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STUDIES ON THE PURIFICATION AND CHARACTERIZATION OF MALATE DEHYDROGENASE FROM *MYCOBACTERIUM PHLEI*

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Summary

Malate dehydrogenase (L-malate:NAD^+ oxidoreductase, EC 1.1.1.37) was purified from *Mycobacterium phlei* using $(NH_4)_2SO_4$ precipitation followed by chromatography on Sephadex G-200, DEAE-cellulose and DEAE Sephadex A-50. The purified preparation homogeneous on column chromatography, polyacrylamide gel electrophoresis and sodium dodecyl sulphate gel electrophoresis had a molecular weight of 86 860. The native enzyme was composed of four subunits of equal molecular weight (21 550) and was thermostable upto 50°C for 15 min. Some kinetic constants of the enzyme were determined. Tyrosine and isoleucine were identified as the N- and C-terminal amino acids respectively. The effects of various activators and inhibitors on the activity of the purified enzyme were studied. The purified enzyme exhibited maximum excitation and emission at 278 and 345 nm respectively. Amino acid composition of the enzyme was determined. Treatment of the enzyme with acid and urea resulted in dissociation of the enzyme followed by loss of catalytic activity. The dissociated enzyme could however be reconstituted by bringing the pH back to neutrality or by removal of urea from the enzyme solution.

Introduction

The metabolism of malate in the genus *Mycobacterium* has been a subject of interest to many investigators [1—8]. In general, saprophytic bacteria have a more active metabolism than avirulent *M. tuberculosis* $H_{37}Ra$. The virulent strain *M. tuberculosis* $H_{37}Rv$ is the least active thus showing an inverse relationship between metabolic activity and virulence. Studies by Seshadri [5] indicate the presence in fast growing species of mycobacteria like *M. smegmatis*, *M.*

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fortuitum and *M. smegmatis* 607, of the NADP-linked malic enzyme (EC 1.1.1.40), while the NAD^+-dependent malate dehydrogenase (L-malate:NAD^+ oxidoreductase EC 1.1.1.37) is not detectable. The reverse is the case with the slow growers like *M. tuberculosis* $H_{37}Rv$ and *M. tuberculosis* $H_{37}Ra$, BCG, *M. kansasii* and *M. scrofulaceum*. However, all species investigated have malate-vitamin K reductase (EC 1.1.99) activity which is considerably more in fast growers than in the slow growers. The activity of malic enzyme in *M. smegmatis* and *M. tuberculosis* $H_{37}Rv$ has been correlated with the NADPH levels of these organisms [9] and that of malate dehydrogenase with their lipid content [5].

Reddy et al. [6] have shown variations in the type of dehydrogenase that initiates malate oxidation in the respiratory chain of *M. tuberculosis* $H_{37}Rv$, the slow growing human pathogenic strain of tubercle bacilli and of *M. smegmatis* and *M. phlei*, the fast growing saprophytes. The malate oxidase system designated as "MAL_{NAD} pathway" [6] in which malate oxidation is mediated by the NAD^+-dependent malate dehydrogenase but not by FAD-dependent malate-vitamin K reductase is characteristic of *M. tuberculosis* $H_{37}Rv$. *M. smegmatis* has a different malate oxidase system designated as "MAL_{FAD} pathway" [6] in which malate oxidation is carried out exclusively by the FAD-dependent malate-vitamin K reductase because of the absence of the NAD^+-dependent malate dehydrogenase in this organism. The system which functions in *M. phlei* is a mixed one designated as "$MAL_{NAD+FAD}$ pathway" [6] in which both the NAD^+ and FAD-dependent dehydrogenases participate. The presence of malate dehydrogenase in slow growing species and its absence in fast-growing species is an interesting feature of the metabolism of malate in the genus *Mycobacterium*. Hence it was thought worthwhile to purify and characterize malate dehydrogenase from *M. phlei* a species in which malate is oxidized both by NAD^+ and FAD-dependent dehydrogenases.

Materials and Methods

Materials

Unless otherwise stated all chemicals were of analytical reagent grade. NAD^+, NADH, tris(hydroxymethyl)aminomethane, cytochrome *c*, lysozyme, ribonuclease, ovalbumin, bovine serum albumin, L-asparagine, L-malic acid, oxaloacetic acid, *p*-chloromercuribenzoate, iodoacetic acid, β-mercaptoethanol, urea, sodium dodecyl sulphate, Blue Dextran, Naphthol Blue Black, Coomassie Brilliant Blue-R, DEAE-cellulose, Sephadex G-200, DEAE-Sephadex A-50, dansyl chloride and riboflavin were obtained from Sigma Chemical Company, St. Louis, Mo., U.S.A. Casaminoacids were purchased from Difco Laboratories, Detroit, Michigan, U.S.A. and Aquacide from Calbiochem, Switzerland. Acrylamide, *N,N'*-methylene bisacrylamide and *N,N,N',N'*-tetramethyl ethylene diamine were products of Canalco Inc. Rockville, Md., U.S.A.

