Purification and Characterization of Glutamate Dehydrogenase from *Halobacterium* of the Dead Sea†

Wolfgang Leicht, Moshe M. Werber,‡ and Henryk Eisenberg*

ABSTRACT: Glutamate dehydrogenase from *Halobacterium* of the Dead Sea has been purified and found to be homogeneous by sodium dodecyl sulfate ($NaDodSO_4$) gel electrophoresis. An average molecular weight of 212 000 has been determined by two methods: (a) sedimentation equilibrium; (b) sedimentation velocity and diffusion coefficient. The subunit molecular weight, determined by $NaDodSO_4$ gel electrophoresis, is 53 500. This suggests that the enzyme is a tetramer. The oligomer has a sedimentation constant $s_{20,w}$ = 8.48 (±0.09) S (analytical centrifuge) and a diffusion coefficient $D_{20,w} = 3.34 (\pm 0.06) \times 10^{-7}$ cm^2/s (laser light scattering). Amino acid analysis shows a 20 mol % excess of acidic over basic amino acids (uncorrected for amide content) and 9 mol % (corrected). By circular dichroism, the native enzyme is found to be almost 60% helical. In the absence of salt it is irreversibly inactivated and most of the secondary structure is lost, whereas it is stable for at last several months at room temperature in the presence of multimolar concentrations of salt. Sulfate ions stabilize the enzyme at lower concentrations than chloride ions. The energy of inactivation accompanying the process of inactivation at high temperatures is of the order of 65 kcal/mol. The optimal NaCl concentration for the reaction occurs at 1.1 M, and the K_m values of the various substrates and coenzymes under these conditions are: 2-oxoglutarate, 3 mM; ammonium, 16.6 mM; NADPH, 0.024 mM; glutamate, 14.2 mM; $NADP^+$, 0.061 mM. It is believed that the halophilic glutamate dehydrogenase resembles more closely homologous enzymes from plant than from bacterial sources.

The ability of living organisms to survive in media containing high salt concentrations or to require salt for their optimal growth has been termed halophilism (Larsen, 1973). Whereas no animals are known who live in salt solutions higher in molarity than ordinary sea water, halophilism was observed in plants, in molds, and in particular in bacteria.

One of the primary questions in halophilism is the mechanism of adaptation of the organism to the extreme environment. Thus, in halophytes, which might be defined as plants which are able to complete their life cycle at salinities from at least 0.3 M up to nearly molar concentrations of sodium chloride in the culture medium (Flowers et al., 1977), the high salt concentration is restricted to the vacuoles and is much lower in the cytoplasm.

The mechanism of salt tolerance in halophilic bacteria seems to be quite different from that described for halophytes. In bacteria, salt is not excluded from the cytoplasm, but the enzymes are modified to be able to function at high salt concentration and, furthermore, they are unable to maintain their integrity in the absence of salt. (For a review see: Lanyi, 1974). This is consistent with the observation that *Halobacteria* are obligate salt-requiring organisms (Gibbons, 1974), whereas no convincing evidence is found for obligate halophytes (Flowers et al., 1977).

Glutamate dehydrogenase is a well-studied enzyme and has been isolated from a large number of sources (for recent reviews, see: Smith et al., 1975; Sund et al., 1975; Eisenberg et al., 1976). It was therefore of interest to study this particular enzyme from halophilic bacteria and to compare it to glutamate dehydrogenases from other sources.

In this work, we have isolated and characterized a glutamate dehydrogenase from an obligate halophilic bacterium, *Halobacterium* of the Dead Sea. The number of subunits of the enzyme, its structural and spectral properties, as well as its kinetic parameters and stability are analyzed in light of the features pertinent to halophilism as well as with respect to the high degree of resemblance between halophilic and procaryotic organisms (Bayley and Morton, 1978).

Experimental Section

Materials. Nucleotides, 2-oxoglutarate, and Coomassie brilliant blue were purchased from Sigma. DEAE[1]-cellulose was from Whatman (DE-52) and Sepharose from Pharmacia. All other chemicals were of analytical grade.

Solutions. Buffer A: 5 mM EDTA, 10 mM sodium phosphate, pH 6.5; buffer B: 4 M sodium chloride, 50 mM sodium phosphate, pH 6.5 (density 1.152 g/mL and relative viscosity 1.419 at 20 °C); buffer C: 50 mM sodium phosphate, pH 6.5.

For all solutions, distilled or double-distilled water was used. Buffers containing molar concentrations of salt were filtered through a Millipore filter (0.45 μm).

Purification Procedure. All steps were performed at room temperature, if not otherwise indicated. The bacteria—*Halobacterium* of the Dead Sea—were grown as previously described by Mevarech et al. (1976).

Osmolytic Breaking. The packed bacteria were suspended in distilled water (100 mL/13 g of bacteria) and stirred vigorously for 15 min. To stop the osmolytic breaking, the suspension was saturated up to 50% with ammonium sulfate by adding solid salt. For better sedimentation of nucleic acids, manganese chloride (2 mmol/13 g of bacteria) was added.

† From the Polymer Department, The Weizmann Institute of Science, Rehovot, Israel. *Received February 21, 1978.* This work was supported by a grant from the Stiftung Volkswagenwerk and a stipendium (to W.L.) from the Minerva Foundation.

