Purification and molecular properties of malate dehydrogenase from the marine diatom *Nitzschia alba*

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Malate dehydrogenase (EC 1.1.1.37) was purified to homogeneity from the marine diatom *Nitzschia alba*. The purification steps consisted of $(\text{NH}_4)_2\text{SO}_4$ precipitation, ion-exchange chromatography, Blue Sepharose affinity chromatography and gel filtration. A typical procedure provided 685-fold purification with 58% yield. The M_r of the holoenzyme was estimated to be 322 000 by gel filtration and 316 000 by ultracentrifugation. The enzyme migrated as a single polypeptide spot on two-dimensional polyacrylamide-gel electrophoresis with an M_r of 38 500, suggesting that the holoenzyme consists of eight identical subunits. This is the first case where malate dehydrogenase has been shown to be a homo-octamer; malate dehydrogenases from other sources are predominantly homodimers, with two homotetramers reported so far. The amino acid composition of the enzyme was determined and the *N*-terminal sequence of the subunit polypeptide was found to be Arg-Lys-Val-Ala-Val-Met-Gly-Ala-Ala-Gly-Gly-Ile-Gly-Gln-Pro-Leu-Ser-Leu-Leu-Leu-Lys-Leu-Ser-Pro-Gln-Val-Thr-Glu-Leu-Ser-Lys-Tyr-. For the first 21 amino acid residues, near-identical sequences were reported for the enzymes isolated from pig heart, *Escherichia coli*, yeast and watermelon. Other physicochemical and catalytic properties, such as sedimentation coefficient, partial specific volume, Stokes radius, excitation and emission maxima, Michaelis constants, pH optima, pH stability range and activation energy, of this enzyme are also presented.

INTRODUCTION

The NAD^+ -dependent malate dehydrogenase (MDH) (L-malate: NAD^+ oxidoreductase, EC 1.1.1.37) is a widely distributed enzyme. Since the enzyme participates in the citric acid cycle, it exists in all aerobic organisms. So far MDH has been purified to apparent homogeneity and well characterized from various organisms, including bacteria [1–5], fungi [6,7], animals [8–13] and higher plants [14–19].

With two exceptions, all malate dehydrogenases purified from different sources are dimers and the M_r values of these enzymes are in the range of 60 000–70 000 [20]. In *Bacillus subtilis* and *Neurospora crassa*, however, the enzymes were shown to be tetramers with the native M_r values of 148 000 and 56 000 respectively [2,6]. In crude extracts it was shown that MDH might exist as oligomeric forms with the M_r much higher than the usual 60 000–70 000, as indicated by non-denaturing polyacrylamide/starch-gel electrophoresis and gel-filtration column chromatography [21]. These high- M_r MDH species, usually in addition to the normal- M_r form of the enzyme, have been reported in bacteria [1,2], fungi [6], animal mitochondria [22] and, most commonly, in higher plants [21,23,24]. These results indicated that the higher- M_r species of MDH in higher plants might result from aggregation of low- M_r species. Since none of the high- M_r species has been purified to homogeneity, the native conformation of these MDH enzymes remained uncertain.

Despite the fact that the diatoms are the most important primary producer in the biosphere [25], the organisms are not well characterized biochemically. In the present study the MDH was purified to homogeneity

from the apochlorotic marine diatom *Nitzschia alba*. The enzyme was extensively characterized, and the results indicate that the usual active form of malate dehydrogenase in *N. alba* consists of eight identical subunits; the enzyme is the only octameric MDH reported so far.

MATERIALS AND METHODS

Plant materials and chemicals

The marine diatom *Nitzschia alba* was obtained from Professor B. Volcani, Scripps Institution of Oceanography, University of California at San Diego, San Diego, CA, U.S.A. The culture stocks were maintained at 25 °C in 1% tryptone-enriched natural sea water [26].

The sources of chemicals were as follows: Sepharose CL-6B, DEAE-Sepharose CL-6B, Blue Sepharose CL-6B and SDS/polyacrylamide-gel-electrophoresis low- M_r marker kit were purchased from Pharmacia Fine Chemicals. Yeast alcohol dehydrogenase, rabbit muscle fructose-bisphosphate aldolase, bovine serum albumin, bovine erythrocyte carbonic anhydrase, bovine liver catalase, horse spleen ferritin, pig thyroglobulin, sweet-potato β -amylase, bovine fibrinogen, β - NAD^+ , β - NADH , Tris, SDS, L-malic acid, DL-malic acid, phenazine methosulphate and Nitro Blue Tetrazolium were purchased from Sigma Chemical Co. Acrylamide, bisacrylamide, Biolytes and protein assay kit were obtained from Bio-Rad Laboratories. All other reagents were from Merck and of reagent grade or better.

