, a purification method is described for preparing an electrophoretically homogeneous protein from a particulate fraction of *Mycobacterium* sp. strain Takeo; some properties of this purified enzyme are also presented.

Materials and Methods

Materials. Chemicals were purchased as follows: FMN, and L-malic acid from Kyowa Hakko Co.; protamine sulfate (Grade II) and ovalbumin (Grade III) from Sigma Chemical Co.; beef liver catalase, rabbit muscle pyruvate kinase, yeast alcohol dehydrogenase, pancreatic trypsin, soybean trypsin inhibitor, and horse heart cytochrome *c* (molecular weight standards) from C.F. Boehringer and Soehne GmbH; Sephadex G-100 and G-200 from Pharmacia Fine Chemicals; Carrier ampholyte from LKB-Productor; and vitamin K-1 and vitamin K-3 from Tokyo Kasei Co. Beef heart cardiolipin was donated by Sumitomo Chemical Co.

Bacterial growth. Mycobacterium sp. strain Takeo was grown as described previously [1].

Enzyme assay. The reaction mixture contained: 80 μmol of potassium phosphate buffer (pH 7.0)/12.5 nmol of FAD/a mixture of 0.12 mg of beef heart cardiolipin and 0.8 nmol of vitamin K-3 in 20 μl of ethanol/appropriate amounts of the enzyme/and 43 nmol of 2,6-dichlorophenolindophenol in a volume of 1.55 ml. The reaction was started with 20 μmol of L-malate (0.05 ml, neutralized with KOH) and reduction of 2,6-dichlorophenolindophenol followed at 600 nm using a Hitachi 323 recording spectrophotometer. A molar extinction coefficient of $20.6 \cdot 10^3$ was used for 2,6-dichlorophenolindophenol at 600 nm [13]. A unit of the enzyme activity is defined as the amount of the enzyme which catalyses the reduction of 1 μmol of 2,6-dichlorophenolindophenol per min at 20°C. Specific activity is expressed as μmol of 2,6-dichlorophenolindophenol per min per mg of protein.

Isoelectric focusing. Isoelectric focusing was performed for 66 h at 0—4°C with 1% Ampholine (pH 6—8) in the presence of Triton X-100 in an 8100 LKB column according to the method of Vesterberg [14]. The pH of each fraction was measured with a Toa Dempa HM-5A pH meter equipped with a GC-195 multielectrode.

Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis. Electrophoresis was carried out by the method of Weber and Osborn [15], and protein

staining by the technique of Chrambach et al. [16]. For estimation of the molecular weight, pyruvate kinase (57 200), ovalbumin (44 000), alcohol dehydrogenase (37 000), trypsin (23 300), and horse heart cytochrome *c* (12 400) were used as standards.

Amino acid analyses. Amino acid analyses were made using a JLC-6 AS automatic analyzer with a single column. About 150 μg of the purified enzyme was exhaustively dialyzed against distilled water and hydrolyzed with 6 M HCl at 110°C in vacuo for 24, 48, and 72 h. Cystine and cysteine residues were determined as cysteic acid after performic acid oxidation [17]. Free cysteine residue was analyzed by the method of Boyer [18] in the presence and absence of 6 M guanidine · HCl. Tryptophan was determined as described by Edelhoch [19].

Molecular weight. The molecular weight was estimated according to the method of Andrews [20] using Sephadex G-100 and G-200 columns (2.5 × 40 cm). Standard proteins used were yeast alcohol dehydrogenase (144 000), rabbit muscle glyceraldehyde-3-phosphate dehydrogenase (135 000), bovine serum albumin (68 000), ovalbumin, trypsin inhibitor (21 500), and cytochrome *c*. The molecular weight was also determined by the method of Martin and Ames [21]. A sample (0.1 ml) was layered on top of a 4.7 ml linear gradient of sucrose (5—20%). Centrifugation was carried out for 22 h at 3—5°C using a Hitachi 55P-2 centrifuge with a Hitachi RPS-40 rotor. Yeast alcohol dehydrogenase and beef liver catalase were used as internal standards.

Protein. Protein concentration was determined by the method of Lowry et al. [22] with bovine serum albumin as standard.