‡ Present address: Biochemistry-Biophysics Program, Washington State University, Pullman, Wash. 99164.

[1] Abbreviations used are: DEAE, diethylaminoethyl; $NaDodSO_4$, sodium dodecyl sulfate; EDTA, (ethylenedinitrilo)tetraacetic acid; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol; NADP, nicotinamide adenine dinucleotide phosphate; NADPH, reduced NADP.

0006-2960/78/0417-4004$01.00/0

Ammonium Sulfate Precipitation. After stirring (1 h), the suspension was centrifuged (1400*g*; 20 min). The sediment was extracted by suspending it with distilled water (10 volumes/volume of sediment). After stirring (15 min), the suspension was saturated up to 60% with ammonium sulfate, stirred for another 60 min, and centrifuged as above. The supernatants were combined.

Extraction with Sepharose. Sepharose 6B was added to the suspension, stirred for 10 min, and centrifuged. This procedure was repeated twice with the supernatants. A total of 120 g of gel equilibrated with ammonium sulfate, at pH 6.5, was used for the extraction of proteins from 50 g of bacteria paste. The combined supernatants contained less than 5% of enzyme activity. The gel was washed by centrifugation with buffer A, saturated with 60% ammonium sulfate. The washed gel was packed in a wide column (6 × 20 cm) and eluted stepwise with buffer A saturated up to 30% with ammonium sulfate.

Fractionation on Sepharose. Enzymatically active fractions were again brought to 60% saturated ammonium sulfate and applied to a Sepharose column (4.5 × 36.5 cm) which had previously been equilibrated with buffer A saturated up to 60% ammonium sulfate. The enzyme was eluted by a decreasing concentration gradient (60 to 30%) of ammonium sulfate in the same buffer.

Chromatography on DEAE-Cellulose. The fractions containing the enzymatic activity were combined and applied to a DEAE-cellulose column (2.5 × 70 cm), which had been equilibrated in the same way as the Sepharose column. Contaminating material was washed off with 320 mL of buffer A containing 0.4 M sodium chloride. The enzyme was eluted by a linear sodium chloride concentration gradient (total volume 1 L) from 0 to 1 M in the presence of 0.4 M sodium sulfate and buffer A.

Affinity Chromatography. 8-(6-Aminohexyl)aminoadenosine-2′-phospho-5′-bisphospho(adenine)ribose-Sepharose (5′-bisphospho-2′-AMP-Sepharose) and 8-(6-aminohexyl)-amino-NADP$^+$-Sepharose (NADP$^+$-Sepharose) with about 0.7 μmol nucleotide bound/g of wet gel were prepared as previously described by Leicht (1978) and packed in 10-mL syringes. A charcoal–Celite column was also prepared according to Murdock and Koeppe (1964) in a 5-mL syringe. Care was taken to rinse all columns thoroughly with distilled water and buffer B before they were equilibrated with buffer A containing 1 M sodium sulfate. The enzymatically active fractions eluted from the DEAE-cellulose column were saturated up to a concentration of 1 M with solid sodium sulfate and passed through the 5′-bisphospho-2′-AMP–Sepharose. The column was washed with buffer A containing 1 M sodium sulfate prior to the enzyme elution by a step of 10 mL of washing buffer containing 2 mM NADPH. The effluent was passed directly over the charcoal–Celite column. The nucleotide-free enzyme was then adsorbed on the NADP$^+$–Sepharose column, from which it was desorbed by two superimposed concentration gradients consisting of a decreasing gradient of sodium sulfate from 1 to 0 M and an increasing gradient of sodium chloride up to 4 M. The enzymatic-active fractions were combined, dialyzed against buffer B, and concentrated by vacuum dialysis.

Enzymatic Assay. Enzymatic assays were performed in either 1 or 1.5 mL containing 0.1 mM NADPH, 30 mM 2-oxoglutarate, 1 M sodium chloride, 0.1 M ammonium chloride, 0.1 M Tris, pH 7.8. The oxidation of NADPH was started with enzyme solution and followed at 366 nm in an "Eppendorf" photometer equipped with a thermostated cell holder at 30 °C. One enzyme unit is defined as the conversion of 1 μmol of substrate in 1 min under the assay conditions.

Determination of Kinetic Constants. Enzymatic assays were performed at 30 °C as described above. For determination of the kinetic constants of NADPH concentrations were varied between 10 and 360 μM, for 2-oxoglutarate between 0.17 and 30 mM, and in the case of ammonium ions between 7.6 and 260 mM. The concentrations of the two other components were kept constant at values several-fold higher than their respective K_m values: NADPH, 120 μM; 2-oxoglutarate, 30 mM; NH_4^+, 100 mM. For the determination of the kinetic constants of NADP$^+$ the concentrations were varied between 1.5 and 240 μM, and in the case of glutamate between 1.7 and 50.5 mM. The concentration of the other component was kept constant at values several-fold higher than its respective K_m value: NADP,$^+$ 240 μM; glutamate, 50.5 mM. The data were analyzed by means of Michaelis–Menten plots.