Purification of MDH from *N. alba*

All purification steps were carried out at 0–4 °C. Unless otherwise specified, the buffer used in the

Abbreviation used: MDH, malate dehydrogenase.

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purification procedures was 0.05 M-sodium phosphate buffer, pH 6.50, containing 1 mM-2-mercaptoethanol and 1 mM-EDTA. All centrifugation steps were performed at 10000 *g* for 10 min.

Extraction and precipitation with $(\text{NH}_4)_2\text{SO}_4$. For each purification a culture vessel containing 15 litres of enriched natural sea water was inoculated with pre-grown exponential-phase cells. The culture was grown at 29 °C with slow stirring. When the culture reached 10^6 cells/ml, the cells were harvested by centrifugation and washed twice with buffer. The sample (approx. 25 g of cells) was homogenized in 4 vol. of buffer with an ultrasonicator. The homogenate was centrifuged and the supernatant was collected as the crude extract.

Solid $(\text{NH}_4)_2\text{SO}_4$ was added to the crude extract until it reached 80 % saturation. After 50 min of continuous stirring, the precipitated protein collected by centrifugation was dissolved in minimum amount of buffer, and dialysed against the buffer for 3 h.

Ion-exchange column chromatography. The dialysed preparation was clarified by centrifugation and the protein solution was applied on a DEAE-Sepharose CL-6B column (2.6 cm $\times$ 10 cm) that had been pre-equilibrated with buffer. MDH activity was adsorbed on the column and the unbound proteins were washed off by 1 bed volume of buffer. After washing, the column was developed by a 500 ml linear gradient of 0.2–0.4 M-NaCl prepared in the same buffer. The fractions containing the MDH activity were pooled. The sample was then desalted and concentrated to a final volume of 2 ml by ultra-filtration on an Amicon YM 10 membrane.

Affinity chromatography. The enzyme solution was applied on a Blue Sepharose CL-6B column (1 cm $\times$ 10 cm) that had been pre-equilibrated with buffer. The column was washed with 15 ml of buffer and then eluted with a linear gradient of NaCl formed with two 25 ml portions of buffer containing 0.05 M- and 0.3 M-NaCl respectively. The fractions containing enzyme activity were pooled, desalted and concentrated as described above.

Gel filtration on a Sepharose CL-6B column. The sample obtained in the previous step was applied on a pre-equilibrated Sepharose CL-6B column (1.6 cm $\times$ 100 cm). Elution by upward flow was carried out at a flow rate of 25 ml/h. Purified enzyme was concentrated by ultra-filtration and used immediately or stored at –20 °C 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-sodium phosphate buffer, pH 6.5, 0.15 mM-NADH, 0.15 mM-oxaloacetate, 1 mM-2-mercaptoethanol and 1 mM-EDTA. An absorption coefficient of $6.32 \text{ mm}^{-1} \cdot \text{cm}^{-1}$ was used for NADH [27]. Assays of enzyme activities were carried out at 20–25 °C (ambient temperature).

Protein content of crude fraction was determined by the method of Lowry *et al.* [28], with bovine serum albumin as standard, whereas that of purified enzymes was assayed by the method of Bradford [29]. Protein elution profiles for the chromatographic columns were determined from the absorbance reading at 280 nm unless otherwise specified.

Determination of the M_r values 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 cm $\times$ 100 cm). The elution volumes of the standards were determined with a u.v. monitor and that of the MDH was determined by activity assays. The M_r of the native enzyme was then calculated by the method of Andrews [30]. The subunit M_r of the MDH was determined by SDS/polyacrylamide-gel electrophoresis as described by Weber & Osborn [31] in Laemmli's slab-gel system [32].