Phospholipid-phosphorus. Phospholipid was extracted three times with chloroform/methanol (2 : 1, v/v) from the purified enzyme and the extracted solution was evaporated in vacuo. The residue was dissolved in chloroform and then applied to a silica gel plate (Merck). The developing solvent was chloroform/methanol/water (65 : 25 : 4, v/v). A spot of phospholipid detected by iodine vapour was cut out and the phosphorus content was determined after wet ashing by the method of Bartlett [23].

Circular dichroism spectrum. The circular dichroism spectrum was measured with a JASCO J-20 spectropolarimeter using a 0.63 mm quartz cell at 23°C. The spectrum data are expressed in terms of mean residue ellipticity, $[\theta]$. The mean residue weight of the enzyme was calculated to be 106 on the basis of amino acid composition.

Preparation of phospholipid dispersion. The method of Saha et al. [24] was used. Dried cardiolipin (10 mg) or cardiolipin (10 mg)/vitamin K-1 (1 mg) mixture was suspended in 5 ml of 10 mM Tris · HCl buffer (pH 7.4)/1 mM EDTA. Sonication was performed in an ice bath for 15 min under N_2 gas and used in the experiments shown in Table II.

Results

Solubilization of FAD-dependent malate dehydrogenase

The enzyme predominantly located in the particulate fraction was not solubilized by simple washing or sonication with 0.15 M KCl, 0.25 M sucrose, or 10 mM EDTA. These results suggest that the enzyme is an "integral protein" according to Singer and Nicolson [25], in contrast to that of *M. phlei* [26].

Other treatments with detergents or phospholipase A were also ineffective for preparing the active solubilized enzyme.

In the previous paper [2], the enzyme was solubilized and then partially purified from an acetone powder of the particulate fraction, but this technique did not produce a phospholipid-depleted enzyme. Therefore, other solubilization methods were examined and it was found that treatment of the particulate fraction with *n*-butanol, followed by ether/ethanol, to produce "butanol powder" produced the required phospholipid-depleted enzyme. The optimal conditions for the preparation of this butanol powder were as follows: temperature (15—20°C), duration of mixing with *n*-butanol (5—10 min), ratio of *n*-butanol to the particulate fraction precipitated by $(NH_4)_2SO_4$ (10 ml/g), and treatment with ether/ethanol (1 : 1, v/v) at 0—10°C (5—10 min).

Purification of the enzyme

All steps were carried out at 0—4°C unless otherwise stated and $(NH_4)_2SO_4$ fractionations were performed at pH 6.2 with addition of ammonia water. The buffer used in the purification procedure was 10 mM potassium phosphate buffer (pH 7.0) unless otherwise indicated.

Step 1. Cell-free homogenate. Washed cells were ground together with two times wights of quartz sand, and after addition of buffer, the homogenate was centrifuged at 5100 × *g*.

Step 2. Particulate fraction. Solid $(NH_4)_2SO_4$ (35% saturation) was added to the supernatant and the mixture stirred for 1 h and then allowed to stand overnight. The precipitate, after centrifugation at 12 800 × *g* was dialyzed overnight against the buffer. Dialyzed solution was centrifuged at 170 000 × *g* for 40 min and the precipitate was resuspended in the buffer plus 0.25 M sucrose/0.15 M KCl and centrifuged at 170 000 × *g*. The washed particulate fraction was homogenized with buffer and precipitated by solid $(NH_4)_2SO_4$ (30% saturation).

Step 3. Butanol powder. The precipitate, obtained at 12 800 × *g*, was vigorously stirred with *n*-butanol at 20°C in a mixer. The temperature was maintained by addition of crushed solid CO_2. The preparation was washed with *n*-butanol and then acetone on a Buchner funnel and the powder was dried under reduced pressure. The powder was vigorously stirred again with ethanol/ether (1 : 1, v/v) and filtered; after being washed with ether, the powder was dried under reduced pressure. This powder was designated as "butanol powder".

Step 4. Solubilization and $(NH_4)_2SO_4$ fractionation. The enzyme was solubilized from butanol powder by homogenizing with 20 times (v/w) of buffer. After stirring, the homogenate was centrifuged at 12 800 × *g*. The supernatant solution was precipitated by addition of solid $(NH_4)_2SO_4$ (60% saturation). The precipitate was centrifuged down at 12 800 × *g*, and then dialyzed against buffer.