Determination of Kinetics of Inactivation. The kinetics of inactivation of halophilic glutamate dehydrogenase were determined in the absence of substrate and coenzyme. To a solution containing the indicated salt concentration and 10 mM in buffer of sodium phosphate (pH 7.4), an aliquot of enzyme was added—final concentration between 3 and 7 μg/mL. At various times, aliquots were withdrawn and assayed in the standard assay system. Plots of the logarithm of the residual activity against time were linear, and the rate constant of inactivation was obtained from the slope of these plots. In the experiments at temperatures higher than 60 °C, several samples were incubated in 4.3 M sodium chloride (10 mM phosphate buffer, pH 7.3) at the desired temperature. At various times, samples were transferred to ice, thus terminating the inactivation process. The results were analyzed as above.

Extinction Coefficient. From the spectra of purified halophilic glutamate dehydrogenase, recorded on a Zeiss M4Q3 spectrophotometer, in high (buffer B) and low salt (buffer C) the conversion factor at 280 nm was calculated. This was done by diluting a stock solution of enzyme (3.27 mg/mL in buffer B) into 8 mL of buffer C which was placed in a cuvette with a path length of 4 cm. The exact dilution factor was determined by weight. The nitrogen content of samples with known absorption at 280 nm in low salt (buffer C) was determined with a micro-Kjeldahl method according to Ehrlich-Rogozinski (1972). The nitrogen content of the enzyme was calculated from its amino acid composition.

Circular Dichroism. Circular dichroic spectra were recorded on a Cary 60 spectropolarimeter equipped with an accessory for circular dichroic measurements. Quartz cells (0.1 and 1 cm) were used for the far- and near-ultraviolet spectral range. The spectra were recorded at room temperature. To calculate the molar ellipticities $[\theta]_{MRW}$, a mean residue weight (MRW) of 108 was obtained from the amino acid composition. An estimate for the α-helical fraction (f_H) of the protein structure was made according to Chen and Yang (1971).

Fluorescence Spectra. Emission fluorescence spectra were obtained on a Perkin-Elmer fluorescence spectrophotometer Model MPF-36 at 25 or 35 °C. The excitation wavelength was 295 nm, which yields only the tryptophan fluorescence. The slit widths on the excitation and emission monochromators were 7 and 8 nm, respectively. The concentration of protein was 10.8 μg/mL. The fluorescence efficiency of the sample relative to that of a tryptophan solution is expressed as R_{Trp} (Cowgill, 1968).

Protein Determination. Protein concentration during the purification procedure was determined according to Schaffner and Weissmann (1973). For calibration, purified halophilic glutamate dehydrogenase was used as standard.

Gel Electrophoresis. Sodium dodecyl sulfate–polyacrylamide slab gel electrophoresis was performed according to

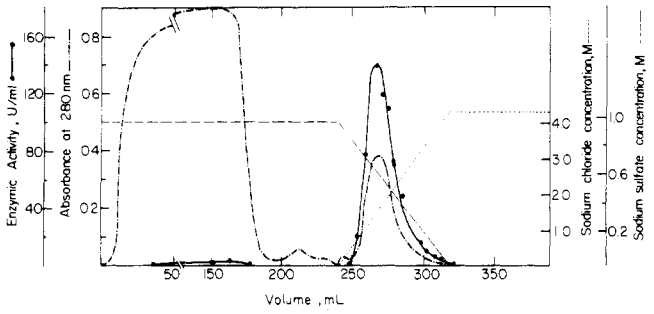

FIGURE 1: Affinity chromatography on 5′-bisphospho-2′-AMP- and $NADP^+$-Sepharose. The eluent from the DEAE-cellulose was applied to an 8-mL mononucleotide-Sepharose column and washed with 7 column volumes containing 1 M sodium sulfate. The enzyme was eluted by a 10-mL step of washing solution containing 2 mM NADPH. After removal of the nucleotide by passing through a charcoal-Celite column, the enzyme was loaded on the $NADP^+$-Sepharose column. After washing with 3 column volumes of the sodium sulfate buffer, the enzyme was eluted by a gradient (45 × 45 g) consisting of the washing buffer and a sodium chloride buffer (4.3 M, 50 mM phosphate, pH 6.5). The flow rate was 30 mL/h: (- • -) absorption at 280 nm, (● - ●) enzymatic activity, (– –) sodium sulfate concentration, (- - -) sodium chloride concentration.

Maizel (1971). Ten percent gels with an overlayered 5% spacer gel were used for analyzing protein samples. Current at a constant voltage of 150 V was maintained for 2 h.

Apparent Specific Volume. Purified glutamate dehydrogenase (3–4 mg/mL) was dialyzed several days in a rotating cylinder dialyzer at room temperature. The densities of the enzyme solution and its dialysate were measured in a Digital Precision density meter DMA 82D equipped with an external measuring cell DMA 601 (Anton Parr KG, Austria), in a room which was kept at 23 °C between 40 and 50% relative humidity. The time of exposure of the sample to the open air was minimized in order to prevent evaporation. The apparent specific "volume", ϕ', required in the evaluation of the ultracentrifugation results (Casassa and Eisenberg, 1964; Eisenberg, 1976), was determined in buffer B.

Analytical Ultracentrifuge. Sedimentation equilibrium and sedimentation velocity experiments were performed in the Beckman Model E ultracentrifuge. For sedimentation equilibrium runs, speeds of 10 000 and 12 000 rpm were used in the meniscus-depletion methods of Yphantis (1964). The protein concentration gradient was traced by interference optics and the UV scanner at 280 nm. Protein samples and their dialysates were run at 23 °C in a 12-mm cell housing with a double-sector aluminum-filled epon centerpiece with sapphire windows (initial concentration 0.5 mg/mL in buffer B). The interference plates were read on a Nikon shadowgraph Model 6C (Nippon-Kogaku, K.K., Japan) with an attached bidirectional digital counter interfaced to a Hewlett-Packard 9810-A desk calculator.