Culturing of the micro-organism

Mycobacteria are strict aerobes and grow at 37°C. Shake culture with vigorous aeration is the preferred method for growing the bacteria in bulk quantities in a relatively short time. In the present study the medium of Brodie and Gray [10] with 0.2% Tween-80 was used. *M. phlei* was grown on a rotary

shaker to the mid-log phase (24 h). Cell suspension (40%) in 0.1 M Tris · HCl buffer, pH 7.4 was sonicated for 5 min in a MSE ultrasonic disintegrator at 16 KHz. Cellular debris and unbroken cells were centrifuged off at 20 000 × *g* for 30 min. In preliminary studies (data not given here) this cell-free extract of *M. phlei* was fractionated into particulate and supernatant fractions by spinning at 144 000 × *g* for 90 min and it was observed that the activity of malate dehydrogenase was present exclusively in the supernatant fraction. However, the fractionation of cell-free extract did not yield a significant degree of purity in the enzyme activity, so the cell-free extract itself was used as the source of enzyme.

Assay of malate dehydrogenase

The assay was carried out spectrophotometrically by following the reduction of oxalacetate to malate. Because of the interference of NADH oxidase with the estimation of malate dehydrogenase, the enzyme was assayed at pH 9.5, at which NADH oxidase is completely inhibited and malate dehydrogenase shows optimal activity. The standard assay mixture in a volume of 3.0 ml contained the following in μmol:glycine/NaOH buffer pH 9.5, 150; NADH, 0.40; freshly prepared oxalacetate 0.50 and the enzyme.

Gel electrophoresis

Polyacrylamide gel electrophoresis was used to monitor the purity of the enzyme and was performed according to the procedure of Davis [11]. Aliquots containing 50—200 μg of protein were subjected to electrophoresis in 7% gel. Electrophoresis was conducted for one and a half hours in Tris/glycine buffer, pH 8.3, using a constant current of 4 mA per tube. The gels were stained with Amido Schwartz and destained mechanically with 7% (v/v) acetic acid. Sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis was carried out according to the method of Weber and Osborn [12]. Different marker proteins were denatured in phosphate buffer 0.01 M, pH 7.0, containing 2% SDS and 1% β-mercaptoethanol at 37°C for 2 h. Polyacrylamide gels for 7% gel were prepared in presence of 0.2% SDS. The proteins were subjected to electrophoresis for two hours in phosphate buffer, pH 7.0, ionic strength 0.01 M containing 0.1% SDS using a current of 4 mA per tube. The gels were stained with Coomassie Blue for 4 h and destained mechanically in a mixture of methanol (5%) and acetic acid (7.5%).

Purification of malate dehydrogenase

(a) Ammonium sulphate fractionation. The crude homogenate was saturated to 65% by addition of solid $(NH_4)_2SO_4$. The precipitates were removed 3 h later by centrifugation at 20 000 × *g* for 45 min. The supernatant fluid was adjusted to 95% saturation by adding $(NH_4)_2SO_4$. The precipitates were collected 3 h later by centrifugation at 20 000 × *g* for 45 min. The precipitates thus obtained were dissolved in a minimum amount of Tris · HCl buffer, 0.1 M, pH 7.4 and dialyzed at 4°C against the same buffer for 24 h with at least 4 changes.

(b) Sephadex G-200 column chromatography. The dialyzed sample was applied to a column (2.7 × 90.0 cm) of Sephadex G-200 which had been pre-equilibrated with Tris · HCl buffer, 0.1 M, pH 7.4. The column was developed

with the same buffer and the peak fractions showing enzyme activity (Fig. 1) were pooled and concentrated by the use of Aquacide.

(c) DEAE-cellulose chromatography. The concentrated protein sample from the above step was dialyzed against Tris · HCl buffer, 0.05 M, pH 8.5 for 6 h with at least 2 changes of buffer. This protein sample was then charged on to a DEAE-cellulose column (1.8 × 32.0 cm) pre-equilibrated with Tris · HCl buffer, 0.05 M, pH 8.5 and washed with the same buffer. A linear gradient of 0.05—0.4 M NaCl was used to elute the column. Fractions of 2.5 ml were collected at a flow rate of 20 ml per hour and monitored for protein and enzyme activity. Fractions containing maximum activity of the enzyme (Fig. 1) were pooled and concentrated to a smaller volume using Aquacide.

