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PROPERTIES OF MALATE DEHYDROGENASE ISOLATED FROM THE MARINE DIATOM *CYLINDROTHECA FUSIFORMIS*

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Key Word Index—*Cylindrotheca fusiformis*; diatom; malate dehydrogenase, purification; properties.

Abstract—Malate dehydrogenase (EC 1.1.1.37) was purified to homogeneity from the marine diatom *Cylindrotheca fusiformis*. A typical experiment provided 353-fold purification with 26% yield. The purified enzyme migrated as a single polypeptide band on SDS-polyacrylamide gel electrophoresis and with M_r of 38 500. Whereas the M_r of the holoenzyme was estimated to be 311 000 by gel filtration, suggesting that the enzyme consists of eight subunits with similar M_r. The holoenzyme molecule shows a Stokes radius of 60 Å. The malate dehydrogenase exhibits pH optima at pH 6.0 for oxaloacetate reduction and pH 10.0 for malate oxidation. It is relatively stable in buffers with pH ranged from 6.0 to 8.5. The enzyme was sensitive to elevated temperature and the activation energy was calculated as 5.64 kcal/mol. The amino-acid composition of the enzyme was determined and the partial specific volume of the enzyme were calculated to be 0.736. The *N*-terminal sequence of the subunit polypeptide was determined to be Arg-Lys-Val-Ala-Val-Leu-Gly-Ala-Gly-Gly-Gly-Ile-Gly-Gln-Pro-Leu-Ser-Leu-Leu-Leu-Lys-Leu-Ser-, near identical sequences were reported for the malate dehydrogenases isolated from *Escherichia coli*, watermelon, and porcine heart.

INTRODUCTION

The NAD^+ dependent malate dehydrogenase (L-malate: NAD^+ oxidoreductase, EC 1.1.1.37, abbreviated as MDH) catalyses the following reaction *in vitro* and *in vivo*: malate + $NAD^+ \rightleftharpoons$ oxaloacetate + NADH + H^+. In higher plants, MDH, together with phosphoenolpyruvate carboxylase and malic enzyme-decarboxylating, regulate the synthesis and oxidation of malate which plays essential roles in plant metabolism. For instance, it has been emphasised that malate regulates the nitrate assimilation pathway [1], maintains the ionic balance and pH [2, 3], and functions as a mobile storage form of carbon dioxide and reducing equivalents in plant cells [4]. Besides, since the reaction catalysed by MDH is a key step in the citric acid cycle, MDH exists in all aerobic organism. So far the enzyme had been purified and characterized from various organisms including bacteria [5–9], fungi [10, 11] animals [12–17] and higher plants [18–24].

Diatoms, as a whole, contribute *ca* 20 to 25% of the world primary production, they are the most important primary producer in the biosphere [25]. However, the biochemistry of the organisms are not well understood. In the present studies, the MDH of the marine diatom *Cylindrotheca fusiformis* was purified to homogeneity and characterized with respect to various physicochemical and catalytic properties.

RESULTS

Purification and homogeneity of the enzyme

Malate dehydrogenase in *Cylindrotheca fusiformis* was purified to homogeneity as judged by SDS-polyacrylamide gel electrophoresis. The purification procedure employed routinely provided greater than 25% of recovery with more than 350-fold of purification. A typical purification experiment is summarized in Table 1. After the

Table 1: Purification of malate dehydrogenase from *C. fusiformis*

Purification step	Volume (ml)	Protein conc. (mg/ml)	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Recovery (%)	Purification (fold)
Crude extract	169	1.72	291	600	2.06	100	1.0
Ammonium sulphate (30 to 85%)	39	3.94	154	448	2.91	74.7	1.4
DEAE-Sepharose CL-6B	53	0.128	6.78	315	46.4	52.5	22.5
Sepharose CL-6B	21	0.048	1.01	276	274	46.0	133
AMP-Sepharose	40	0.0064	0.26	178	695	29.7	337
Sepharose CL-6B	19	0.0112	0.21	155	728	25.8	353

consecutive steps of ammonium sulphate fractionation, DEAE-Sepharose ion-exchange chromatography, gel filtration on Sepharose CL-6B column, AMP-Sepharose affinity chromatography and a final gel filtration column, the recovery was 25.8% with 353-fold of purification. A total of 213 μg of purified enzyme was obtained from 30 l of cultured cells.