Sedimentation velocities of the enzyme were conducted at 56 000 rpm at 20 °C with samples of different concentrations in buffer B, in cells as previously described above (quartz instead of sapphire windows), in a four-hole rotor.

Light Scattering. Homogeneous protein solutions at different concentrations were carefully freed from dust by filtration through a Millipore filter (0.22 μm) and centrifugation (68 000 rpm for 1.5 h). Quasielastic laser light-scattering experiments were performed in a modified (Jolly and Eisenberg, 1976) Malvern Photon Correlator (Malvern Molecular Analyzer 4300, Precision Devices & Systems Ltd., Malvern, U.K.). A Malvern RR 95 scaling and clipping extender unit was used to compute a prescaled autocorrelation function of the scattered light fluctuation according to Koppel (1972). The accumulated result was analyzed in a Hewlett-Packard 9810-A calculator using the cumulant fit method described by Jakeman et al. (1972). A single exponential fit with a decay time τ at an angle θ of 90° gave the translational diffusion coefficient $D_{20,s}$ in the solvent used, buffer B. An argon laser (Spectra Physics Model 165) with an emission wavelength of 514.5 nm was used as a light source.

TABLE I: Purification of Glutamate Dehydrogenase from *Halobacterium* of the Dead Sea.[a]

	total protein (mg)	total enz act. (units)	sp act. (units/mg)	deg of purif-icat	recov-ery (%)
$(NH_4)_2SO_4$ ppt	11400	8270	0.72	1	100
fractionat on Sepharose by a $(NH_4)_2SO_4$ grad	1850	5860	3.2	4.4	71
chromatogr on DEAE-cellulose	28	5500	27	37.5	66
affin chromatogr	14.5	4800	330	460	58

[a] Starting from 18 L of bacterial suspension which yield 130 g of packed bacteria.

Results

Isolation and Criteria for Homogeneity. Batch adsorption and chromatography of halophilic proteins on Sepharose and DEAE-cellulose in the presence of high sulfate concentrations were based on observations previously described by Mevarech et al. (1976). In the case of halophilic glutamate dehydrogenase, either Sepharose 4B or 6B could be used with identical results.

Recently, it was shown (Leicht, 1978) that halophilic glutamate dehydrogenase could be bound biospecifically to nucleotide–Sepharose in the presence of sulfate ions. Thus, it is possible to elute the enzyme by lowering the sulfate concentration. The increasing sodium chloride concentration gradient was superimposed to prevent inactivation due to the lowering of ionic strength. The enzyme could also be eluted in a conventional way by adding coenzyme to the irrigating buffer. However, optimal results were obtained when both methods were combined in the described time-saving on-line procedure. A typical elution pattern is shown in Figure 1.

The purification procedure, which resulted in 14 mg of purified protein (better than 98% purity, as judged from sodium dodecyl sulfate gel electrophoresis) from 130 g of packed wet bacteria, is summarized in Table I. Evidence for homogeneity is also provided by the observation of a symmetrical boundary in sedimentation velocity studies.

Amino Acid Analysis. The recovery of the amino acids was found to be 98%. The high excess, 20 mol % before correction and 9 mol % after correction for amide content, of acidic amino acids over the basic amino acids is the most characteristic feature of the amino acid composition (Table II). The values given are the mean of two sets of analyses from different enzyme samples, which were hydrolyzed in 6 N HCl for 22 and 72 h.

Ultraviolet Absorption. The extinction coefficient $E_{280}^{0.1\%}$ of halophilic glutamate dehydrogenase in high salt was found to be 1.24. Calculation was performed on the basis of nitrogen analysis of samples in buffer C using the nitrogen content as determined from amino acid analysis. A correction factor for different absorbancies of the protein in high and low salt concentrations was taken into account as described under the Experimental Section.

In distinction to the behavior observed for halophilic malate dehydrogenase (Mevarech et al., 1977), the absorbance in-

TABLE II: Amino Acid Analysis of Halophilic Glutamate Dehydrogenase.

amino acid	mol %	proposed no. of res/subunit[d]
Asp	12.8	50
Glu	13.8	54
Gly	9.7	47–48
Ala	10.9	54
Val[a]	8.1	40
Leu	6.8	33–34
Ile[a]	4.8	23–24
Ser[a]	5.3	26
Thr[a]	6.9	30
Cys[c]	0.6	3
Met	0.9	4
Pro	4.0	20
Phe	3.0	15
Tyr	4.5	22
His	0.6	3
Lys	2.5	13
Arg	3.9	19
Trp[b]	1.5	7–8
NH_3	11	54

[a] Values for valine, isoleucine, serine, and threonine were corrected using factors given by Reeck (1970). [b] Tryptophan was determined according to the method of Edelhoch (1967). [c] Cysteine was determined by hydrolysis in dimethyl sulfoxide according to Spencer and Wold (1969). [d] A subunit molecular weight of 53 000 was assumed.

creases when glutamate dehydrogenase is transferred from the active state in 4 M NaCl into low salt or 6 M guanidine hydrochloride.

