Purification and Chemical Characterization of Malate Dehydrogenase of *Bacillus subtilis*

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In an attempt to elucidate the relationships between some pyridine nucleotide-dependent dehydrogenases in *Bacillus subtilis*, such as alanine dehydrogenase, malate dehydrogenase, lactate dehydrogenase, and glutamate dehydrogenase, these enzymes are being purified and characterized with respect to their chemical, serological, and enzymic properties. In two previous papers (1, 2), the purification and properties of alanine dehydrogenase have been reported. This paper elaborates the isolation and crystallization of malate dehydrogenase (L-malate:NAD oxidoreductase, EC 1.1.1.37) as well as its molecular weight and amino acid composition.

Although malate dehydrogenase activity in microorganisms is usually higher than that of several other dehydrogenases which participate in the tricarboxylic acid cycle, it has not yet, to the author’s knowledge, been purified from bacteria, nor have its chemical, serological, and enzymic properties.

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*B. subtilis* strain 60-180 (a derivative of the Marburg strain), as well as other Marburg derivatives, produces malate dehydrogenase “constitutively”: the malate dehydrogenase activity is much stronger than that of alanine dehydrogenase and lactate dehydrogenase if the latter enzymes are not induced or derepressed. The enzyme can therefore be obtained in large quantities from bacteria grown in any growth medium.

**EXPERIMENTAL PROCEDURE**

**Organism and Culture Medium**—*B. subtilis* strain 60-180, a prototroph, was grown in minimal glucose medium (3).

**Buffers**—The buffers used were phosphate buffers (Na₂HPO₄·KH₂PO₄) and Tris buffer (Tris·HCl).

**Ion Exchange Agents and Calcium Phosphate Gel**—These were the same as described in a previous paper (1).

**Dehydrogenase Assay**—The dehydrogenase activity (malate dehydrogenase, lactate dehydrogenase, and alanine dehydrogenase) was measured in 0.05 M phosphate buffer (pH 7.7) containing 0.001 M EDTA and it was eluted with increasing concentrations of NaCl, from 0 to 0.4 M. The gradient was produced by adding phosphate buffer containing 0.5 M NaCl to a mixing chamber which contained 250 ml of phosphate buffer.

**Estimation of Protein**—At various stages of purification, protein was assayed by the method of Lowry et al. (4), with crystalline bovine serum albumin as standard, or in nucleic acid-free preparations by the absorbance at 280 nm, based on ε₁% cm = 10. Purified crystalline malate dehydrogenase has ε₁% cm = 6.6 at neutral pH (see below).

**EXPERIMENTS AND RESULTS**

**Isolation and Crystallization of Malate Dehydrogenase**

**Growth and Preparation of Cells with High Malate Dehydrogenase Content**—Strain 60-180 was grown in minimal glucose medium in 300-liter batches at 44–45°C with aeration. When an inoculum of 2 x 10⁵ cells per ml was used, the culture reached a titer of about 10⁹ cells per ml after 16 to 18 hours. The cells were harvested in a Sanyo centrifuge and dried by acetone cooled to −20°C. About 260 g of acetone-dried cells were obtained.

**Extraction and Preliminary Fractionation with Ammonium Sulfate**—These steps were the same as those used for the preparation of alanine dehydrogenase (1). From 10 g of acetone-dried cells, about 1.5 g of protein which contained 10⁴ units of malate dehydrogenase, 10⁷ units of lactate dehydrogenase, and less than 5 x 10⁶ units of alanine dehydrogenase were obtained after the fractionation with 45 to 65% saturation of ammonium sulfate (1).

All subsequent procedures, including chromatography and dialysis, were carried out in the cold.

**Calcium Phosphate Gel Column Chromatography**—The material obtained in the preceding step was repurified with saturated ammonium sulfate, and the precipitate was suspended in phosphate buffer containing 0.01 M phosphate buffer (pH 7.7) and dialyzed against the same buffer. The dialyzed material was placed on a calcium phosphate gel column (2 x 35 cm) buffered with 0.01 M phosphate buffer (pH 6.8), and was eluted with phosphate buffer (pH 6.8) at concentrations which were gradually increased from 0.01 M to 0.2 M. The gradient was produced by adding phosphate buffer containing 0.5 M NaCl.