(d) DEAE-Sephadex A-50 chromatography. The concentrated enzyme solution from the DEAE-cellulose column was dialyzed against Tris · HCl buffer, 0.05 M, pH 9.0, for 6 h with at least 2 changes of buffer. This protein sample was then charged on to a DEAE-Sephadex A-50 column (1.7 × 30 cm) pre-equilibrated with Tris · HCl buffer 0.05 M, pH 9.0. A linear gradient of 0.05—0.4 M NaCl was used to elute the protein from the column. Fractions of 2.5 ml were collected at a flow rate of 20 ml per hour and those with maximum enzyme activity (Fig. 1) were pooled and concentrated using Aquacide.

The procedure outlined above resulted in a 180-fold purification with 18.95% yield of the enzyme. The purity of the enzyme was checked at each stage of purification using polyacrylamide gel electrophoresis and the results are presented in Fig. 2. Enzyme activity and degree of purification are given in Table I.

Measurement of molecular weight

The molecular weight of the electrophoretically homogeneous preparation was determined by chromatography on Sephadex G-200 using cytochrome *c* (11 700), ribonuclease 'A' (13 700), α-chymotrypsinogen 'A' (25 700), pepsin (34 000), ovalbumin (43 000), bovine serum albumin (69 000), alkaline phosphatase (80 000) and bovine serum albumin (dimer, 138 000) as standard proteins. SDS gel electrophoresis was also utilized to determine molecular weight using cytochrome *c* (11 700), trypsin (23 280), alpha-chymotrypsinogen 'A' (25 700), pepsinogen (40 000), ovalbumin (43 000) and bovine serum albumin (69 000) as standard proteins.

TABLE I

PURIFICATION OF *M. PHLEI* MALATE DEHYDROGENASE

Cells grown on a rotary shaker on Brodie's and Gray's medium were used. Preparation of cell free extract and purification of the enzyme were as described in the text. The purification obtained in a typical experiment is represented in the table. 1 unit of the enzyme = the amount of the enzyme that converts 1 μmol of NADH to NAD^+. Specific activity = No. of μmol of NADH converted to NAD^+ per min per mg protein.

Step	Total protein (mg)	Total activity (units)	Specific activity	Purification (-fold)
Crude	5692.20	1375.39	0.24	—
65—95% $(NH_4)_2SO_4$ fraction	528.60	870.61	1.64	6.80
Sephadex	60.00	724.50	12.07	50.00
DEAE-cellulose	16.50	480.41	29.11	120.40
DEAE-Sephadex	6.00	260.82	43.47	180.00

End group analysis

The N-terminal residue of malate dehydrogenase was identified by the method of Gray [13] using dansyl chloride and the C-terminal residue was identified by the standard procedure of Ambler [14] using carboxypeptidase A.

Amino acid analysis

Hydrolysis of malate dehydrogenase was carried out as described by Moore and Stein [15]. Samples were dialyzed exhaustively against distilled water and hydrolyzed for 24 h with constant-boiling HCl at 110°C. Aliquots of the hydrolyzed fraction were subjected to chromatographic analysis in the amino acid analyser for determining the amino acid composition. Corrections were made for loss or incomplete hydrolysis as described by Piez et al. [16]. The concentration of cysteine and methionine was determined after performic acid oxidation by the method of Moore [17]. Tryptophan was estimated by spectrophotometric [18] and colorimetric methods [19].

Reversible inactivation of the enzyme by acid and urea

Effect of acid and urea treatment on the structure of malate dehydrogenase of *M. phlei* was investigated and the method of Munkres [20] was followed for studying the inactivation and reactivation of the enzyme. Purified enzyme solution 0.03—0.06% was dialyzed at 4°C against buffer containing 10 mM sodium citrate, 50 mM NaCl, 1 mM EDTA and 1 mM β-mercaptoethanol at a final pH of 6.8, (buffer A). To the enzyme solution added 1 M citric acid to lower the pH of the buffer to 2.8. The same amount of NaCl was added to control enzyme solution and incubated at 22°C. The samples of the acidic and controlled solution were removed at intervals and diluted with 50 mM sodium phosphate buffer pH 7.0 (with 0.1% bovine serum albumin) in ice bath. For reactivation of the activity acidic solution is neutralized to pH 6.8 with 1 M NaOH and NaCl solution is added to the control solution. Both solutions are incubated at 22°C. Samples were removed at intervals and diluted as described above. Assay of the diluted enzyme was carried out immediately after the above procedure was completed.