Determination of Stokes radius and sedimentation coefficient

The Stokes radius was determined by gel-filtration techniques on Sepharose CL-6B gels. The column was calibrated with the following protein standards: bovine fibrinogen (10.7 nm), thyroglobulin (8.5 nm), horse spleen ferritin (6.1 nm), rabbit muscle aldolase (4.81 nm) and bovine serum albumin (3.5 nm). The Stokes radius of the MDH was calculated by the method of Monty & Siegel [33].

The sedimentation coefficient of the enzyme was determined by linear-sucrose-density-gradient ultra-centrifugation as described by Martin & Ames [34]. The centrifugation was carried out at 150000 *g* for 36 h at 4 °C in a Beckman SW41 Ti rotor. Concentrated enzyme preparations, at a final volume of 150 μ l each, were layered on 11 ml 5–20 % (w/v) sucrose gradients made in 50 mM-sodium phosphate buffer, pH 6.5, containing 15 % (v/v) glycerol, 1 mM-2-mercaptoethanol, 1 mM-EDTA and 0.2 M-NaCl. After centrifugation, 0.2 ml fractions were collected starting from the bottom of the gradient. The migration position of the enzyme was determined by measuring its activity, and those of the standard proteins were determined by the Bradford protein assay [29]. The sedimentation coefficient of the enzyme was obtained from the standard curve constructed by plotting the sedimentation coefficients versus the migration distances (elution volumes, V_e) of the standards. Additionally, it has been reported that the empirical relationship between the sedimentation coefficient and the M_r is $S = 0.00242 M_r^{0.67}$ [35], and therefore a second standard curve was constructed by plotting the M_r values as a function of the sedimentation coefficients to estimate the M_r of the enzyme.

Electrophoresis procedures

Two-dimensional gel electrophoresis (isoelectric focusing and SDS/polyacrylamide-gel electrophoresis) was performed by using the procedure of O'Farrell [36] with 10 % separating gels on the second-dimensional runs. After electrophoresis, the gels were stained overnight with 0.05 % Coomassie Brilliant Blue R-250 in 30 % (v/v) methanol/10 % (w/v) trichloroacetic acid/10 % (v/v) acetic acid before destaining.

pH optima, pH stabilities and heat stabilities

The pH optima of the purified enzyme were determined by assaying MDH activity over the pH range 4.0–10.0 with the following buffers in the reaction mixture: 0.1 M-sodium acetate buffer, pH 4.0–5.5; 0.1 M-sodium phosphate buffer, pH 5.5–7.5; 0.1 M-Tris/HCl buffer, pH 7.5–9.0; 0.1 M-glycine/NaOH buffer, pH 9.0–10.0. The pH stabilities of the enzyme were obtained by incubating the enzyme at 25 °C in the above buffers of different pH values for 90 min and then the remaining activities were determined by the standard assays. For heat-stability studies, 1 ml sample of enzymes in 0.1 M-sodium phosphate buffer, pH 6.5, were incubated in sealed test tubes at various temperatures. Portions (10–20 μ l) were removed at 2–10 min intervals, cooled on ice for 1 min and assayed immediately. The activation energy of the purified enzyme was determined by assaying the rate of oxaloacetate reduction at temperatures ranged from 10 to 35 °C and the value was calculated by the Arrhenius plot ($\log k$ versus $1/T$, where k is the rate constant and T is the temperature in K).

K_m values

The determination of K_m values for the MDH was performed by 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, 1–6 mM; NAD^+ , 0.5–2 mM; oxaloacetate, 50–150 μ M; NADH, 75–200 μ M. K_m values were calculated from double-reciprocal plots ($1/v$ versus $1/[S]$, where $[S]$ is concentration of substrate) and secondary plots (slopes versus $1/[S]$ and intercepts versus $1/[S]$) [37].

Determination of the amino acid composition and the N-terminal sequence

For amino acid composition analysis, purified enzyme sample was washed in double-distilled water and concentrated by ultrafiltration. The sample was then hydrolysed in 6 M-HCl at 110 °C for 24 h *in vacuo*. The acid lysate was dried under vacuum in a desiccator containing conc. H_2SO_4 and KOH pellets as desiccants. The amino acid composition of the sample was then determined with an automatic amino acid analyser (Beckman model 6300) [38]. The tryptophan content of the protein was determined by the method of Spande & Witkop [39]. Subsequently, the partial specific volume of the enzyme was calculated from the amino acid composition as described by Zamyatin [40]. Automated Edman degradations [41] were performed on an Applied Biosystems gas/liquid-phase model 470A sequencer with an on-line 120A phenylthiohydantoin analyser in accordance with the manufacturer's specifications, and the data were analysed by a Waters 740 data module and an Applied Biosystems model 700 data module simultaneously. The resulting amino acid phenylthiohydantoin derivatives were analysed on an Applied Biosystems phenylthiohydantoin C_{18} reverse-phase cartridge column (2.1 mm $\times$ 22 cm).

