Malate Dehydrogenase Isolated from Extremely Halophilic Bacteria of the Dead Sea. 1. Purification and Molecular Characterization†

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ABSTRACT: The complete purification of malate dehydrogenase (EC 1.1.1.37) from extremely halophilic bacteria of the Dead Sea is described. The purification procedure includes (a) precipitation by ammonium sulfate, (b) fractionation on Sepharose 4B using a decreasing concentration gradient of ammonium sulfate, (c) gel permeation chromatography on Sephadex G-100, (d) chromatography on hydroxylapatite, and (e) affinity chromatography on 8-(6-aminohexyl)amino-NAD^+-Sepharose at 4.26 M NaCl. The absorption and fluorescence spectra of the native and denatured enzyme were measured, and the extinction coefficient at 280 nm in 4.26 M NaCl was found to be 0.803 cm^2 mg^{-1}. The amino acid composition analysis showed an excess of 10.4 mol % of acidic amino acids. The apparent specific "volume" ϕ' of the active enzyme at 4.26 M NaCl was found to be 0.680 ± 0.015 mL/g. The molecular weight of the native enzyme was found to be 84 000 ± 4000 determined in 4.26 M NaCl from equilibrium sedimentation data. The molecular weight of the subunits is 39 000 as measured by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Thus, the native enzyme is composed of two subunits.

Extremely halophilic bacteria live under conditions very unusual for life in general. It was reported (Ginzburg et al., 1970) that in a species *Halobacterium* isolated from the Dead Sea, the intracellular salt concentration exceeds 4 M KCl and 2 M NaCl. The biochemical machinery of these bacteria appears to be adapted to function at very high salt concentrations. Since most of the conventional (nonhalophilic) enzymes cannot function under such conditions, one might expect that special structural features will characterize the halophilic enzymes (Lanyi, 1974).

Investigations on structural properties of enzymes from extremely halophilic bacteria have suffered from the absence of suitable enzyme purification procedures. The fact that halophilic enzymes are rapidly inactivated at low salt concentration has reduced the applicability of certain conventional fractionation techniques which function only at low ionic strength. A previous attempt to purify halophilic malate dehydrogenase (Holmes and Halvorson, 1965) led to very low recoveries. Two new techniques which have been recently introduced enable us to undertake the purification of halophilic malate dehydrogenase (EC 1.1.1.37) to homogeneity in high yields. The first technique is based on the observation that halophilic enzymes are adsorbed to polysaccharide gels at 2.6 M $(NH_4)_2SO_4$ (Mevarech et al., 1976). The second technique is affinity chromatography of dehydrogenases on 8-(6-aminohexyl)amino-NAD^+-Sepharose (Lee and Kaplan, 1975). We found that this affinity gel binds halophilic MDH[1] at ionic strengths as high as 4.3 M NaCl and can thus be applied in the purification of this enzyme.

The availability of milligram quantities of pure halophilic malate dehydrogenase opened the way for a detailed investigation on the relation between the structure and the function of this enzyme. We report here some basic molecular properties of the purified halophilic malate dehydrogenase. In part 2 (see following paper in this issue), we present an analysis of the effect of salt concentration on the structure and catalytic function of the enzyme.

Materials and Methods

Chemicals. Oxaloacetic acid and NADH were products of Sigma. All salts employed were of analytical grade. Sepharose 4B and Sephadex G-100 (Pharmacia) and DEAE-cellulose DE 52 (Whatman) were used. Hydroxylapatite was prepared according to Levin (1962). 8-(6-Aminohexyl)amino-NAD^+-Sepharose was kindly provided by Dr. Chi-Yu Lee (Lee and Kaplan, 1975).

Solutions. The standard buffer solution contains 4.26 M NaCl, 0.01 M sodium phosphate. The pH of the solution was adjusted to 7.3 (Metrohm pH meter Model E-300B, Metrohm EA120U combined glass electrode).

Ammonium sulfate solutions: solution A, 1.95 M $(NH_4)_2SO_4$, 50 mM sodium phosphate, pH 6.6; solution B, 2.6 M $(NH_4)_2SO_4$, 50 mM sodium phosphate, pH 6.6; solution C, 1.3 M $(NH_4)_2SO_4$, 50 mM sodium phosphate, pH 6.6.