For the denaturation of the enzyme by urea, enzyme solution (0.03—0.06%) was dialyzed against buffer A. To this enzyme in buffer added urea solution to a final concentration of 8 M and incubated at 22°C for 30 min. Sodium phosphate buffer 50 mM pH 7.0 containing 0.1% bovine serum albumin was used for the dilution of enzyme solution. Dilution and dialysis was carried out at 4°C.

Results

Homogeneity of the preparation

When the preparation from DEAE-Sephadex column was subjected to polyacrylamide gel electrophoresis at pH 8.3 (Fig. 2) only one band was observed. Identical results were obtained with polyacrylamide gel electrophoresis performed in triplicate and at varying pH (5.0, 8.3, 9.5) with the protein concentration ranging from 50 to 200 μg, suggesting homogeneity with respect to charge. Gel chromatography of the same preparation in Tris · HCl buffer 0.1

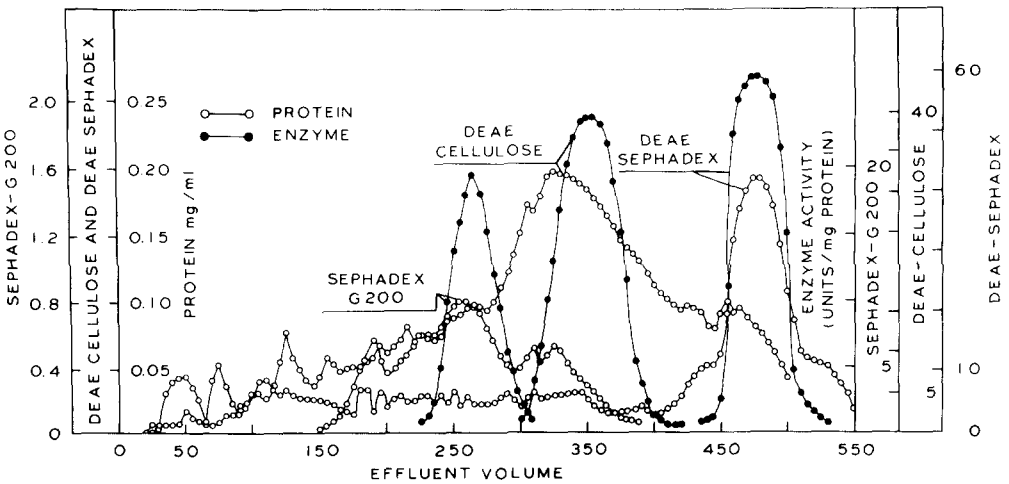

Fig. 1. Chromatographic profiles of malate dehydrogenase from *M. phlei* on Sephadex G-200, DEAE-cellulose and DEAE-Sephadex. Details of the experiments are given in the text.

M, pH 7.4 on Sephadex G-200 column gave a single symmetrical peak. Furthermore, only one band was observed during sodium dodecyl sulphate polyacrylamide gel electrophoresis establishing the size homogeneity of the preparation.