Step 5. Protamine sulfate. The dialyzed solution was adjusted to 10 mg of protein/ml and nucleic acids and acidic proteins were precipitated by addition a quarter volume of 1% protamine sulfate in buffer. After stirring, the precipitate was removed by centrifugation at 13 000 × *g*.

Step 6. Hydroxyapatite column. The supernatant solution was adjusted to 0.2 M with respect to phosphate concentration and applied to a hydroxyapatite column (2 × 30 cm) previously equilibrated with 0.2 M potassium phos-

phate buffer (pH 6.6). The column was washed with 100 ml of the equilibrating buffer, and then eluted with a linear gradient of 0—0.7 M NaCl. Enzymatically active fractions were combined.

Step 7. Calcium phosphate gel column. Hydroxyapatite eluate was diluted 2.5 times with cold water, and then applied to a column of calcium phosphate gel deposited on cellulose [26] (1.5 × 20 cm) equilibrated with 0.15 M sodium phosphate buffer, pH 6.6. After washing the column with this buffer, the enzyme was eluted with a linear gradient of 0—0.35 M sodium phosphate buffer (pH 6.6). The enzymatically active fractions were combined and concentrated to a small volume and the buffer was exchanged with 0.3 M potassium phosphate (pH 6.6) in a collodion bag. This procedure is summarized in Table I.

Purity

The purified enzyme was subjected to SDS-polyacrylamide gel electrophoresis as described under Materials and Methods. Under the result shown in Fig. 1 the enzyme was homogeneous with regard to protein component.

Analyses and requirements of cofactors

Phospholipid-phosphorus content of the purified enzyme analyzed as described under Materials and Methods, was found to be 0.23 μg of phosphorus/mg of enzyme protein. The absorption spectrum exhibits an absorption maximum at 280 nm with a shoulder at 290 nm; other absorption maxima corresponding to FAD and vitamin K were not found.

The requirement of cofactors for the enzyme activity was examined and in the absence of FAD, no reduction of 2,6-dichlorophenolindophenol was observed, while in the absence of either cardiolipin or vitamin K-3, only about 3% of the maximal enzyme activity was found. In the absence of two or three cofactors, no measurable activity was observed.

Stability

When the purified enzyme (1 mg or 6 mg of protein/ml) was kept in 0.3 M potassium phosphate buffer (pH 6.6) at −20°C for 2 weeks, about 20% of the

TABLE I

PURIFICATION OF FAD-DEPENDENT MALATE DEHYDROGENASE FROM *MYCOBACTERIUM* SP. STRAIN TAKEO

Step		Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Yield (%)
1.	Cell-free homogenate	19 300	4280	0.22	100
2a.	Particulate fraction	11 900	3950	0.33	92
2b.	Washed particulate fraction	8 700	3780	0.42	88
3.	Butanol powder	—	1520	—	36
4.	Solubilized enzyme from powder	530	850	1.60	20
5.	Protamine sulfate supernatant	134	780	5.82	18
6.	Hydroxyapatite eluate	1.92	346	180	8
7.	Calcium phosphate eluate	0.70	217	310	5

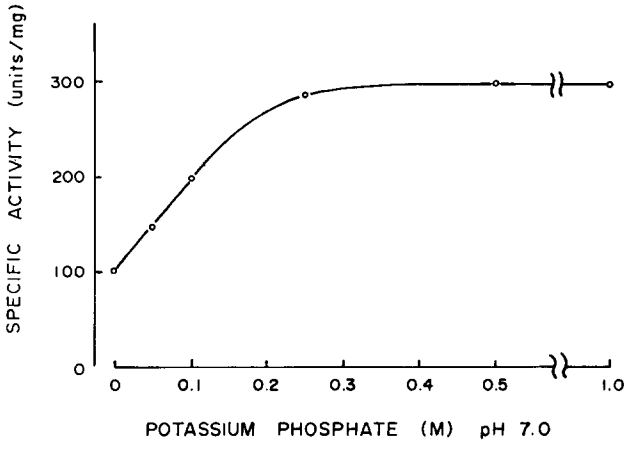

Fig. 1. SDS polyacrylamide gel electrophoresis of purified FAD-dependent malate dehydrogenase. Purified enzyme (10 μg) was subjected to the electrophoresis and stained as described under Materials and Methods. BPB, Bromophenol Blue.