Sedimentation Velocity. The sedimentation velocities (in buffer B) of halophilic glutamate dehydrogenase were measured at five different enzyme concentrations between 0.21 and 0.65 mg/mL. As no dependence of the apparent sedimentation constant on the protein concentration was observed, the average value $s_{20,s} = 3.77$ (±0.04) S was found. From this we calculated $s_{20,w} = 8.48$ (±0.09) S by standard procedures. This is a purely hypothetical state, as the enzyme does not maintain its native structure in pure water.

Diffusion Coefficient. The diffusion coefficients (in buffer B) at four different protein concentrations were determined. In the relatively small range used (0.21–0.35 mg/mL), no concentration dependence was observed. The average value of $D_{20,s}$ was 2.35 (± 0.04) × 10^{-7} cm^2/s; $D_{20,w}$ was calculated by standard procedures to be 3.34 (± 0.06) × 10^{-7} cm^2/s. As referred to above for $s_{20,w}$, this is also a hypothetical state.

Molecular Weight. The apparent specific volume of halophilic glutamate dehydrogenase in 4 M sodium chloride (buffer B) was found to be $\phi' = 0.707 \pm 0.005$ mL/g.

A molecular weight of 213 000 ± 5800 was calculated from sedimentation equilibrium experiments as described. Using interference optics, the plots r^2 vs. ln c were linear and showed a random error between 0.2 and 0.3%.

Molecular weights of the holoenzyme were also determined from the Svedberg equation:

$$M_{app} = \frac{s_{20,s}RT}{D_{20,s}(1 - \phi'\rho)}$$

We derived a value of 211 200 ± 5900 which is in good agreement with the result from equilibrium sedimentation.

In polyacrylamide slab-gel electrophoresis in the presence of $NaDodSO_4$, a subunit molecular weight of 53 500 was found using as reference proteins bovine serum albumin, catalase, bovine glutamate dehydrogenase, ovalbumin, and halophilic malate dehydrogenase (Mevarech et al., 1977). Thus, the enzyme seems to be composed of four subunits of identical size.

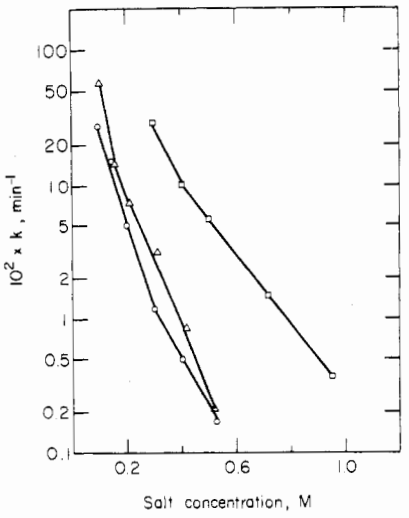

FIGURE 2: Dependence of the rates of inactivation of halophilic glutamate dehydrogenase. Rates of inactivation were determined at various salt concentrations buffered with 10 mM sodium phosphate, pH 7.3, using 3–7 μg/mL enzyme: (○ - ○) sodium chloride, 35 °C; (□ - □) sodium chloride, 45 °C; (△ - △) ammonium sulfate, 45 °C.

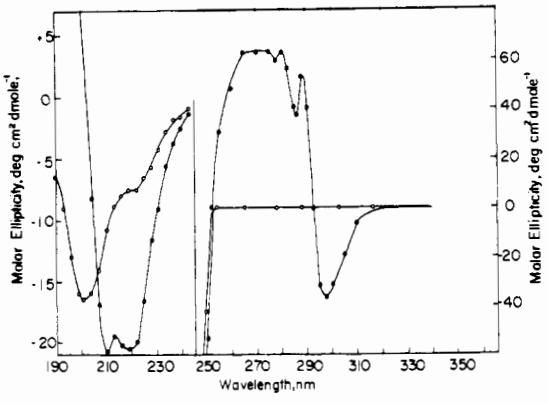

FIGURE 3: Circular dichroism of halophilic glutamate dehydrogenase in high (buffer B, ●) and low salt (buffer C, ○). The concentrations of glutamate dehydrogenase were 0.69 mg/mL at high salt and 0.46 mg/mL at low salt.

Stability Properties. The dependence of the rate of inactivation of halophilic glutamate dehydrogenase on the concentration of sodium chloride and ammonium sulfate at two temperatures is shown in Figure 2. It is evident that ammonium sulfate is efficient in stabilizing the native conformation of glutamate dehydrogenase at lower concentrations than sodium chloride. An increase of 10 °C causes the rate of inactivation in 0.5 M sodium chloride to increase 25-fold. However, at high concentrations of sodium chloride (4.3 M) the enzyme was stable at least for several hours at 65 °C. Above this temperature, the rate of inactivation at this salt concentration increased even more rapidly with temperature than at low salt concentration. Thus, in 4.3 M sodium chloride, an increase of temperature of 5 °C caused a 20-fold increase in the rate of inactivation. The energy of activation accompanying the process of inactivation in the range between 65 and 75 °C was found to be of the order of 65 kcal/mol.