All solutions were filtered through 0.45-μm Millipore filters.

Bacterial Growth Conditions. The bacteria of the species *Halobacterium* isolated from the Dead Sea (Ginzburg et al., 1970) were a gift from Drs. M. and B. Z. Ginzburg. The growing conditions were as previously described (Mevarech et al., 1976).

Enzymatic Assay. Enzymatic activity was measured in 1 mL of the standard buffer containing 0.1 mM NADH and 0.25 mM oxaloacetate. The oxidation of NADH was followed at 340 nm with a Zeiss PMQII spectrophotometer equipped with a linear-to-log converter and a recorder. Enzymatic activity is expressed in international units.

Protein Determination. Protein content was determined by the modified biuret method (Koch and Putnam, 1971).

Spectral Analysis. Absorption spectra were measured with a Zeiss PMQII spectrophotometer. Fluorescence corrected

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[1] Abbreviations used are: NAD^+, nicotinamide adenine dinucleotide oxidized form; NADH, nicotinamide adenine dinucleotide reduced form; MDH, malate dehydrogenase; DEAE, diethylaminoethyl; $NaDodSO_4$, sodium dodecyl sulfate, GuHCl, guanidine hydrochloride; IU, international units.

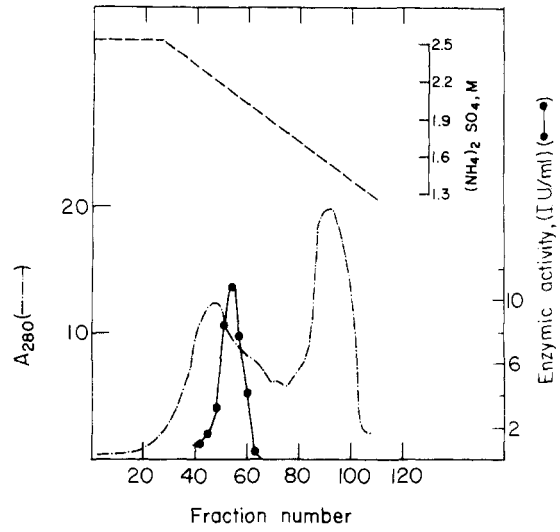

FIGURE 1: Ammonium sulfate fractionation on a column of Sepharose 4B. Three grams of protein was loaded on a column of 3.4 × 2.0 cm. The chromatogram was developed first with 400 mL of solution B (see Materials and Methods) and then with a decreasing linear gradient made up of 500 mL of solution B and 530 mL of solution C. The flow rate was 40 mL/h. Fraction volume was 10 mL.

excitation and emission spectra were obtained with a fluorescence spectrophotometer Model MPF-36 (Perkin-Elmer).

Amino Acid Analysis. Three milliliters of pure halophilic MDH (1.2 mg/mL) was dialyzed against three changes of 250 mL of 1 mM sodium phosphate buffer, pH 7. The protein solution was then divided into three 1-mL samples. Hydrolysis was performed for 24, 48 and 72 h in an evacuated sealed ampule at 110 °C, after addition of 1 mL of 12 N HCl to each sample. To correct for free ammonia contamination in the solvents, two identical blanks were run with 1 mL of the dialysate buffer. The analysis was performed with a Beckman Model 121 automatic amino acid analyzer. The half-cystine content was determined after oxidation by dimethyl sulfoxide according to the method of Spencer and Wold (1969), and analyzed as cysteic acid with the use of the amino acid analyzer.

Tryptophan was determined by the spectrophotometric method of Edelhoch (1967).

Nitrogen Determination. Nitrogen was determined according to the micro Kjeldahl method (Ehrlich-Rogozinski, 1972).

Partial Specific Volume Determination. Purified MDH (7 mg/mL) was dialyzed against standard buffer for 5 days at 25 °C. The densities of the enzyme solution and of the dialysate were measured by a Digital Precision Density Meter DMA 02D equipped with an External Measuring Cell DMA 601 (Anton Paar, K.G., Austria). The apparent specific "volume", ϕ' (Casassa and Eisenberg, 1964; Eisenberg, 1976) was calculated according to

$$\phi' = \left(1 - \frac{\rho_s - \rho_0}{c}\right)/\rho_0$$

where ρ_s is the density of the enzyme solution, ρ_0 is the density of the solvent mixture in equilibrium with enzyme solution, and c is the concentration of the enzyme expressed in grams/milliliter.