M_r *determination*

The subunit M_r for the enzyme was estimated using 10 and 12 5% SDS-polyacrylamide gels. In both cases the M_r of the MDH subunits was estimated as 38 500 ± 300. The M_r of native MDH was estimated by gel filtration on a Sepharose CL-6B column. The elution volume of the enzyme remained constant in pH from 6.0 to 8.5 and in the presence of up to 0 3 M of NaCl The M_r of the holoenzyme was calculated to be 311 000 ± 700 from its relative elution volume (V_e/V_o) and those of the marker proteins with known M_rs In consideration with the subunit M_r, it is likely that the MDH from *C fusiformis* is a octamer of similar sized subunits.

pH optima and stabilities

The pH optima for oxaloacetate reduction and malate oxidation were found to be 6 0 and 10.0, respectively. The stability of the enzyme between pH 4.0 and 10 0 was investigated after 90 min incubation at 25° and subsequent assay of the rate of oxaloacetate reduction at the optimal pH. The MDH was very stable between pH 6.5 to 8.5 Therefore, the marked activity changes in this region observed in the pH optimum experiments are not due to denaturation, but to the ionization of specific groups of the enzyme or substrates involved in the catalytic process. Outside this range, the enzyme activity decreased sharply after 90 min of incubation

Heat stabilities

When the enzyme was incubated at superoptimal temperatures, i.e. 40 and 45°, its activity respectively decreased by 17 and 92% after a 10 min period. In separate experiments, the enzymatic activities for oxaloacetate reduction were determined at various temperatures and the activation energy (E_a) was then calculated as 5 64 kcal/mole by an Arrhenius plot

Kinetic constants

K_ms were determined for the MDH for the following four substrates· L-malate, oxaloacetate, NAD^+ and NADH and values are presented in Table 2 in which other properties of the enzyme are also summarized

Amino acid composition and N-*terminal sequence*

Homogeneous protein was subjected to acid hydrolysis and the amino acid composition is shown in Table 3 The *N*-terminal amino-acid sequence of the enzyme is determined to be Arg-Lys-Val-Ala-Val-Leu-Gly-Ala-Gly-Gly-Gly-Ile-Gly-Gln-Pro-Leu-Ser-Leu-Leu-Leu-Lys-Leu-Ser-Near identical sequences were found for malate dehydrogenases isolated from *Nitzchia alba* (unpublished data), porcine heart [26], *E coli* [27], yeast, watermelon [28].

DISCUSSION

In the present study some molecular properties of the malate dehydrogenase from *Cylindrotheca fusiformis* were investigated. The enzyme shows only one active form, its M_r corresponds to 311 000 on gel filtration column chromatography. The elution profile does not change between 0 and 0.3 M NaCl, pH 6 0 to 8.5 Under extreme conditions it can either associate into high M_r aggregates or dissociate into another inactive form which eluted near the bed volume on gel filtration column (data not shown) Therefore, our data suggested that the high M_r form of MDH from *C. fusiformis* is not the result of aggregation SDS polyacrylamide gel electrophoresis and *N*-terminal analysis indicate that the MDH from *C. fusiformis* consists of only one kind of polypeptide with a M_r of 38 500. In consideration of the M_r of the native enzyme, we

Table 2 Physical and catalytic properties of malate dehydrogenase from *C fusiformis*

Physical properties	
M_r	
Holoenzyme. gel filtration	311 000 ± 7 000*
Subunit. SDS-gel electrophotesis	38 500 ± 300*
Number of subunits	8
Partial specific volume (υ)	0 736
Stokes radius	60 Å
Catalytic properties	
K_m values (M)	
L-Malate	$1\,33 \times 10^{-3}$
Oxaloacetate	$5\,61 \times 10^{-5}$
NAD^+	$1\,02 \times 10^{-3}$
NADH	$3\,24 \times 10^{-4}$
pH optima	
Malate oxidation	10.0
Oxaloacetate reduction	6 0
pH stability range	6 0 to 8 50
Activation energy (E_a)	5 64