Fig. 2. Changes of specific activity upon dilution in potassium phosphate buffer. 5 μl of the purified enzyme (0.22 μg) in 0.3 M potassium phosphate buffer, pH 6.6, were diluted in the 95 μl of various concentrations of potassium phosphate buffer, pH 7.0. 10 μl of the diluted enzyme was assayed for enzyme activity. Undiluted enzyme has a specific activity of 300.

enzyme activity was lost. Enzyme solutions (2.2 μg/ml) were allowed to stand for 24 h at 0°C and about 90 and 20% of the enzyme activity were lost in 0.1 and 0.3 M potassium phosphate buffer (pH 6.6), respectively. Addition of sucrose (0.1—1 M) or glycerol (5—20%) did not stabilize the enzyme.

Changes of enzyme activity in different media

Imai and Brodie [11] reported that when the enzyme of *M. phlei* was diluted in the media containing either KCl, glycerol, Tris · HCl buffer, phospholipid, vitamin K-1 or these combinations and then assayed immediately, the specific activity of the enzyme was changed. Similar changes of the specific activity of the enzyme were observed in this study; a decrease by dilution into lower than 0.3 M potassium phosphate buffer (pH 7.0) and no change above 0.3 M as compared with the undiluted enzyme (specific activity; 300) (Fig. 2). Similarly, the specific activity was decreased upon dilution into KCl (below 1.0 M)/50 mM potassium phosphate (pH 7.0).

The specific activity was slightly increased when the enzyme was diluted into

TABLE II

EFFECT OF DILUTION ON FAD-DEPENDENT MALATE DEHYDROGENASE

5 μl of the purified enzyme (0.22 μg) in 0.3 M potassium phosphate, pH 6.6, were diluted in 95 μl of the medium described in the table. All media contained 0.1 M potassium phosphate, pH 6.8. 10 μl of the diluted enzyme was assayed immediately for enzyme activity.

Dilution medium	Specific activity
Buffer alone	200
Cardiolipin (5 μg)	243
Cardiolipin (5 μg)/FAD (0.05 μg)	280
Cardiolipin (5 μg)/FAD (5 μg)	357
Cardiolipin (5 μg)/vitamin K-1 (0.5 μg)	226
Cardiolipin (5 μg)/vitamin K-1 (0.5 μg)/FAD (0.05 μg)	283
Cardiolipin (5 μg)/vitamin K-1 (0.5 μg)/FAD (5 μg)	373
FAD (5 μg)	269

a medium containing cardiolipin (5 μg) and FAD (5 μg), or cardiolipin (5 μg), vitamin K-1 (0.5 μg) and FAD (5 μg) as shown in Table II. Dilution into other media resulted in a decrease of the specific activity. Sucrose and glycerol did not affect the specific activity of the enzyme.

Molecular weight

The molecular weight of the enzyme (51 000—55 000) was estimated by gel chromatography, sucrose density gradient centrifugation, and SDS-polyacrylamide gel electrophoresis (Table III). Further, SDS-polyacrylamide gel electrophoresis even in the presence of 8 M urea produced one protein band and the enzyme is thus assumed to be made up of a single polypeptide chain. The molecular activity (units/μmol of enzyme) of the enzyme under the assay condition, was calculated to be 16 000—19 000, using a molecular weight of 53 000.

Isoelectric point

Isoelectric focusing was performed in the presence of 0.05% Triton X-100, which prevented precipitation of the enzyme and the isoelectric point was found to be pH 6.9.