Analytical Ultracentrifuge. The molecular weight of the halophilic MDH was determined by equilibrium sedimentation using the meniscus depletion method (Yphantis, 1964). The sedimentation experiment was conducted in a Beckman Model E analytical ultracentrifuge at 24 000 rpm at 20 °C. The protein concentration gradient was traced by interference optics using a pulsed laser light source (Paul and Yphantis, 1972a,b). The analysis of data was performed with the aid of a computer program kindly provided to us by Drs. Roark and Yphantis.

Sedimentation velocity measurements were conducted at 56 000 rpm at 20 °C. The enzyme (0.78 mg/mL) was dissolved in 4.26 M NaCl, 0.01 M sodium phosphate, pH 7.3. The protein boundary movement was examined by an absorption scanner at 280 nm. $s_{20,w}$ was calculated by using the measured values of the density of the solvent, 1.162 g/mL, and its viscosity relative to water, 1.515.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis. Polyacrylamide gel electrophoresis in the presence of $NaDodSO_4$ was performed according to Weber and Osborn (1969). For the determination of the subunit molecular weight, the following protein markers have been used: bovine serum albumin, bovine glutamate dehydrogenase, ovalbumin, rabbit muscle glyceraldehyde dehydrogenase, and horse heart cytochrome *c*.

Results

Purification. All operations were performed at room temperature except where otherwise indicated.

Step 1: Preparation of Extract. Fifty-five grams of wet bacteria was suspended in 140 mL of standard buffer. The suspension was cooled to 0 °C and sonicated for 5 min in a Branson LS-75 sonicator (4 A). The extract contained 175 mL.

Step 2: Ammonium Sulfate Fractionation. The crude extract was dialyzed twice against 10 volumes of solution A, each time for 8 h, and then centrifuged in a MSE preparative ultracentrifuge, rotor 8 × 50, at 30 000 rpm for 1 h at 20 °C. The supernatant was collected (153 mL). Solid ammonium sulfate (0.15 g to 1 mL of extract) was added slowly. After addition, the solution was stirred for an extra hour and then centrifuged as above. The supernatant was collected (138 mL). The precipitate was suspended in 100 mL of solution B, stirred 1 h and centrifuged as above. The supernatant was collected (99 mL) and combined with the previous one.

Step 3a: Sepharose-Ammonium Sulfate Chromatography. The combined supernatants were loaded on a Sepharose 4B column which was previously equilibrated with solution B. The column was then washed with solution B. Then, a decreasing linear gradient of ammonium sulfate was applied. The chromatogram is given in Figure 1. The active fractions were pooled (180 mL).

Step 3b: Concentration Prior to Gel Permeation Chromatography. The pooled fractions of step 3a (in ammonium sulfate solution) were applied on a small DEAE-cellulose column (packed in a 10-mL syringe) which was preequilibrated with solution B. All the malate dehydrogenase was adsorbed to the gel. The enzyme was eluted with a solution containing 2.0 M NaCl, 50 mM sodium phosphate, pH 7.3. All the enzyme was collected in 20 mL and dialyzed against standard buffer.

Step 4: Recycling Technique in Gel-Permeation Chromatography. The concentrated enzyme from step 3b was applied to a Sephadex G-100 column which was equilibrated with standard buffer. The flow rate was maintained constant with a peristaltic pump. When MDH started to emerge from the column, the outlet tube was connected to the inlet one, and the enzyme was recycled. This procedure was repeated, resulting in three cycles of MDH through the column. The chromatogram of the third cycle is given in Figure 2. The active fractions were pooled (230 mL).