*Mean ± standard error of mean from three independent experiments

Table 3. Amino acid composition of malate dehydrogenase from *C. fusiformis*

Amino acid	Mol (%)	Number of residues per subunit
Aspartic acid/asparagine	12 9	42.8
Threonine	6.3	24.4
Serine	5.9	26.4
Glutamic acid/glutamine	10.4	10.4
Proline	6.6	25.8
Glycine	6.5	44.0
Alanine	6.1	33 5
Valine	8.2	32.4
Methionine	1 7	4.8
Isoleucine	4 4	14.6
Leucine	8.2	28.2
Tyrosine	4 2	9.7
Phenylalanine	5 0	12.5
Histidine	2 1	6 4
Lysine	6.7	19.6
Arginine	4.1	10.3
Half-cystine	0.8	3.3
Tryptophan*	0 5	1.0
Total	100 6	350.1

*Determined by the methods of Spande and Witkop [44].

suggest that the MDH from *C. fusiformis* is composed of eight subunits. With two exceptions, all MDHs purified from different sources are dimers and the M_r of these enzymes were in the range 60000 to 70000 [29]. In *Bacillus subtilis* and *Neurospora crassa*, however, the enzyme were shown to be tetramers with native M_rs of 148000 and 56000, respectively [5, 10]. In the crude extract, it was shown that MDH might exist as oligomeric forms with M_rs much higher than the usual 60000 to 70000 as indicated by nondenaturing polyacrylamide/-starch gel electrophoresis and gel filtration column chromatography [30]. These high M_r MDH species, usually in addition to the normal M_r form of the enzyme, were reported in bacteria [5, 6], fungi [10], animal mitochondria [31], and, most commonly, in higher plants [30, 32, 33]. However, none of the high M_r MDH had been purified to homogeneity, therefore the native conformation of the MDHs remained uncertain and the higher M_r species of MDH in higher plants might result from aggregation of low M_r species. In fact, it has been shown that the glyoxysomal MDH isozymes in castor bean were found to exist in "some sort of complex or aggregate" [23]. In contrast, our data imply that the high M_r form of MDH from *C. fusiformis* is not the result of aggregation. The data suggests that the native *C. fusiformis* MDH is octameric and in a higher M_r form, which is different from the MDHs purified from all other species previously reported An octameric MDH was also observed in another marine diatom, *Nitzschia alba* (unpublished data). It is interesting to investigate whether high M_r forms of MDH are common features in marine diatoms, and the significance of their existence.

A list of physicochemical and catalytic properties of the *C. fusiformis* MDH have been determined and summarized in Table 2. No substantial difference was observed among the *C. fusiformis* MDH and the same enzyme purified from other sources. For instance, the pH optima of the *C. fusiformis* MDH are comparable with the values obtained for the same enzyme from *B. subtilis* [5], *Schizosaccharomyces pombe* [11], beef heart mitochondria [12] and spinach leaf microbodies [20]. The optima pH range of these enzymes are 9.0 to 10.0 for malate oxidation, and 7.0 to 8.0 for oxaloacetate reduction. A slightly lower pH optimum (6.0) for oxaloacetate reduction is observed in *C. fusiformis* MDH.

The activation energy of MDH were calculated for several species, including *H. lanuginosa*, *M. puisillus* [9], *S. pombe* [11], *N. crassa* [34], and watermelon [14]. The values were reported to be in the range 5 to 10 kcal/mol whereas the value for *C. fusiformis* MDH has been determined as 5.64 kcal/mol.

From the amino acid composition of MDH from *C. fusiformis*, it is found that the enzyme molecule contains relatively large amounts of aspartic acid (or asparagine) glycine, alanine and valine but very little methionine, cysteine and tryptophan (Table 3). Similar feature was also found in the MDH obtained from other sources [8] except *Mycobacterium phlei* in which a large amount of methionine was reported [35]. Further comparison reveals that a greater degree of similarity was found between *C. fusiformis* and animal sources than bateria and fungi [8].

The *N*-terminal sequence of *C. fusiformis* MDH was compared with that of the MDH from porcine heart mitochondria [26], *E. coli* [27], yeast and watermelon [28]. The data indicate that a highly conservative region exists in the *N*-terminal of all MDHs There are at least 17 identical residues in the first 21 *N*-terminal residues among the enzymes from different sources. Recently, the three-dimensional structure of porcine heart mitochonerial MDH was determined by X-ray diffraction [36]. The result indicated that the *N*-terminal sequence (residues 1 to 22) contains one β-conformation (residues 1 to 5), one α-helix (residues 11 to 22) and one co-enzyme binding domain (residues 6 to 10). The α-helix is found to reside in the interface between the subunits and which may be important for subunit–subunit interaction of the enzyme molecule. Since there is a high degree of homology of *N*-terminal sequence between porcine heart mitochondrial MDH and *C. fusiformis* MDH, it is conceivable that similar functional domains would exist in the same region.