RESULTS

Purification and homogeneity of the enzyme

MDH of *N. alba* was purified to homogeneity. The purification procedure employed was proven to be highly efficient. It routinely provided higher than 50 % recovery

Fig. 1. Purification of the MDH from *N. alba* by the consecutive chromatography steps: (a) ion-exchange, (b) affinity column and (c) gel filtration

(a) Elution profile of *N. alba* MDH and protein from a DEAE-Sepharose CL-6B column. A 165 mg sample of protein in 40 ml of buffer was applied on the column. The enzyme was bound on the gel and eluted from the column after the application of a 500 ml linear gradient of 0.2–0.4 M-NaCl. (b) Elution profile of *N. alba* MDH and protein from an affinity column of Blue Sepharose CL-6B. After the column had been washed with buffer to remove unbound proteins, the enzyme was eluted by an application of a 70 ml linear gradient of 0.05–0.3 M-NaCl. (c) Elution profile of *N. alba* MDH and protein from a gel-filtration column of Sepharose CL-6B. The approx. 1.5 ml sample containing 183 μ g of protein concentrated from the previous step was applied on the column, which was then chromatographed with a flow rate of 18 ml/h. $\circ$, MDH activity; $\bullet$, A_{280} ; arrows indicate where the salt gradients were applied.

with greater than 600-fold purification of the enzyme from the crude extract. After the consecutive steps of $(\text{NH}_4)_2\text{SO}_4$ fractionation, DEAE-Sepharose ion-exchange chromatography, Blue Sepharose affinity chromatography and gel filtration on a Sepharose CL-6B column, in a typical purification the recovery was 58.9 % with 685-fold purification, and the yield was 151 μ g of enzyme from 15 litres of cultured cells. Fig. 1 shows that the elution profiles of the enzyme from the above columns

Table 1. Purification of MDH from *N. alba*

For experimental details see the text.

Purification step	Volume (ml)	Concn. of protein (mg/ml)	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Recovery (%)	Purification (fold)
Crude extract	65	2.70*	175.4	305.5	1.74	100	1.0
(NH ₄) ₂ SO ₄ (0–80 % satn.)	41	4.03*	165.25	299.4	1.81	98	1.04
DEAE-Sepharose CL-6B	67.5	0.0189†	1.28	257.2	200.94	84.2	115.5
Blue Sepharose CL-6B	23.5	0.00779†	0.183	193.4	1056.83	63.3	607
Sepharose CL-6B	32	0.00471†	0.151	179.9	1191.39	58.9	685

* Assay by the method of Lowry *et al.* [28].

† Assay by the method of Bradford [29].

Fig. 2. Two-dimensional polyacrylamide-gel electrophoresis of purified *N. alba* MDH

Abbreviations: IEF, isoelectric focusing; SDS/PAGE, SDS/polyacrylamide-gel electrophoresis.

and a typical purification experiment is summarized in Table 1. The final enzyme preparation migrated as a single spot on two-dimensional polyacrylamide gels and no trace of contaminant was detected with Coomassie Brilliant Blue staining (Fig. 2). These data suggested that MDH in *N. alba* contained one kind of subunit with identical isoelectric point and M_r .

M_r values and other physical properties

The M_r of native MDH was determined by gel filtration on a Sepharose CL-6B column. The elution volume of the enzyme remained constant in the presence of up to 0.3 M-NaCl. At higher salt concentrations the holoenzyme was found to be partially dissociated into a low- M_r form that did not show enzymic activity. The M_r of the holoenzyme was calculated to be 322000 ± 22200 from its relative elution volume (V_e/V_0) and those of the marker proteins of known M_r values (Fig. 3a). The M_r of the subunit polypeptide chain of the enzyme was determined by SDS/polyacrylamide-gel electrophoresis [31,32]. Only one band was observed on the gels and its

M_r was calculated to be 38500 ± 500 from the standard curve constructed by plotting the distances migrated and the known M_r values of the marker proteins (Fig. 3b). As a second approach the M_r of the native enzyme was calculated to be 316000 from its sedimentation coefficient, which was determined to be 12.2 S (Figs. 4a and 4b). From consideration of the M_r of the holoenzyme, it is most likely that the MDH from *N. alba* is a homo-octamer. Some other physical properties of the enzyme are summarized in Table 2.