Step 5: Hydroxylapatite Chromatography. The active

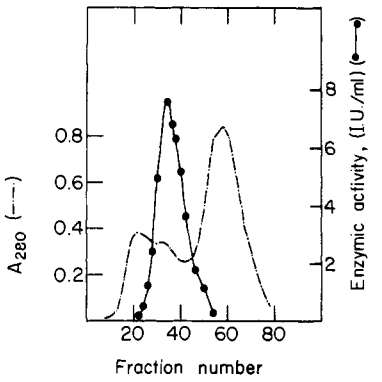

FIGURE 2: Gel filtration chromatography on Sephadex G-100. The chromatogram presents the third cycle of the recycling procedure (for details see Step 6 under Results). The column which was used was Pharmacia Type K 50/100 with two flow adaptors. Column dimensions were 5 × 86 cm. The flow rate was 30 mL/h. The fraction volume was 10 mL.

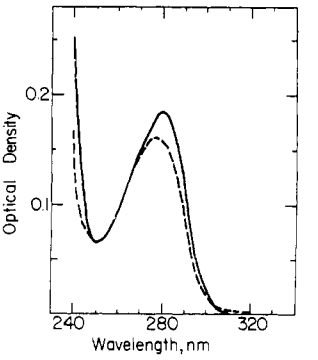

FIGURE 4: Absorption spectra. Enzyme solution (5.75 mg/mL) in standard buffer was diluted gravimetrically 1:100 into (a) standard buffer (—), (b) 10 mM sodium phosphate, pH 7.3 (- - -). Measurements were performed in cuvettes of 4-cm light path 1 min after dilution (after 1 min, no change with time was observed). The spectrum of enzyme in 6 M GuHCl is practically identical to that of the enzyme in low NaCl concentration.

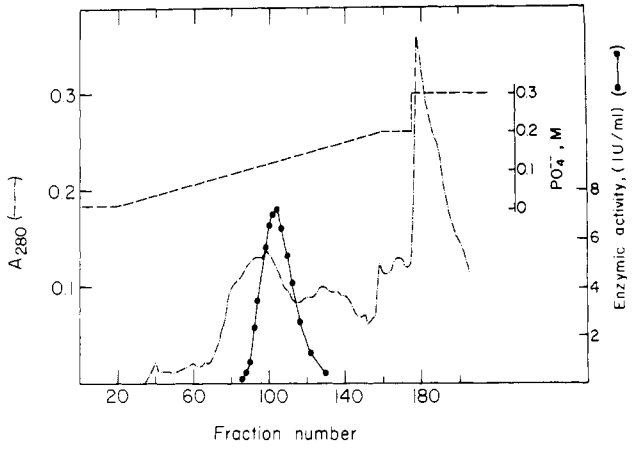

FIGURE 3: Chromatography on hydroxylapatite. Eighty-three milligrams of protein dissolved in 230 mL of standard buffer (see Materials and Methods) was loaded on a column of 1.9 × 20 cm. The chromatogram was developed by increasing the phosphate concentration gradient, made up from 500 mL of standard buffer and 500 mL of 4.26 M NaCl, 0.2 M sodium phosphate, pH 7.3. The flow rate was 20 mL/h, and the fraction volume 6.7 mL.

TABLE I: Summary of Purification of Halophilic MDH.

	Vol (mL)	Total act. (IU)	Total protein (mg)	Sp act. (IU/mg)	Yield (%)	Fold Purification
Crude extract	174	1230	5100	0.24	100	1
Ammonium sulfate fractionation	237	1270	2900	0.44		1.8
Sepharose-ammonium sulfate	180	900	470	1.93	73	8
Gel-permeation chromatography	230	700	83	8.5	57	35
Hydroxylapatite chromatography	150	627	21.6	29	51	120
Affinity chromatography	21.5	587	5.7	104	48	430

fractions from step 4 were loaded on a hydroxylapatite column which had been equilibrated with standard buffer. The column was then eluted by an increasing phosphate concentration gradient. The chromatogram is shown in Figure 3. The active fractions were pooled (150 mL).

Step 6: Affinity Chromatography. The active fractions from the hydroxylapatite chromatography step were loaded on a 10-mL column of NAD^+-Sepharose equilibrated with standard buffer. The column was washed until no absorption of the effluent was observed; then, a step of standard buffer containing 0.1 mM NADH was applied. The active fractions were pooled (21.3 mL).

The purification degrees and the recoveries of each step are summarized in Table I. The $NaDodSO_4$ gel electrophoresis pattern of a sample taken from step 6 gave a single band.