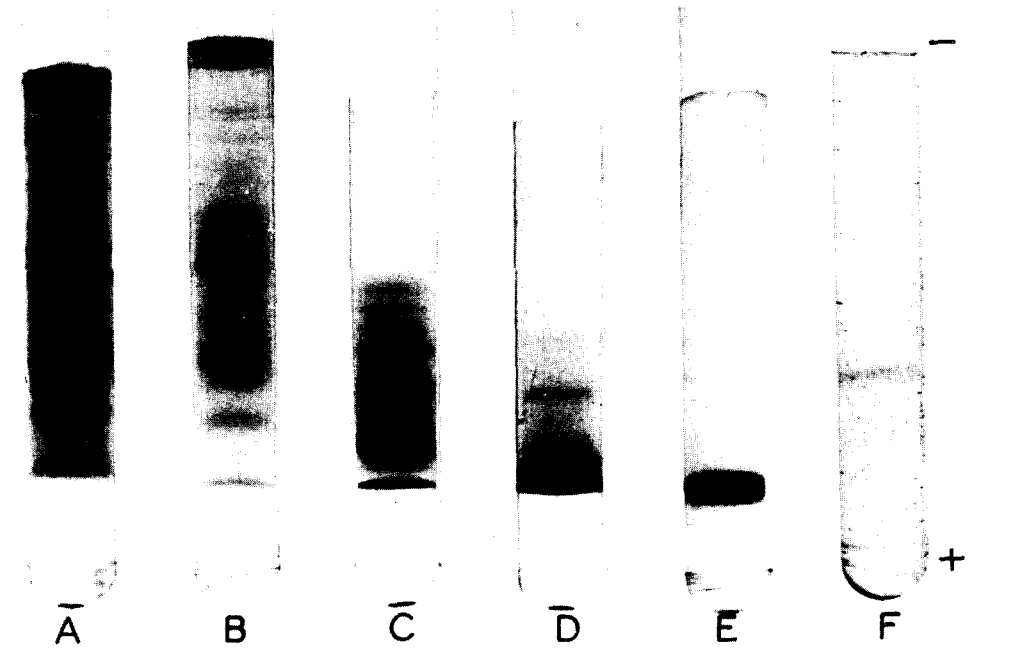

Fig. 2. Electrophoretic pattern of protein at various stages of purification. At each stage of purification, aliquots containing approx. 50—200 μg protein were subjected to polyacrylamide disc gel electrophoresis. Protein was electrophoresed for 90 min using Tris/glycine buffer. Details of electrophoresis are given in the text. A, crude extract; B, 65—95% ammonium sulphate fraction; C, Sephadex G-200 elute; D, DEAE-cellulose eluate; E, DEAE-Sephadex eluate. Sodium dodecyl sulphate polyacrylamide gel electrophoresis of malate dehydrogenase was carried out at pH 7.0 (F) as described in the text.

Stability of the enzyme

The dilute enzyme preparation obtained from the DEAE-Sephadex column was found to be stable at 20°C for at least 6 weeks. Even at room temperature (25°C) not more than 20% of the activity was lost within 2 weeks. This stability of the enzyme appeared to be independent of heterologous concentrations since even in the crude cell-free extract left at room temperature (25°C) for two weeks not more than 25% loss of activity was observed.

Molecular weight

Molecular weight of malate dehydrogenase was determined as outlined in the section Materials and Methods. From the elution profile the molecular weight of the purified enzyme was calculated to be 86 860. SDS gel electrophoresis was also utilized to determine molecular weight. The R_m value of malate dehydrogenase corresponded to a molecular weight of 21 550, which is about one fourth of the molecular weight of the enzyme calculated from gel filtration. This indicates that the malate dehydrogenase of *M. phlei* is composed of four subunits which are of equal molecular weight. Thus the molecular weight of malate dehydrogenase with SDS polyacrylamide gel electrophoresis comes out to be 86 200.

Activity of malate dehydrogenase as a function of pH

The effect of pH on the ability of malate dehydrogenase to oxidize malate to oxalacetate and to reduce oxalacetate to malate was investigated over a pH range of 4.0—10.5. Maximum conversion of malate to oxalacetate was observed at pH 9.0. However, the enzyme showed a broad pH optimum (9.2—9.6) for reduction of oxalacetate to malate.

Thermostability of the enzyme

The thermostability of malate dehydrogenase was investigated by incubating the diluted enzyme in Tris · HCl buffer for 15 min at the indicated temperature (Fig. 3). The purified enzyme preparation retained its activity completely upto 50°C but a sharp decline occurred at higher temperatures. At 55°C the enzyme had only 60% activity while at 60°C only about 25% activity was observed.

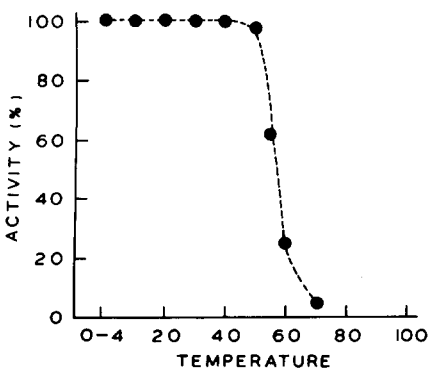

Fig. 3. Effect of temperature on the activity of malate dehydrogenase. Diluted enzyme samples were incubated for 15 min at the indicated temperature ± 0.5.

These results are comparable with those observed by Murphey et al. [21] for malate dehydrogenase preparations from *Bacillus subtilis* and *Escherichia coli*.