Catalytic properties

K_m values were determined for the enzyme for the following four substrates: L-malate, oxaloacetate, NAD⁺ and NADH at pH 8.0. The kinetic parameters were obtained by standard assays except that the substrate concentrations were varied. By the use of double-reciprocal plots and secondary plots the K_m values for L-malate, oxaloacetate, NAD⁺ and NADH were determined and are listed in Table 3. The dependence of the rates of oxaloacetate reduction and malate oxidation catalysed by the MDH on the pH of the reaction mixture was determined in the range pH 4.0–10.0. The pH optima for oxaloacetate reduction and malate oxidation were found to be 6.5 and 9.5 respectively. The stability of the enzyme between pH 4.0 and 10.0 was investigated after 60 min incubation at 20 °C and subsequent assay of oxaloacetate reduction at the optimal pH. The MDH was very stable in the range pH 5.5–8.5. Therefore the marked activity changes in this region observed in the pH-optimum experiments are probably due not 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 60 min of incubation. When the enzyme was incubated at superoptimal temperatures, i.e. 35 °C and 40 °C, its activity decreased by 55 % and 97 % respectively after a 10 min period. In the same experiments the activation energy (E_a) for oxaloacetate reduction by the enzyme in the standard assay was also determined and calculated to be 24.7 kJ/mol (5.91 kcal/mol) by Arrhenius plot. The catalytic properties of the MDH from *N. alba* are summarized in Table 3.

Amino acid composition and N-terminal sequence

Homogeneous protein was subjected to acid hydrolysis and the amino acid composition was determined. Table

Fig. 3. Determination of the M_r values of *N. alba* MDH (a) and its subunit (b)

(a) M_r determination of purified *N. alba* MDH by gel filtration on a Sepharose CL-6B column that had been pre-equilibrated in 50 mM-sodium phosphate buffer, pH 6.5, containing 100 mM-NaCl. Approx. 180 units of MDH were applied on the column, which was calibrated with the following standards: pig thyroglobulin (M_r 669000), sweet-potato β -amylase (M_r 200000), yeast alcohol dehydrogenase (M_r 150000), bovine serum albumin (M_r 67000) and bovine erythrocyte carbonic anhydrase (M_r 29000). (b) Subunit- M_r determination for *N. alba* MDH. The purified enzyme was treated with SDS and electrophoresed in SDS/12%-polyacrylamide gels alongside the M_r markers: rabbit muscle phosphorylase b (M_r 98000), bovine serum albumin (M_r 67000), ovalbumin (M_r 45000), bovine erythrocyte carbonic anhydrase (M_r 29000), soya-bean trypsin inhibitor (M_r 20100) and bovine milk α -lactalbumin (M_r 14400). The gels were stained with Coomassie Brilliant Blue and the mobilities relative to the front (R_F) were calculated. Arrows indicate the positions of the enzyme or its subunit.

Fig. 4. Determination of sedimentation coefficient (a) and M_r (b) of purified *N. alba* MDH by sucrose-density-gradient ultracentrifugation

The standards were ($s_{20,w}$, M_r): pig thyroglobulin (19.2 S, 669000), bovine liver catalase (11.3 S, 232000), yeast alcohol dehydrogenase (7.4 S, 150000) and bovine serum albumin (4.3 S, 67000). The positions of *N. alba* MDH are indicated by the arrows. The sedimentation coefficient of the MDH was determined to be 12.2 S from the calibration curve (a). Subsequently its M_r was calculated to be 337000, as indicated on the calibration curve (b), which was constructed according to the empirical equation of Young [35].