Preparation of Coenzyme-Free MDH. It is extremely difficult to release the bound NADH from the enzyme by dialysis, even when dialysis is performed against 1 mM sodium phosphate (in which the enzyme is readily inactivated). The best way for the elimination of the bound NADH was to adsorb the enzyme dissolved in the "standard buffer" on a hydroxylapatite column (5 mL bed volume) and to wash the column with 250 mL of standard buffer. The enzyme was then eluted in a rather concentrated form by a solution containing 4.3 M NaCl, 0.3 M sodium phosphate, pH 7.3.

Spectral Properties. The absorption spectra of the halophilic MDH were measured under three different conditions: (a) at 4.26 M NaCl, 10 mM sodium phosphate, pH 7.3; (b) at 42.6 mM NaCl, 10 mM sodium phosphate, pH 7.3; (c) At 6 M GuHCl, 20 mM sodium phosphate, pH 6.5. The enzyme remained active only at the higher concentration of NaCl, whereas at the low NaCl concentration and in GuHCl the enzyme was inactivated in less than 1 min. The corresponding spectra are given in Figure 4. It is seen that the enzyme inactivation leads to a considerable change in the spectrum. The maximum is shifted from 280 nm in the active enzyme to 276 nm and the specific absorption is decreased. The blue shift of the spectrum and the decrease in the absorbance are probably due to the exposure of the tryptophans from an hydrophobic environment to the water. This interpretation is supported by the observation of a major change in the fluorescence emission spectrum of the enzyme upon inactivation at 42.6 mM NaCl (see Figure 5). The maximum of the emission spectrum is shifted from 328 nm in the active enzyme to 350 nm in the inactive form and the quantum yield is considerably decreased. The emission spectra of the native enzyme are almost the same when excited at either 280 or 295 nm. It seems thus that the emission spectrum results mainly from tryptophan fluorescence. The decrease of tryptophan fluorescence upon inactivation is accompanied by the appearance of the fluorescence from tyrosines at 308 nm as a shoulder in the fluorescence band.

The extinction coefficient, $\epsilon_{280nm}^{0.1\%} = 0.803\ cm^2\ mg^{-1}$, was

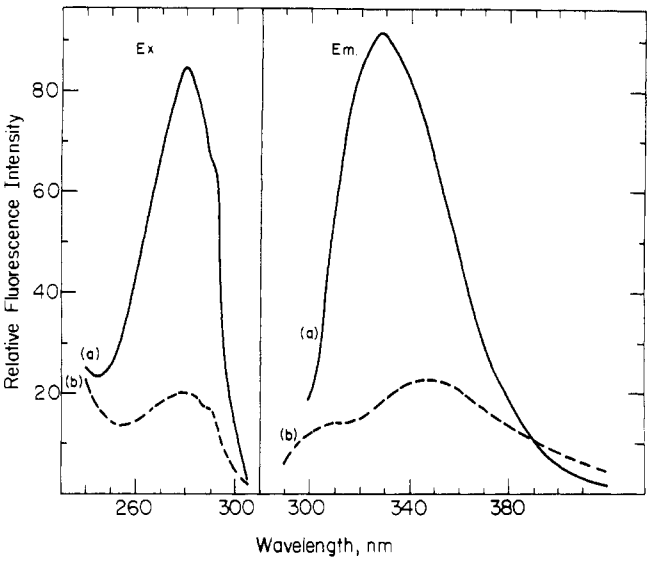

FIGURE 5: Fluorescence spectra. Enzyme solution (OD_{280} 4.45) in standard buffer was diluted 1:100 into (a) standard buffer (—), (b) 10 mM sodium phosphate, pH 7.3 (- - -). Excitation wavelength for emission spectra was 280 nm. Emission wavelength for excitation spectra was 328 nm for a and 350 nm for b.

determined from the absorbance at 280 nm and the nitrogen content. The percent of nitrogen in the enzyme was calculated from the amino acids composition.

Amino Acid Composition. Table II presents the amino acid composition obtained for the halophilic MDH. The data are compared with the previously published amino acid composition of bulk proteins of extreme halophilic bacteria (Reistad, 1970).