**DEAE-Sephadex Column Chromatography**—The dialyzed solution obtained in the previous step was placed on a DEAE-Sephadex column (1 × 30 cm) buffered with 0.02 M phosphate buffer (pH 7.7) containing 0.001 M EDTA, and dialyzed against the same buffer. The yield of malate dehydrogenase was 9 x 10⁸ units in about 200 mg of protein.

The bulk of the malate dehydrogenase fraction was precipitated with ammonium sulfate at 90 to 95% saturation. The precipitate was resuspended in about 10 ml of 0.01 M phosphate buffer (pH 7.7) containing 0.001 M EDTA, and dialyzed against the same buffer. The yield of malate dehydrogenase was 9 x 10⁸ units in about 200 mg of protein.

**DEAE-Sephadex Column Chromatography**—The dialyzed solution obtained in the previous step was placed on a DEAE-Sephadex column (1 × 30 cm) buffered with 0.02 M phosphate buffer (pH 7.7) containing 0.001 M EDTA, and dialyzed against the same buffer. The yield of malate dehydrogenase was 9 x 10⁸ units in about 200 mg of protein.

A typical elution pattern is shown in Fig. 1. This procedure removed the bulk of yellow material which contaminated the malate dehydrogenase fraction of the previous step. The malate dehydrogenase fraction was precipitated with ammonium sulfate at 90 to 95% saturation, redissolved in several milliliters of 0.01 M phosphate buffer (pH 7.7), and dialyzed against the same buffer
1. Partially purified sample (malate dehydrogenase fraction from a calcium phosphate column, about 200 mg of protein) was placed on a DEAE-Sephadex column (1 × 30 cm) and eluted with increasing concentrations of NaCl. ---, absorbance; ----, malate dehydrogenase activity.

2. Partially purified malate dehydrogenase (purified through a DEAE-Sephadex column, about 40 mg of protein) was placed on an Ecteola cellulose column (1 × 30 cm) and eluted with gradually increasing NaCl from 0 to 0.3 M. A typical elution pattern is shown in Fig. 2.

3. The malate dehydrogenase fraction was collected by precipitation with ammonium sulfate at 90 to 95% saturation and redisolved in the smallest feasible amount of 0.05 M phosphate buffer (pH 7.7) containing 0.001 M EDTA. After a small amount of insoluble material had been discarded by centrifugation, saturated ammonium sulfate solution was added until the solution became slightly turbid. The crystallization was accomplished after the solution had been kept overnight in the cold. Recrystallization could be performed without a significant loss in activity. Needle-like crystals were obtained (Fig. 3). About 9 mg of crystalline enzyme (3 × 10⁶ units) were obtained from 10 g of acetone-dried cells.

All of the following experiments were carried out with malate dehydrogenase recrystallized at least three times.

Measurement of Sedimentation Coefficient and Molecular Weight

Sedimentation—Ultracentrifugation experiments were carried out in a Spinco model J centrifuge by Dr. D. J. Cummings. The protein was dissolved in 0.1 M phosphate buffer, pH 7.7, at a concentration of 0.3%. Schlieren patterns of the enzyme showed single sedimentation boundaries.

The sedimentation constant (s₂₀,₅₀) was calculated as 6.15 S.

Molecular Weight—The molecular weight of the enzyme was determined by the sedimentation equilibrium method (5) with the use of Rayleigh interference optics in a Spinco model E centrifuge.

The protein was dissolved in one of the following solvents: Solvent I, 0.05 M NaH₂PO₄-KH₂PO₄ (pH 7.7); II, 0.05 M Tris-HCl (pH 8.8) containing 5 × 10⁻⁴ M NADH and 0.1 M NaCl; III, 0.1 M Tris-HCl (pH 8.8). The initial concentration of the protein was 0.015 to 0.008%.

Molecular weight can be given by the equation (5)

\[ M = \frac{2.303(d \log \Delta'y/dr)mRT}{r_w \omega^2 (1 - \rho)} \]

where \( R \) = gas constant, \( T \) = absolute temperature, \( m \) = magnification factor, \( r_w \) = radius to middle point of fringes, \( \rho \) = partial specific volume of protein, \( \omega \) = angular velocity, and \( d \log \Delta'y/dr \) = slope of logarithm of fringe displacement versus radius.