Conformational Changes Accompanying Inactivation. The circular dichroic spectrum in high salt (buffer B) showed a high content (58%) of α-helical structure (Figure 3). This percentage dropped to 17% when sodium chloride was absent

TABLE III: Kinetic Parameters for the Substrates of Halophilic Glutamate Dehydrogenase at Various Salt Concentrations.

salt and concn	kinetic parameters	2-oxo-glutarate	substrate and coenzyme			
			NH_4^+	NADPH	glutamate[a]	$NADP^+$ [a]
NaCl, 1M	K_m, mM	3.0	16.6	0.024	14.2	0.061
	$10^{-6}k_{cat}$, s^{-1}	1.70	1.17	1.37	0.10	0.10
Na_2SO_4, 0.474 M	K_m, mM			0.014		
	$10^{-6}k_{cat}$, s^{-1}			1.05		
Na_2SO_4, 1.74 M	K_m, mM			0.018		
	$10^{-6}k_{cat}$, s^{-1}			0.32		

[a] Oxidative deamination direction of the reaction.

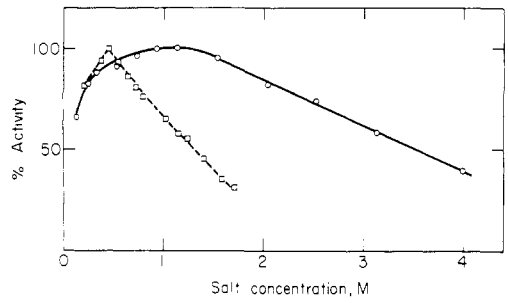

FIGURE 4: Salt concentration-activity profiles of halophilic glutamate dehydrogenase. The experiments were performed in 0.1 M Tris-HCl buffer, pH 7.8, at 30 °C. The activity was measured in the reductive amination direction: (○ - ○) sodium chloride; (□ - - - □) sodium sulfate.

(buffer C). In the near ultraviolet range, the dichroic spectral bands due to the aromatic amino acids were completely lost upon transferring the halophilic enzyme to low salt.

Tryptophan fluorescence emission spectra (not shown) of glutamate dehydrogenase were recorded at 4.0 and 0.125 M NaCl at 35 °C. A 38% quenching of the fluorescence intensity accompanied by a red shift of the maximum of 8 nm (from 335 to 343 nm) was observed upon lowering the salt concentration. The quantum yield relative to tryptophan R_{Trp} (Cowgill, 1968) decreases from 0.60 to 0.40. These two features—decrease in quantum yield and red shift—are characteristic of processes of exposure of tryptophan residues to a polar environment.

Kinetic Properties. The dependence of the activity of glutamate dehydrogenase on the concentration of sodium chloride and sodium sulfate is presented in Figure 4. The optimal salt concentration seems to be determined by the ionic strength, since the optimum activity in the case of sodium sulfate occurred at a concentration of 0.45 M, i.e., an ionic strength of 1.35, to be compared to 1.1 M in the case of sodium chloride.

The kinetic parameters (K_m and k_{cat}) of the substrate(s) and coenzymes of both reductive amination and oxidative deamination were compared (Table III) under various salt conditions. There seems to be little, if any, dependence of the K_m values upon the nature or concentration of salts. Most of the effect on the activity of changing salts or their concentration appears in the k_{cat} parameter.

The effect of several known modifiers of bovine glutamate dehydrogenase activity was tested on the activity of halophilic glutamate dehydrogenase. Neither ADP nor GTP up to concentrations of 0.95 and 1.3 mM, respectively, had any effect on the activity of halophilic glutamate dehydrogenase. On the other hand, 2′,5′-ADP was found to cause 50% inhibition at 0.75 mM. The activity with NADH as coenzyme was less than 1% of that with the native coenzyme NADPH.

Discussion

A characteristic feature of many proteins from halophilic bacteria is their instability at low concentrations (<0.5 M) of neutral salts. This instability has interfered with attempts to purify enzymes from halophilic sources, because most of the common purification techniques are performed under conditions which cause halophilic proteins and enzymes to lose their native structure and activity. However, some halophilic enzymes, such as ornithine carbamoyl transferase from *H. salinarium* (Dundas, 1972) could be protected from inactivation in solutions of low ionic strength by the addition of substrate. Other halophilic enzymes like malate dehydrogenase from *H. salinarium* (Holmes and Halvorson, 1965) and isocitrate dehydrogenase from *H. cutirubrum* (Hubbard and Miller, 1969) were purified under low salt conditions. In these cases, the purified inactive enzymes could be reactivated (with small total yields) by dialyzing them against high salt solution.

For halophilic glutamate dehydrogenase (which is composed of a larger number of subunits), no conditions could be found, under which the salt-dependent inactivation could be reversed. Therefore, the purification technique used has been based on the adsorption of the halophilic proteins to unsubstituted Sepharose and to DEAE-cellulose (Mevarech et al., 1976) at high ammonium sulfate concentrations. For the final purification, an affinity chromatography procedure was used which can be applied at high ionic strength.

The conclusion that halophilic glutamate dehydrogenase is a tetramer was based on the subunit molecular weight, which was determined by $NaDodSO_4$ gel electrophoresis. However, the empirically calibrated method can give erroneous values for molecular weights of halophilic proteins (Werber and Mevarech, 1978). The error depends on the low combined content of glycine, arginine, histidine, and lysine residues. It can be estimated following considerations of Mattice et al. (1976) that this error is less than 9% in the present case, and therefore it is not likely to alter the conclusion with respect to the stoichiometry of subunits in the halophilic glutamate dehydrogenase.