Amino acid composition

The amino acid composition of the enzyme is presented in Table IV. Titration of the enzyme with *p*-chloromercuribenzoate showed the presence of 1 mol of cysteine residue/mol of the enzyme. The polarity of enzyme protein

TABLE III

ESTIMATES OF THE MOLECULAR WEIGHT OF FAD-DEPENDENT MALATE DEHYDROGENASE

Method	Molecular weight
Sephadex G-100 gel chromatography	51 000—52 000
Sephadex G-200 gel chromatography	53 000—55 000
Sucrose density gradient centrifugation	51 000—55 000
SDS polyacrylamide gel electrophoresis	51 000—53 000

TABLE IV

AMINO ACID COMPOSITION OF FAD-DEPENDENT MALATE DEHYDROGENASE

Amino acid	Residues per 53 000 g of protein
Lysine	8.3
Histidine	32.7
Arginine	21.1
X *	7.7
Aspartic acid	38.2
Threonine **	24.9
Serine **	80.7
Glutamic acid	69.9
Proline	15.2
Glycine	76.7
Alanine	44.5
Half-cystine ***	2.7
Valine	25.9
Methionine	0
Isoleucine †	13.0
Leucine †	24.3
Tyrosine	6.9
Phenylalanine	11.9
Tryptophan ††	7.0

* Unidentified amino acid residue. This value was calculated from ninhydrin color of ornithine.
** Based on extrapolation to zero time of hydrolysis.
*** Determined after performic acid oxidation.
† Values obtained after 72 h of hydrolysis.
†† Determined spectrophotometrically.

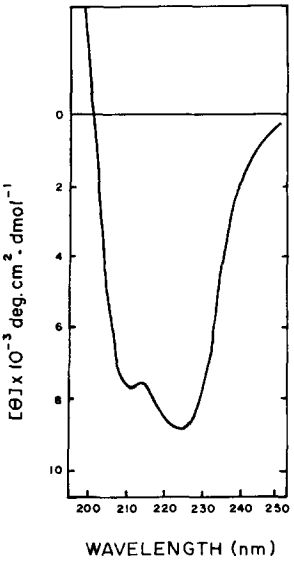

Fig. 3. Far-ultraviolet circular dichroism spectrum of FAD-dependent malate dehydrogenase. The purified enzyme (90 μg) was contained in 0.3 ml of 0.3 M potassium phosphate buffer, pH 6.6.

was 55% [27], which is an exceptionally high value among membrane-bound enzymes.

Circular dichroism spectrum

The circular dichroism spectrum of the purified enzyme was measured (Fig. 3) and α-helix content and β-structure were calculated to be 24 and 0%, by the method of Chen et al. [28].

Discussion

Three half cystines were found in the amino acid composition of the enzyme. One of them was titratable by *p*-chloromercuribenzoate and simultaneously lost its activity, and binding of FAD to the enzyme · cardiolipin complex was not observed in the presence of *p*-chloromercuribenzoate (unpublished observations), indicating the presence of the cysteine residue of the binding site of the enzyme. The participation of sulfhydryl group in the binding of flavins to enzymes has been reported in flavoproteins [29—32]. Hydroxylysine was found to be present in the enzyme of *M. phlei* [11]. In our elution profiles of the amino acid analysis of the enzyme of *Mycobacterium* sp. strain Takeo, an unknown peak eluted between histidine and lysine was observed and it was determined to be due to ornithine from coincidence of the peak with that of authentic ornithine but not with that of hydroxylysine under three different elution conditions. The presence of ornithine in the amino acid composition has been reported in an iron-sulfur protein of a *Pseudomonas* [33] and superoxide dismutase of *Pseudomonas ovalis* [34]. As for the polarity of the enzyme protein, including ornithine to hydrophilic amino acid, it was exceptionally high (55%) compared to that of the typical membrane-bound enzyme [27] and that of *M. phlei* [11]. High values, however, have been reported in cytochrome b_5 of rabbit hepatic microsome [35]. In this protein, presence of a hydrophobic segment is shown, through which the protein is attached to the membrane.

The specific activity of the enzyme was decreased upon dilution into a low concentration of potassium phosphate buffer. This suggests two possibilities; aggregation-disaggregation phenomena as reported in *M. phlei* enzyme [11], and a conformational change including partial denaturation. Similarly, changes of the specific activity found in the presence of the cofactor(s) (Table II) may be interpreted as conformational change(s) of the enzyme when complexed with these cofactors.

The present and previous studies clearly indicate that the properties of FAD-dependent malate dehydrogenase obtained from *Mycobacterium* sp. strain Takeo are different to those of the enzyme from *M. phlei*, except that both molecular weights are approx. 53 000.

The formation of complexes of the purified enzyme with cardiolipin, FAD, vitamin K-1 will be reported in the near future.

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