Michaelis constants

Michaelis constants were determined for oxalacetate, NADH, L-malate and NAD^+ by extrapolation of the straight line obtained in the double reciprocal plots in the non-inhibitory range of substrates. Higher concentrations of oxalacetate and malate resulted in inhibition of enzyme activity. There was no evidence of an anomalous increase in initial velocity with high concentrations of L-malate as reported by Davies and Kun [22] and Siegel and Englard [23]. Michaelis constants obtained for malate dehydrogenase from *M. phlei* were as follows: Oxalacetate, $4.43 \cdot 10^{-5}$ M; NADH, $2.86 \cdot 10^{-5}$ M; L-malate, $8.33 \cdot 10^{-4}$ M and NAD^+, $4.59 \cdot 10^{-4}$ M.

Yoshida [24] reported Michaelis constants for oxalacetate, NADH, L-malate and NAD^+ for the malate dehydrogenase of *B. subtilis* as $6.1 \cdot 10^{-5}$, $2.7 \cdot 10^{-5}$, $9.0 \cdot 10^{-4}$ and $1.4 \cdot 10^{-4}$ M respectively. Suppression of the reaction at higher concentrations of oxalacetate and malate has been observed by various investigators [24,25]. The values for Michaelis constants for malate dehydrogenase of *M. phlei* are comparable with those reported by Grimm and Doherty [26] for the malate dehydrogenases of bovine heart mitochondrial and supernatant fraction.

Effect of sulphydryl activators and inhibitors on the catalytic activity of malate dehydrogenase

Cysteine, 2-mercaptoethanol and reduced glutathione in a wide range of concentrations ($3.33 \cdot 10^{-5}$—$1.66 \cdot 10^{-1}$ M) were found to activate the enzyme. Different concentrations of thiol group effectors were incubated with 0.4 μg of the enzyme separately, for 5 min prior to addition of the substrate and the reaction was followed at pH 9.5. Cysteine showed the most prominent effects followed by reduced glutathione and 2-mercaptoethanol, indicating a requirement of thiol groups for the catalytic function of the enzyme. At a concentration of $3.33 \cdot 10^{-2}$ M, cysteine increased the activity of the enzyme by 50% while at the same concentration only 20% and 40% increase was observed with β-mercaptoethanol and reduced glutathione respectively.

Among inhibitors of the sulphydryl groups Hg^{2+} was found to be the most potent, followed by *p*-chloromercuribenzoate and iodoacetate. Hg^{2+} at a concentration of $3.33 \cdot 10^{-2}$ M completely inhibited the enzyme, while *p*-chloromercuribenzoate and iodoacetate at the same concentrations inhibited by 74.0% and 70.0% respectively.

Effect of metal ions on the activity of purified malate dehydrogenase

Effect of different metal ions in a wide range of concentrations ($3.33 \cdot 10^{-5}$ —$3.33 \cdot 10^{-2}$ M) on the activity of the enzyme (0.4 μg in each case) was studied at pH 9.5. None of the monovalent ions affected the activity of the enzyme significantly. However, a slight increase in activity was observed at higher concentrations ($3.33 \cdot 10^{-3}$ M) of all the three ions (Na^+, K^+, NH_4^+). The effect of some divalent cations like Zn^{2+}, Mg^{2+}, Mn^{2+}, Cu^{2+}; Ni^{2+} and Co^{2+} was investigated. All these ions acted as inhibitors of the enzyme. Mn^{2+} was observed to

be the most potent inhibitor and even at a concentration of $3.33 \cdot 10^{-4}$ M, completely blocked activity. Cu^{2+} at a concentration of $3.33 \cdot 10^{-4}$ M inhibited activity by 40% but no inhibition was observed at higher concentrations. At a concentration of $3.33 \cdot 10^{-2}$ M Zn^{2+}, Ni^{2+} and Co^{2+} inhibited the activity by 33, 57 and 61% respectively. Yoshida [24] reported inhibition of the activity of malate dehydrogenase of *B. subtilis* by Mg^{2+}, Ca^{2+} and Zn^{2+} at concentration of 10^{-2}, 10^{-2} and $2 \cdot 10^{-3}$ M respectively. Inhibition of malate dehydrogenase of human erythrocytes by various bivalent ions has also been reported by Shrago and Falcone [25].

End group analysis

The N-terminal and C-terminal residues of malate dehydrogenase were identified as tyrosine and isoleucine. The presence of single terminal residues indicates that all four polypeptide chains of the enzyme had the same terminal structure.

Optical properties

The ultraviolet absorption spectra of malate dehydrogenase from *M. phlei* showed an absorption maximum at 278 nm and a trough near 255 nm. The fluorescence spectra of malate dehydrogenase showed maximum excitation at 278 nm and an emission maximum at 345 nm indicating the presence of tryptophan.