Partial Specific Volume and Molecular Weight. In solutions containing three or more components, interactions between the components might largely influence the value of the apparent partial specific volumes. It is thus essential to measure the density increment, $(\partial\rho/\partial c)_\mu$, of the protein at constant chemical potential, μ, of the diffusible solutes (Casassa and Eisenberg, 1964; Eisenberg, 1976). In analogy with two-component systems, an apparent specific quantity, ϕ', may be defined by

$$\left(\frac{\partial\rho}{\partial c}\right)_\mu \equiv 1 - \phi'\rho$$

Reisler and Eisenberg (1969) and Reisler et al. (1977) have shown that ϕ' in solutions of proteins (and also of nucleic acids) strongly depends on salt concentration. At the low enzyme concentrations studied the differential increment $(\partial\rho/\partial c)_\mu$ may be replaced by $\{(\rho_s - \rho_0)/c\}_\mu$. Conditions of constant potential can be obtained by effective dialysis of the protein solution against the solvent and measuring the difference between the densities of the protein solution and the solvent. The $(\partial\rho/\partial c)_\mu$ value of the native halophilic malate dehydrogenase at 4.26 M NaCl, 10 mM sodium phosphate, pH 7.3, was found to be 0.211 ± 0.011; thus, $\phi' = 0.680 \pm 0.015$. Four independent measurements were performed and the relatively large standard deviation is due to the fact that only small amounts of enzyme were available at this stage. Furthermore, the high concentration of salt renders the experiments very difficult.

From the analysis of sedimentation equilibrium of the native enzyme in 4.26 M NaCl, 10 mM sodium phosphate, pH 7.3, the molecular weight was calculated with the use of the experimental value of ϕ' at this salt concentration. The protein was found to be homogeneous and experiments at different initial protein concentration (between 0.1 and 0.8 mg/mL) resulted in the same molecular weight. We find for the native state of the halophilic malate dehydrogenase a molecular weight of 84 000 ± 4000.

TABLE II: Amino Acid Composition of Halophilic MDH.

Amino acid	Exptl Value[a]	Proposed	mol % Halophilic MDH	mol % Halophilic Bulk[b]
Lys	10.1 ± 0.1	10	2.6	2.6
His	9.1 ± 0.1	9	2.3	2.0
Arg	18.1 ± 0.3	18	4.7	5.1
Asp	54.2 ± 0.4	54	14.1	13.9
Glu	49.9 ± 0.1	50	13.1	12.9
N (as NH_2)[c]	32.4 ± 0.2	32	8.4	6.6
Thr	19.1 ± 0.2	19	5.0	6.7
Ser	21.3 ± 0.2	21	5.5	4.9
Pro	16.5 ± 0.4	17	4.4	3.9
Gly	40.9 ± 0.2	41	10.7	8.6
Ala	38.7 ± 0.1	39	10.2	10.1
Val	33.8 ± 0.4	34	8.9	8.6
Met	5.2 ± 0.3	5	1.3	1.7
Ile	18 ± 0.4	18	4.7	4.4
Leu	25.3 ± 0.3	25	6.5	7.5
Tyr	9.7 ± 0.2	10	2.6	2.5
Phe	10.1 ± 0.1	10	2.6	3.0
½-Cys[d]	0	0	0	0.8
Trp[e]	3	3	0.8	0.7

[a] Amino acid residues per 40 000 daltons. Values are calculated from the mean of three measurements. [b] Taken from Reistad (1970). [c] Measured as ammonia. Values presented are calculated after subtraction of blank ammonia (<3%) (see Materials and Methods). [d] Determined after oxidation according to Spencer and Wold (1969). [e] Determination by the method of Edelhoch (1964).

Sedimentation Coefficient. We found s_{20} at 4.26 M NaCl, 0.01 M sodium phosphate, pH 7.3, to be 2.27 S, independent of concentration (within experimental error of 1%) in the protein concentration range between 0.5 and 1.1 mg/mL. From this value, $s_{20,w}$ was calculated by standard procedures to be 5.22 S.

Sodium Dodecyl Sulfate Electrophoresis. Banding of the MDH in $NaDodSO_4$–polyacrylamide gel electrophoresis was used for the determination of the molecular weight of the subunit. The enzyme appears to be pure and the molecular weight of the subunit is 39 000. We conclude from these measurements that in the native state the enzyme is composed of two subunits.