4 shows the amino acid composition of the MDH from *N. alba*. From these data an M_r of 39000 was calculated for the subunit polypeptide. This is in good agreement with the M_r value of 38500 as determined by SDS/polyacrylamide-gel electrophoresis. Table 5 gives the *N*-terminal amino acid sequence of the enzyme. Nearly identical sequences for MDH enzymes from pig heart [42], *E. coli* [43], yeast and watermelon [44] are also shown.

DISCUSSION

In the present study some molecular properties of a homogeneous preparation of the MDH from *N. alba* were investigated. Homogeneity was confirmed by several methods, including two-dimensional gel electrophoresis and *N*-terminal sequence determination. These criteria rule out the possibility of interference by contaminating macromolecules and of undefined enzyme subforms, such as are frequently observed in other MDH

Table 2. Physical properties of MDH from *N. alba*

M_r of holoenzyme	
Gel filtration	322 000 ± 22 200*
Ultracentrifugation	316 000 ± 8200*
M_r of subunit	
SDS/polyacrylamide-gel electrophoresis	38 500 ± 500*
Amino acid composition	39 000
No. of subunits/molecule	8
Sedimentation coefficient ($s_{20,w}$)	12.2 ± 0.2 S*
Partial specific volume ($\bar{v}$)†	0.750 ml/g
Stokes radius	6.0 nm
Excitation maximum‡	280 nm
Emission maximum‡	339 nm

* Mean ± S.E.M. for at least two determinations.

† The partial specific volume was calculated on the basis of the amino acid composition [41].

‡ Determined with a Hitachi F-400 fluorescence spectrophotometer.

Table 3. Catalytic properties of MDH from *N. alba*

K_m values	
L-Malate	1.33×10^{-3} M
Oxaloacetate	5.61×10^{-5} M
NAD ⁺	1.02×10^{-3} M
NADH	3.24×10^{-4} M
pH optima	
Malate → oxaloacetate	9.50
Oxaloacetate → malate	6.50
Activation energy (E_a)	24.7 kJ/mol

preparations [45,46]. MDH isoenzymes of different M_r values have been reported from a variety of species, particularly in higher plants. Habig & Racusen reported two forms of MDH from wax-bean leaves with M_r values of 69 000 and 275 000 [21]. The larger form was dissociable to active units of M_r 69 000 by high salt or urea concentrations. MDH isoenzymes with different M_r values have also been reported from corn root tips [24] and soya-bean leaves [47]. Furthermore, the glyoxysomal MDH isoenzymes of castor bean were also found to exist in 'some sort of complex or aggregate' [37]. Hitherto, however, none of the higher- M_r forms of MDH was purified to homogeneity.

The purified MDH from *N. alba* shows only one active form, and its M_r corresponds to 322 000 on gel-filtration column chromatography. The elution profile does not change in the range 0–0.3 M-NaCl or in the range pH 6.0–8.5. Therefore our data imply that the high- M_r form of MDH from *N. alba* is not the result of aggregation. As a second approach, the M_r of the enzyme was determined to be 316 000 by ultracentrifugation (Fig. 4). These data strongly suggest that the native *N. alba* MDH is a higher- M_r form that is different from the MDH enzymes purified from all other species previously reported. A high- M_r MDH has also been observed in another marine diatom, *Cylindrotheca fusiformis* (A. Y. Yueh, C.-S. Chung & Y.-K. Lai, unpublished work). It would be of interest to investigate whether high- M_r forms of MDH are common features in marine diatoms and the significance of their existence.

SDS/polyacrylamide-gel electrophoresis and two-dimensional polyacrylamide-gel electrophoresis show

Table 4. Amino acid composition of MDH from *N. alba*

Amino acid	Amino acid composition	
	(g/100 g of protein)	(no. of residues/subunit)
Aspartic acid + asparagine	5.02	17
Threonine	6.99	25
Serine	6.25	28
Glutamic acid + glutamine	8.94	27
Proline	6.22	25
Glycine	6.30	43
Alanine	8.75	48
Valine	9.91	39
Methionine	0.67	2
Isoleucine	4.93	17
Leucine	9.87	34
Tyrosine	4.60	11
Phenylalanine	4.91	13
Histidine	1.41	4
Lysine	9.20	28
Arginine	4.01	10
Half-cystine	1.57	6
Tryptophan*	0.95	2
Total	100.50	379
Subunit M_r		39 000

* Determined by the method of Spande & Witkop [39].

that the MDH from *N. alba* consists of only one kind of polypeptide with an M_r of 38 500. The data obtained from N-terminal analysis also indicate that the enzyme contains only one kind of polypeptide. From consideration of the M_r of the native enzyme we suggest that the MDH from *N. alba* is composed of eight identical subunits; it is the only octameric MDH so far reported.