EXPERIMENTAL

Plant materials and chemicals. The marine diatom *Cylindrotheca fusiformis* was obtained from Prof. B. Volvani (Scripps Institute of Oceanography, University of California at San Diego). The maintenance of stocks and mass culture of the diatom were carried out as described [37]. The sources of chemicals were as follows: Sepharose CL-6B, DEAE-Sepharose CL-6B, AMP-Sepharose CL-6B, and SDS low M_r marker kit were purchased from Pharmacia. Yeast alcohol dehydrogenase, rabbit muscle aldolase, bovine serum albumin, bovine erythrocyte carbonic anhydrase, bovine liver catalase, horse spleen ferritin, porcine thyroglobulin, sweet potato β-amylase, bovine fibrinogen, β-NAD^+, β-NADH, Tris and SDS were purchased from the Sigma. Acrylamide, bis-acrylamide, Biolytes and protein assay kit were obtained from Bio-Rad. All other reagents were from Merck and of reagent grade or better.

Purification of malate dehydrogenase from C fusiformis All purification steps were carried out at a temp. between 0 to 4°. Unless otherwise specified, the buffer used in the purification procedures was 0.05 M K-phosphate, pH 7.0 containing 1 mM each of 2-mercaptoethanol and EDTA. All centrifugation steps were performed at 10000 *g* for 10 min.

Extraction and precipitation with ammonium sulphate. For each purification preparation, a culture vessel containing 30 l of enriched natural sea-water was inoculated with pre-grown log-phase cells. The culture was grown at 25° with slow stirring and vigorous aeration. When the culture reached 3×10^6 cells/ml, the cells were harvested by centrifugation, washed twice with 4 vol of buffer, and then homogenized in same amount of buffer with ultrasonics. The homogenate was centrifuged and the supernatant was collected as the crude extract.

Solid $(NH_4)_2SO_4$ was added to the crude extract until it reached 35% satn. After 60 min of continuous stirring, the pptd materials were collected by centrifugation and discarded. The supernatant was saved and $(NH_4)_2SO_4$ concn was raised to 80% satn. After another 60 min of continuous stirring, the precipitated protein collected by centrifugation was dissolved in minimum amount of buffer, and dialysed against the buffer for 3 hr.

Ion-exchange column chromatography. The dialysate was clarified by centrifugation and the protein soln was applied onto a DEAE-Sepharose CL-6B column (2.6 × 10 cm) which was pre-equilibrated with buffer. MDH activity was found to be adsorbed onto the column and the unbound proteins were washed off by one bed vol. of buffer. After washing, the column was developed by a 400 ml linear gradient concn of NaCl, 0.1 to 0.5 M prepared in the same buffer. The fractions containing the MDH activity were pooled. The sample was then desalted and concd into a final vol. 2 ml by ultrafiltration on an Amicon YM 10 membrane.

Gel filtration on a Sepharose CL-6B column. The sample obtained in the previous step was applied to a pre-equilibrated Sepharose CL-6B column (1.6 × 100 cm). Elution was by upward flow at 25 ml/hr. Fractions containing MDH activity were pooled, desalted, and concd as previously described.

Affinity chromatography. The enzyme soln was applied onto a AMP-Sepharose column (1 × 3 cm) which was previously equilibrated with buffer. The column was washed with 15 ml of buffer and the bound enzyme was eluted with 0.1 mM NADH in the same buffer. Fractions containing enzyme activity were pooled, concd by ultrafiltration and rechromatographed on the above gel filtration column to remove the final trace of contaminant. Purified enzyme prepns were used immediately or stored at −20° in 50% (v/v) glycerol.