Amino acid composition of malate dehydrogenase

The acid hydrolysate of malate dehydrogenase was analysed for individual amino acids in an automatic Technicon Amino Acid Analyser. Norleucine was used as internal standard for calculating the number of residues of individual amino acids contained in one mole of malate dehydrogenase and the values are given in Table II.

Reversible dissociation of malate dehydrogenase by acid and urea

Effect of acid and urea on the structure of malate dehydrogenase was investigated and for studying the inactivation and reactivation of the enzyme, the method of Munkres [20] was followed. Malate dehydrogenase solution when acidified to pH 2.8 at 22°C completely lost its catalytic activity and no precipitation of the protein was noted (protein concentration of the enzyme solution was 600 μg per ml). Reneutralization of the acidic solution led to reconstitution of the dissociated enzyme. The extent of reactivation was dependent on the length of exposure of the enzyme to acidic conditions. On restoration of pH to neutrality within 1 h after acidification, the enzyme regained about 95% activity. However, if reneutralization was carried out after 6 h of acidification only 60% of the activity could be restored.

Reactivation of malate dehydrogenase after urea denaturation was also carried out according to the method of Munkres [20], 8.0 M urea being used to dissociate the enzyme. In this case reconstitution of the enzyme was observed to be dependent on the manner of urea removal. When the concentration of urea was rapidly reduced by dilution, up to 17% of the original activity was recovered. However, if urea was removed slowly by dialyzing the protein solution in the cold, up to 35% of the original activity was restored.

TABLE II

AMINO ACID COMPOSITION OF MALATE DEHYDROGENASE OF *M. PHLEI*

Malate dehydrogenase was hydrolyzed with 6 M HCl for 24 h at 110°C as described in the text. Average values from duplicate analysis are given. Calculated number of amino acid residues per mole are based on molecular weight of the enzyme as 86 860. Number of residues per mole indicates the nearest integral number to the calculated number.

Amino acid	Malate dehydrogenase	
	Residues per mol	g/100 g protein
Lysine	67.29 (67)	9.84
Histidine	36.70 (37)	5.43
Arginine	14.68 (15)	2.20
Aspartic acid	72.95 (73)	10.73
Threonine	22.22 (22)	3.23
Serine	21.29 (21)	3.08
Glutamic acid	70.73 (71)	10.43
Proline	19.62 (20)	2.94
Glycine	38.80 (39)	5.73
Alanine	67.36 (67)	9.84
Half cystine	6.91 (7)	1.02
Valine	51.60 (52)	7.64
Methionine	49.83 (50)	7.35
Isoleucine	42.01 (42)	6.17
Leucine	46.00 (46)	6.76
Tyrosine	14.30 (14)	2.05
Phenyl alanine	30.58 (31)	4.55
Tryptophan	4.39 (4)	0.58

Discussion

The molecular weight of the purified malate dehydrogenase by Sephadex G-200 gel filtration was calculated to be 86 860, but when disulfide bonds and hydrophobic hydrogen bonds present in the protein were disrupted by treatment with mercaptoethanol and SDS, the R_m value of malate dehydrogenase in SDS polyacrylamide gel electrophoresis corresponded to a molecular weight of 21 550 indicating that the enzyme molecule is composed of four subunits of equal molecular weight. The molecular size of some NAD^+-linked malate dehydrogenases of several major groups of animal, plant and microbial species has been determined by gel filtration [27]. The elution volumes of malate dehydrogenases from all animal and plant sources examined were equal [27] as were those of the enzymes from several microbial species (around 60 000). However, significantly smaller elution volumes corresponding to higher molecular weights were obtained from malate dehydrogenases of certain gram positive bacteria [27] in the order *Eubacteriales* (117 000). The property of gel filtration of the enzymes in any group appears to be independent of heterologous protein concentrations since the elution volume of the enzyme activities in crude cell extracts are identical with the elution volume of the purified crystalline enzymes. To the authors knowledge, no animal, plant or microbial source has so far been reported to have both high and low molecular weight forms of malate dehydrogenase. Also, there is no report of the existence of a form with an intermediate

molecular weight. To the best of our knowledge this is the first report of malate dehydrogenase of intermediate molecular weight, 86 860. However, subunit structure of malate dehydrogenase of *M. phlei* was the same as reported for other larger forms of malate dehydrogenase from different sources. Murphey et al. [27] reported that all low molecular weight forms of malate dehydrogenase from various sources were dimers with subunits of equal molecular weight and the large molecular forms of malate dehydrogenase were tetramers with four subunits.