Several catalytic properties of the *N. alba* MDH have been determined (Table 3). The pH optima of the MDH are comparable with the values obtained for the same enzymes from *B. subtilis* [48], *Schizosaccharomyces pombe* [7], spinach leaf microbodies [18] and bovine heart mitochondria [10]. The pH optima ranges of these enzymes is 9.0–10.0 for the oxaloacetate reduction and 7.0–8.0 for malate oxidation. No apparent difference is found among these species, except for oxaloacetate reduction, a slightly lower pH optimum (6.5) being observed with *N. alba* MDH.

The activation energies of MDH enzymes have been calculated for several species, including *H. lanuginosa*, *M. puissilus* [5], *S. pombe* [7], *Neurospora crassa* [49] and watermelon [37]. The values have been reported to be in the range 20–40 kJ/mol and the value for *N. alba* MDH was determined to be 24.7 kJ/mol.

From the amino acid composition of MDH from *N. alba* it is found that the enzyme molecule contains relatively high amounts of glycine, alanine and valine but very little methionine, cysteine and tryptophan (Table 4). Similar features have also been found in the MDH enzymes obtained from other sources [4] except for that from *Mycobacterium phlei*, in which a large amount of methionine was reported [27]. Additionally, the valine and

Table 5. *N*-Terminal amino acid sequence analysis of MDH from *N. alba*: comparison with the *N*-terminal sequences of MDH enzymes from other sources

Abbreviations: mMDH, mitochondrial MDH; gMDH, glyoxysomal MDH.

Source	<i>N</i> -Terminal amino acid sequence	Reference
<i>N. alba</i> MDH	R K V A V M G A A G G I G Q P L S L L L K L S P Q V T E L S K Y . . .	Present study
Pig heart mMDH	A K V A V L G A S G G I G Q P L S L L L K N S P L V S R L T L Y . . .	[42]
<i>E. coli</i> MDH	M K V A V L G A A G G I G Q A L A L L L K T Q L P S G S E L S L . . .	[43]
Yeast mMDH	Y K V T V L G A G G G I G Q P L S L L L K	[44]
Watermelon mMDH	A T E S V P E R K V A V L G A A G G I G Q P L A L L M K	[44]
Watermelon gMDH	(S) A K G G A P G F K V A I L G A A G G I G Q P L A M L M K	[44]

tyrosine contents of *N. alba* MDH are slightly higher than those of MDH enzymes from other species. Extensive comparison reveals that a higher degree of similarity was found between *N. alba* MDH and the enzymes from animal sources than those from bacteria and fungi [4].

Table 5 shows the *N*-terminal sequences of MDH enzymes for different species including pig heart mitochondria [42], *E. coli* [43], yeast and watermelon [44]. The data indicate that a highly conservative region exists in the *N*-terminal of the enzymes. There are at least 18 identical residues in the first 21 *N*-terminal residues among the enzymes from different sources. The three-dimensional structure of pig heart mitochondrial MDH has been determined by the X-ray-diffraction method [50]. The results indicated that the *N*-terminal sequence (residues 1–22) contains one β -conformation (residues 1–5), one α -helix (residues 11–22) and one coenzyme-binding domain (residues 6–10). The α -helix is found to reside in the interface between the subunits, and may be important for subunit–subunit interaction of the enzyme molecule. Since there is a high degree of homology of *N*-terminal sequence between pig heart mitochondrial MDH and *N. alba* MDH, it is conceivable that similar functional domains would exist in the same region of the *N. alba* MDH. On the other hand, the sequence further downstream (residues 22–32) shows a high degree of diversity among pig heart mitochondrial, *E. coli* and *N. alba* MDH enzymes. It has been shown that this particular region (residues 22–32) contains no significant functional domain [50]. In view of the relationship between structure and function of any given protein, it is conceivable that the first 22 *N*-terminal residues of MDH enzymes may be an important functional domain. Further experiments on sequence analysis and X-ray-diffraction crystallography are necessary for a comprehensive comparison.

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