Enzyme assays and protein determination. MDH activity determined during purification and in the characterization of purified enzyme was measured spectrophotometrically with a Hitachi spectrophotometer equipped with an automatic recorder. Unless otherwise specified, the reaction mixture contained 50 mM K-Pi (pH 7.0), 0.15 mM NADH, 0.15 mM oxaloacetate, 1 mM each of 2-mercaptoethanol and EDTA. An extinction coefficient of 6.32/mM/cm was used for NADH. Assays of enzymatic activities were carried out at 20°. Unless otherwise specified, protein content was determined according to the method of ref. [38] using bovine serum albumin as standard.

Determination of M_r *of the native enzyme and its subunit.* The M_r of the purified enzyme was determined by gel filtration chromatography in a Sepharose CL-6B column (1.6 × 100 cm), with the following M_r standards: porcine thyroglobulin (669000), sweet potato β-amylase (200000), yeast alcohol dehydrogenase (150000), bovine serum albumin (67000) and bovine erythrocyte carbonic anhydrase (29000). These samples were analysed individually at a flow rate of 18 ml/hr. The elution vols of the standards were determined by a UV monitor, and that of the MDH determined by activity assays. The M_r of the native enzyme was then calculated according to the method ref. [39]. The subunit M_r of the MDH was estimated by SDS-polyacrylamide gel electrophoresis as described in ref. [40] in Laemmli's slab gel system [41].

Determination of Stokes radius. The Stokes radius was estimated by gel filtration techniques using Sepharose CL-6B gels. The column was calibrated with the following protein standards: bovine fibrinogen (107 Å), porcine thyroglobulin (85 Å), horse spleen ferritin (61 Å), rabbit muscle aldolase (48.1 Å) and bovine serum albumin (35 Å). The Stokes radius of the MDH was calculated according to the method of ref. [42].

pH optima and stabilities. The pH optima of the purified enzyme were determined by assaying MDH activity over the pH range 4.0 to 10.0 using the following buffers in the reaction mixture: 0.1 M NaOAc, pH 4.0 to 5.5, 0.1 M Na-Pi, pH 5.5 to 7.5, 0.1 M Tris–HCl, pH 7.5 to 9.0 and 0.1 M glycine–NaOH, pH 9.0 to 10.0. The pH stabilities of the enzyme were obtained by incubating the enzyme at 25° in the above buffers of varying pH for 90 min and then the remaining activities were determined by the standard assays.

Heat stabilities and activation energy. For heat stability studies, 1-ml sample of enzymes in 0.05 M K-Pi, pH 7.0 were incubated in sealed test tubes at various temps. Aliquots (10 to 20 μl) were removed at 2 to 10 min intervals and assayed for MDH activity immediately. The activation energy of the purified enzyme was determined by assaying the rate of oxaloacetate reduction at temps ranged from 10 to 35° and the value was calculated by Arrhenius plot ($\log k$ vs $1/T$ where k is the rate constant and T is the temp in K).

Michaelis constants. The estimation of Michaelis constants (K_m) for the MDH was performed using a 6 × 6 array analysis and all assays were carried out in 0.05 M Tris–HCl buffer (pH 8.0). The substrate ranges used were as follows: malate, 0.6 to 2 mM, NAD^+, 1 to 2.5 mM, oxaloacetate, 25 to 125 μM; and NADH, 75 to 200 μM. K_m values were calculated by double reciprocal and secondary plots ($1/v$ vs $1/[S]$).

Determination of the amino acid composition and the N-terminal sequence. For amino acid composition analysis, purified enzyme sample was washed in double dist. H_2O and concd by ultrafiltration. The sample was then hydrolysed in 6 M HCl at 110° for 24 hr *in vacuo*. The acid lysate was dried under vacuum in a desiccator containing concentrated H_2SO_4 and KOH pellets as desiccants. The amino acid composition of the sample was then determined by an automatic amino acid analyser (Beckman Model 6300) according to the method of ref. [43]. The tryptophan content of the protein was determined by the method of ref. [44]. Subsequently, the partial specific vol. of the enzyme was calculated from the amino acid composition as described in ref. [45]. Automated Edman degradations [46] were performed on an Applied Biosystem Inc. (ABI) gas/liquid phase Model 470A Sequencer with an on line 120A phenylthiohydantoin (PTH)-amino acids analyser. The resulting PTH-amino acids were analysed on an ABI PTH-C18 reverse phase cartridge column (2.1 mm × 22 cm) according to the manufacturer's specification and the data were analysed by a Waters 740 data module and an ABI model 900 data module simultaneously.

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