Most of the glutamate dehydrogenases isolated from animals and microorganisms (summarized by Smith et al., 1975) are hexamers with a subunit molecular weight of about 50 000. There is, however, one recent report about a NAD^+-dependent glutamate dehydrogenase from *Chlorella* which is also a tetramer of 180 000 (Meredith and Wilkins, 1977). For glutamate dehydrogenase isolated from pea roots, a molecular weight of 208 000 (Pahlich and Joy, 1970) was found, but the molecular weight of the subunit has not been determined.

The high excess of acidic amino acids over the basic ones was also found for most of the other isolated proteins from halophilic sources: ferredoxin from *H. halobium* (Kerscher et al.,

1976), malate dehydrogenase from *Halobacterium* of the Dead Sea (Mevarech et al., 1977), and ferredoxin from the same source (Werber and Mevarech, 1978). This feature is thought to be characteristic of most halophilic proteins (Lanyi, 1974).

Another typical property of halophilic proteins which we have found to apply to the case of glutamate dehydrogenase is the rather high content of α-helical structure which is partially lost upon salt-dependent inactivation. Similar effects were also observed in the case of the isocitrate dehydrogenase from *H. cutirubrum* (Wulff et al., 1972) and alanine dehydrogenase from *H. salinarium* (Keradjopoulos and Holldorf, 1977).

Additional evidence for conformational changes comes from the fluorescence experiments. Thus, the red shift of the maximum of fluorescence emission, which is observed upon lowering the salt concentration, is indicative of exposure of the tryptophan residues of the enzyme to a more polar environment than the native one. This conclusion is supported by the fact that this shift is accompanied by a drop of one-third in the quantum yield of the fluorescence, which is probably due to quenching by the polar environment of the salt solution. A similar and even larger quenching of fluorescence of an halophilic enzyme, caused by the lowering of salt concentration and inactivation, has also been observed in the case of halophilic malate dehydrogenase (Mevarech et al., 1977). It thus seems that the exposure of tryptophan, and probably other aromatic residues, might be a general property of halophilic enzymes, which is related to the loss of secondary structure and probably reflects a large conformational change that occurs upon lowering of the salt concentration.

As previously reported (Lanyi and Stevenson, 1970; Higa and Cazzulo, 1975; Eisenberg et al., 1978), it seems that salts possessing a salting-out character tend to stabilize the native conformation of halophilic enzymes more than those of the salting-in type. It has been proposed (Mevarech and Neumann, 1977) that this property along with the linearity of the plots of log k of inactivation against salt concentration is indicative of the participation of hydrophobic interactions in the stabilization of the native structure of these enzymes.

It has also been claimed that enzymes from extreme halophilic bacteria possess a thermophilic character (Keradjopoulos and Wulff, 1974; Keradjopoulos and Holldorf, 1977), but this argument has been disputed by other authors (Kim and Fitt, 1977) in view of the low thermal stability of the enzyme in the absence of substrates. We find the thermal stability of glutamate dehydrogenase in the absence of substrate or coenzyme in 4.3 M NaCl remarkable, since even at 65 °C its half-life is about 11 h. Thus, the stability in this case is higher than for mesophilic enzymes, but it is still lower than that of truly thermophilic enzymes (Zuber, 1976). The energy of activation for the denaturation process (65 kcal/mol) is indicative of the large number of interactions that are maintaining the integrity of the protein active conformation (cf. Lanyi and Stevenson, 1970).

The K_m values for the substrates and coenzymes of halophilic glutamate dehydrogenase seem to be very similar to those observed for a variety of different glutamate dehydrogenases (Smith et al., 1975). Moreover, the turnover numbers of the enzyme, assuming four active subunits and 4.25 and 25 s^{-1} for the reductive amination and oxidative deamination, respectively, are also in the range of values observed for other glutamate dehydrogenases when corrected for molecular weight (Frieden, 1959; Corman and Kaplan, 1967). Neither is the K_m value of NADPH significantly dependent on the type and nature of salt concentration, indicating that the changes in the salt conditions do not affect its binding to a large extent.

The effects of salt concentration on the activity of glutamate dehydrogenases from various sources have previously been studied. Chaplin et al. (1965) observed that chloride and sulfate ions caused activation of bovine glutamate dehydrogenase, similarly to the results of this study, except that the maximum activity in this work occurred around 0.2 M for both ions. However, the interpretation given for these effects (Corman and Kaplan, 1967) was that added salt increased the K_m value for the inhibiting NADH, therefore causing activation. Since the present study was performed using NADPH as coenzyme, for which no inhibition has been detected, the activation effects in this case might be real. It should, however, be noted that in a recent study (Blauer and Sund, 1977) it was shown that changes in the degree of binding of NADPH by bovine glutamate dehydrogenase did occur when varying the salt concentration in the range 0–1 M.

It is clear from the data that the coenzymes for the reactions of this enzyme are NADP and NADPH, whereas NADH can support less than 1% of the activity with NADPH. No effect of ADP nor GTP on the enzyme activity was found, and therefore these effectors of bovine glutamate dehydrogenase (Sund, 1976; Eisenberg et al., 1976) do not play a role in the activation of the halophilic enzyme, which may also indicate that it is not subject to a ligand-induced association behavior. The fact that the 2′,5′-ADP does inhibit this glutamate dehydrogenase is in accordance with the ability of the 5′-bisphospho-2′-AMP-Sepharose gel to bind the enzyme, which was exploited for purification purposes (see above). The larger degree of inhibition observed in the NADH-supported reaction is indicative of the weaker binding of NADH as compared to NADPH.