If a given protein solution is homogeneous, plots of \( \log \Delta'y \) against radius should be linear. If protein molecules reversibly dissociate into small subunits at a range of concentration used for measurement, the plots should be curved. Higher d log \( \Delta'y/dr \) values should be obtained at larger rather than smaller \( r \) values. Because the protein solution is more concentrated near the bottom of the cell, greater fraction of the molecules should associate. By contrast, in the upper layer of the cell, the protein solution is more diluted and a greater part of the molecules should be in dissociated form.

Some examples of the relationships between the logarithm of the fringe displacement and the radius obtained from the interference patterns are shown in Fig. 4, A to C.

The interference patterns obtained in Solvents I and II showed no concentration dependence and indicated a high degree of homogeneity of the protein (Fig. 4, A and B). In both solvents the molecular weight was estimated as approximately 150,000 to 146,000, based on a partial specific volume value of 0.741 ml
Fig. 4. The logarithm of the fringe displacement as a function of the distance from the center of the rotor. The data were obtained from the interference pattern of sedimentation equilibrium by comparator reading. Magnification factor (m), 1.884; vertical axis, log (millimeters of fringe displacement X 100); axis of abscissas, millimeters of comparator coordinate. A, initial concentration of the enzyme was 0.012% in 0.05 M Tris-HCl (pH 8.8) containing 5 x 10^{-6} M NADH per g, which was calculated from the amino acid composition of the enzyme according to the method of Cohn and Edsall (6).

In contrast, the interference patterns obtained in Solvent III showed concentration dependence (Fig. 4C). The molecular weight was estimated as approximately 30,000 to 42,000 in the upper layer of the cell and as about 57,000 to 58,000 near the bottom of the cell.

As will be reported in a separate paper, malate dehydrogenase lost its enzymic activity when it was dissolved at low concentrations (less than 0.05%) in alkaline buffer (above pH 8) containing no NADH. The activity was fully recovered after precipitation with ammonium sulfate. While the enzyme was fully active in phosphate buffer at pH 7.7 (Solvent I) or in Tris buffer at pH 8.8 containing 5 x 10^{-6} M NADH (Solvent II), even at concentrations lower than 0.015%, it was almost completely (more than 99%) inactive in Tris buffer at pH 8.8 (Solvent III). Accordingly, the value of 148,000 for the molecular weight corresponds to active protein which dissociates into smaller inactive subunits upon dilution in alkaline buffer containing no NADH.

**Amino Acid Composition**

The crystalline enzyme was dialyzed against water and lyophilized. The weight samples of dried protein were hydrolyzed for 20 hours at 108-110°C in hydrochloric acid of constant boiling point in an evacuated, sealed tube. The hydrolysates were evaporated to dryness under reduced pressure over NaOH. In order to estimate the cysteine (or cystine) content, samples of oxidized protein were subjected to amino acid analysis. The procedure used for the oxidation of the protein with performic acid was essentially that described by Schram, Moore, and Bigwood (7). After oxidation, the reaction mixture was diluted with 10 volumes of cold water and the solution was immediately
### Table I

<table>
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<tr>
<th>Amino acid</th>
<th>Nonoxidized</th>
<th>Oxidized</th>
<th>Amino acid residues</th>
<th>No. of residues per subunit</th>
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<tr>
<td></td>
<td>%</td>
<td>%</td>
<td></td>
<td></td>
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<tr>
<td>Cysteic acid</td>
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<td>None</td>
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<td>Aspartic acid</td>
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<td>7.30 ± 0.10</td>
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<td>5.54 ± 0.26</td>
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<tr>
<td>Glutamic acid</td>
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<td>5.33</td>
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<td>Isoleucine</td>
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<td>Tryosine</td>
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<td>3.21 ± 0.11</td>
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<td>Tryptophan</td>
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<td>120.12</td>
<td>103.70</td>
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**Fig. 5.** Absorption diagram of crystalline malate dehydrogenase. ---, 0.384 mg of protein per ml in 0.05 M phosphate buffer, pH 7.7. ---, 0.321 mg of protein per ml in 0.1 N NaOH. Values of absorbance used for estimation of tryptophan are as follows: $E_{295} = 0.0075; E_{290} = 0.0108; E_{284.4} = 0.327; E_{280} = 0.230$. lyophilized. The samples were dissolved in a small amount of water, and the solution was again lyophilized and hydrolyzed as described above.