Discussion

Difficulties in the purification of halophilic enzymes are encountered in both the crude and the fine fractionation stages. We found that precipitation by organic solvents caused large losses in activity. On the other hand, fractionation by ammonium sulfate is not discriminative enough, since the solubility properties of most of the halophilic proteins in ammonium sulfate are rather similar.

Although the degree of purification in the ammonium sulfate precipitation step is only twofold, it was used here in order to reduce the nonprotein components which might interfere in the later steps. The Sepharose–ammonium sulfate fractionation method (Mevarech et al., 1976) can handle large protein quantities, has a good separation power and is thus the method of choice for the crude stage.

Two additional chromatographic methods, gel filtration chromatography and hydroxylapatite chromatography, were not sufficient for a complete purification. Ion-exchange chromatography could not be used due to inactivation of

TABLE III: Composition of Acidic and Basic Amino Acids of MDH from Various Sources.

Source	Asx[a]	Glx[a]	Arg	Lys	$\frac{Asx + Glx}{Arg + Lys}$	Reference
Halophilic MDH	14.1	13.1	4.7	2.6	3.7	
Pig heart mMDH	9.3	10.1	3.8	11.8	1.2	Thorne (1962)
Beef heart mMDH	3.8	9	2.2	6.1	2.0	Siegel and Engelard (1962)
Beef heart sMDH	10.9	8.8	3.0	10.7	1.4	Siegel and Engelard (1962)
Pseudomonas testosteroni	10.3	8.8	2.6	5.6	2.3	You and Kaplan (1975)
Escherichia coli	7.6	10.9	2.8	6.4	2.0	Murphey et al. (1967)
Thermus aquaticus	8.5	12	6.4	5.2	1.8	Biffen and Williams (1976)
Bacillus subtilis	12.2	15.2	7.2	8.3	1.8	Yoshida (1965)

[a] Includes the amide forms; contents of the free acids Asp and Glu was usually not precisely determined.

halophilic enzymes at low ionic strengths. Only the very powerful affinity chromatography for dehydrogenases (Lee and Kaplan, 1975) led to an homogeneous preparation. The affinity method was used in a rather advanced stage of the purification where there is no competition with other dehydrogenases and thus the capacity of the column is higher. Moreover, at this stage, there is no problem of contamination with other enzymes which also have affinity for the same gel. Although our procedure is rather long, it is reproducible and results in good yields. The procedure was carried out entirely at high salt concentrations necessary to maintain the native enzyme.

After successful purification of milligram quantities of pure halophilic MDH, it became possible to investigate some physical and chemical properties of the enzyme. Special interest and also some difficulties derive from the necessity to determine these at very high salt concentrations at which the active state of the enzyme is maintained.

It is not yet possible to speak of typical properties of halophilic proteins, because only very few halophilic enzymes have been purified and sufficiently characterized (for a review, see Lanyi, 1974). Because these proteins are adapted to function at unusually high salt concentrations, one may, however, expect certain special structural properties. For a long time, it was considered that the unusually high salt tolerance is related to the large excess of acidic amino acids. It is known that the bulk soluble proteins (Reistad, 1970), the ribosomal proteins (Bayley, 1966; Visentin et al., 1972), and some enzymes, e.g., alkaline phosphatase (McParland, 1969), of halophilic bacteria have an excess of acidic amino acids. As seen in Table II, the amino acid composition of the halophilic MDH is very similar to that of the bulk proteins and the excess of negatively charged amino acids is high (10.4 mol %). If we compare the ratio of aspartic and glutamic acids over lysines and arginines in the halophilic MDH (equal to 3.7 and to 2.6 when corrected for amide content) to that of seven different nonhalophilic MDHs (Table III, column 6) we see that it is significantly larger than that of the enzymes from the nonhalophilic sources, for which the precise correction for amide content is not always available.

On the other hand, the molecular weights of the halophilic and the nonhalophilic MDHs are rather similar and they are composed of the same number (two) of subunits. The most pronounced structural difference between the halophilic MDH and the nonhalophilic ones is thus the excess of negatively charged groups in the former case.

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