The pH optima of the malate dehydrogenase of *M. phlei* for oxalacetate and malate (Table III) are comparable with values obtained for malate dehydrogenase from *B. subtilis* [24] human erythrocytes [25] and beef heart mitochondria [26]. It is noteworthy that other enzymes of mycobacteria are also reported to have a high alkaline pH optima [28—31].

Amino acid analysis of malate dehydrogenase from *M. phlei* revealed the presence of 7 molecules of cysteine per mole of the enzyme and the activity of the enzyme was increased by sulphydryl activators and all inhibitors of the sulphydryl groups inhibited the catalytic activity of the malate dehydrogenase indicating the requirement of thiol groups for the catalytic function of the enzyme at the active site. Pyridine nucleotide dehydrogenases generally have free sulphydryl groups [21] and these groupings are required at the NADH binding site of NAD^+ linked dehydrogenases [21,32]. However, Murphey et al. [21] reported the absence of cysteine residues in the malate dehydrogenase of *B. subtilis* raising the question as to the requirement of thiol groups at the NADH-binding site of NAD^+-linked dehydrogenases. Yoshida [24] reported that mercuric chloride at a concentration of 10^{-4} M showed only 15% inhibition of the activity of malate dehydrogenase of *B. subtilis*. *p*-Chloromercuribenzoate at a concentration of 10^{-3} M did not show any inhibition of the same enzyme and the autors reported that since mercuric ions and *p*-chloromercuribenzoate had no significant inhibitory effect on the enzyme activity, the cysteine residue which is found in the enzyme (1 residue per subunit) may not be essential for the en-

TABLE III

MOLECULAR AND CATALYTIC PROPERTIES OF *M. PHLEI* MALATE DEHYDROGENASE

Parameter	Value
Molecular weight	86 860
Molecular weight of subunit	21 550
Number of subunits	4
pH optimum:	
with malate	9.0
with oxalacetate	9.2—9.6
Thermostable up to	50°C
K_m oxalacetate	$4.43 \cdot 10^{-5}$ M
K_m L-malate	$8.33 \cdot 10^{-4}$ M
K_m NADH	$2.86 \cdot 10^{-5}$ M
K_m NAD^+	$4.59 \cdot 10^{-4}$ M
N-terminal residue	tyrosine
C-terminal residue	isoleucine
Excitation maximum	278
Emission maximum	345

zymatic activity [33] unlike the case of malate dehydrogenase of *M. phlei* where mercuric chloride, *p*-chloromercuribenzoate and iodoacetate produce significant inhibition of the enzyme activity confirming the strong need of participation of sulphydryl groups in the catalytic function of the enzyme.

The malate dehydrogenase of *M. phlei* showed excitation and emission maxima at 278 and 345 nm respectively and amino acid analysis revealed the presence of 4 residues of tryptophan per mole of the enzyme. Proteins containing tryptophan exhibit a maximum in their emission spectra between 332 and 346 nm [34]. Thorne [35] reported that mitochondrial malate dehydrogenase was probably deficient in tryptophan, although other workers [26,36] have suggested that the enzyme from beef heart has approximately one residue of this amino acid. Murphey et al. [21] reported the absence of tryptophan in the *B. subtilis* malate dehydrogenase but the enzyme from *E. coli* was reported to contain tryptophan. It exhibited an emission maximum at 330 nm, which may indicate that the tryptophan residues in this protein are in a non-polar environment [37].

Munkres [20] put forward the mechanism of reversible denaturation of malate dehydrogenase of *Neurospora* by acid and urea involving the dissociation of tetramer to subunits with the reassociation being directed by specific non-covalent interaction intrinsic to the amino acid sequence of the polypeptide chains. Murphey et al. [21] also confirmed the dissociation of malate dehydrogenase into subunits by acid and urea. The malate dehydrogenase from *M. phlei* is composed of four subunits and the enzyme can be denatured either by acid or by treatment with high concentration of urea. This denaturation of malate dehydrogenase possibly may be due to the dissociation of the enzyme into subunits. It thus appears that the native enzyme probably may have only one active site and the catalytic monomer is the tetrameric form of the protein. Inactivation followed by dissociation of the protein by acid and urea treatment therefore reveals that for making up a single active site cooperative interaction and folding of the four polypeptide chains is essential. However, whether the active site is made up by the folding of one chain and a mere function of stabilization through interaction is rendered by other three chains or whether the direct participation of residues from all four polypeptide chains is offered in the catalytic function cannot be committed with certainty.

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