The amino acid analysis was performed by use of a Phoenix automatic amino acid analyzer.

Tryptophan was estimated by measuring the extinction at 294.4 and 280 m\(\mu\) in 0.1 m NaOH solution with correction for spurious absorption as described by Goodwin and Morton (8) (Fig. 5). The tryptophan content was estimated to be less than 0.00%. The tyrosine content estimated from the extinction was 7.35%, which agrees with the value obtained by the amino acid analyses, i.e. 7.52%.

The amino acid composition of malate dehydrogenase is presented in Table I.

As will be reported in a separate paper, the number of peptides spots obtained by two-dimensional paper chromatography and electrophoresis after tryptic digestion of malate dehydrogenase was 34. By using this result together with the lysine and arginine contents and the molecular weight of the protein, it can be concluded that the enzyme must consist of four identical (or nearly identical) subunits. Since the subunit would have a
molecular weight of 37,000 and contain 21 lysine and 10 arginine residues, which would give rise to 37 peptides on fingerprinting, this value is in good agreement with the observed number.

The number of amino acid residues per subunit is shown in Table I.

**DISCUSSION**

Highly purified crystalline L-malate dehydrogenase of *B. subtilis* dissolved in a buffer at pH 7.7 or pH 8.8 containing NADH exhibited homogeneous and non-concentration-dependent interference patterns in sedimentation equilibrium, and the molecular weight was estimated as approximately 150,000 to 146,000. On the other hand, when the enzyme was dissolved at low concentration (about 0.01%) in a buffer at pH 8.8 containing no NADH, the interference patterns showed concentration dependence. The molecular weight estimated in the upper layer of the cell was about 40,000, and that near the bottom of the cell was 57,000. This fact indicates that the protein dissociated into smaller (probably four) subunits which were enzymically inactive. NADH protected against the dissociation of the enzyme.

A tryptic hydrolysate of malate dehydrogenase gave 34 peptide spots in fingerprinting, in combination with the molecular weight and the frequencies of lysine and arginine; this finding indicated that *B. subtilis* malate dehydrogenase consists of four identical (or nearly identical) subunits of molecular weight 37,000.

If one assumes that the shape of the active protein is a prolate spheroid, one can estimate its major dimension from the molecular weight of 150,000, the partial specific volume of 0.741 ml per g, and the sedimentation constant of 6.15 S. The axes are then 31 μm and 3.5 μm, respectively.

In animal tissues two kinds of malate dehydrogenase seem to exist. One, apparently cytoplasmic in origin, is easily extractable and has a molecular weight of 52,000 (9). The other, presumably mitochondrial in origin, requires more drastic means of extraction and has a molecular weight of 62,000 (10). Seemingly multiple forms of malate dehydrogenase have also been observed for extracts of *B. subtilis* (11). However, on disk electrophoresis (Canalco model 12) only a single active band was observed in the crude extract as well for the crystalline enzyme.

**SUMMARY**

1. *Bacillus subtilis* malate dehydrogenase (L-malate:NAD oxidoreductase, EC 1.1.1.37) has been purified by column chromatography on calcium phosphate gel, diethylamino ethyl Sephadex, and Ecteola cellulose; it has been obtained in crystalline form.

2. The sedimentation patterns indicated a homogeneous preparation. The sedimentation constant was 6.15 S at a concentration of 0.3% of the protein.

3. The interference patterns of sedimentation equilibrium showed no concentration dependence at pH 7.7 or at pH 8.8 in the presence of reduced nicotinamide adenine dinucleotide, and the molecular weight was estimated as 148,000 ± 2,000. In the absence of reduced nicotinamide adenine dinucleotide at pH 8.8, the protein dissociated into smaller inactive subunits.

4. The enzyme is composed of four identical or nearly identical subunits of molecular weight 37,000. The subunits have the following amino acid composition: Aspo, Thr27, Serta, G1ua8, Pro15, Gly19, Ala29, CysH1, Val55, Met9, Ile23, Leu7, Tyr12, Phe5, Lys*, His9, Arg15, Trp*.

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Purification and Chemical Characterization of Malate Dehydrogenase of *Bacillus subtilis*  
Akira Yoshida


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