In conclusion, the properties which pertain to the halophilic character of proteins and which are common to most of the other halophilic enzymes studied so far are (1) the low-salt inactivation, which is accompanied by a loss of the α-helical structure; (2) the stabilization by salts of the "salting-out" type; and (3) the high content of acidic amino acids.

On the other hand, we have found that at least with respect to the stoichiometry of subunits, halophilic glutamate dehydrogenase resembles more closely analogous enzymes from plants than the procaryotic enzymes. This fits in well with other similarities observed between *Halobacterium* species and plant or other eukaryotic systems, as has also recently been pointed out by Bayley and Morton (1978). Thus, the use of light energy by the purple membrane of *H. halobium* (Oesterhelt and Stoeckenius, 1971), sequence homologies between halophilic and algal ferredoxins (Hase et al., 1977), as well as immunological cross-reactivities between the above-mentioned ferredoxins (Geiger et al., 1978) are all features which relate these organisms with plants rather than with procaryotes. It may therefore be that *Halobacteria* might have evolved in a symbiotic or parasitic relationship with halophytes, via a transfer of genetic information. It is hoped that a combination of future structural and genetic studies might shed light on this question.

Acknowledgments

The authors thank Mrs. Sarah Rogozinski for the nitrogen determinations, Mrs. J. Conu for the amino acid analysis, Dr. Martin Hirshfeld for the instruction to W.L. to operate the ultracentrifuge, Dr. Zvi Kam and Nina Borochov for introducing W.L. to the laser light scattering, Yeheskiel Haik for his skilled help in the determination of the apparent specific

volume, and Dr. M. Mevarech for his stimulating interest in this work.

References

Bayley, S. T., and Morton, R. A. (1978), *Crit. Rev. Microbiol.* (in press).
Blauer, G., and Sund, H. (1977), *Biopolymers 16,* 1053.
Casassa, E. F., and Eisenberg, H. (1964), *Adv. Protein Chem. 19,* 287.
Chaplin, A. E., Huggins, A. K., and Munday, K. A. (1965), *Comp. Biochem. Physiol. 76,* 49.
Chen, Y.-H., and Yang, J. T. (1971), *Biochem. Biophys. Res. Commun. 44,* 1285.
Cohn, E. J., and Edsall, J. T. (1943), Proteins, Amino Acids and Peptides, Reinhold, New York, N.Y., p 372.
Corman, L., and Kaplan, N. O. (1967), *J. Biol. Chem. 242,* 2840.
Cowgill, R. W. (1968), *Biochim. Biophys. Acta 168,* 43.
Dundas, I. E. D. (1972), *Eur. J. Biochem. 27,* 376.
Edelhoch, H. (1967), *Biochemistry 6,* 1948.
Ehrlich-Rogozinski, S. (1972), *Pure Appl. Chem. 29, (1),* 484.
Eisenberg, H. (1976), Biological Macromolecules and Polyelectrolytes in Solution, Oxford, Clarendon Press.
Eisenberg, H., Josephs, R., and Reisler, E. (1976) *Adv. Protein Chem. 30,* 101.
Eisenberg, H., Leicht, W., Mevarech, M., and Werber, M. M. (1978), in Proceedings, 2nd International Symposium on Pyridine Nucleotide-Dependent Dehydrogenases Konstanz, March 1977, Sund, H., Ed., Walter de Gruyter, Berlin, p 368.
Flowers, T. J., Troke, P. F., and Yeo, A. R. (1977), *Annu. Rev. Plant Physiol. 28,* 89.
Frieden, C. (1959), *J. Biol. Chem. 234,* 2891.
Geiger, B., Mevarech, M., and Werber, M. M. (1978), *Eur. J. Biochem. 84,* 449.
Gibbons, N. E. (1974), in Bergey's Manual of Determinative Bacteriology, 8th ed, Buchanan and Gibbons, N. E., Ed., Williams and Williams, Baltimore, Md., p 269.
Hase, T., Wakahayashi, S., Matsubara, H., Kerscher, L., Oesterhelt, D., Rao, K. K., and Hall, D. O. (1977), *FEBS Lett. 77,* 308.
Higa, A., and Cazzulo, J. J. (1975), *Biochem. J. 147,* 267.
Holmes, P. K., and Halvorson, H. O. (1965), *J. Bacteriol. 90,* 312.
Hubbard, J. S., and Miller, A. B. (1969), *J. Bacteriol. 99,* 167.
Jakeman, E., Oliver, G. J., Pike, E. R., and Pusey, P. N. (1972), *Proc. Phys. Soc. London, Sect. B 5,* L93.
Jolly, D., and Eisenberg, H. (1976), *Biopolymers 15,* 61.
Keradjopoulos, D., and Holldorf, A. W. (1977), *Fems. Microbiol. 1,* 179.
Keradjopoulos, D., and Wulff, K. (1974), *Can. J. Biochem. 52,* 1033.
Kerscher, L., Oesterhelt, D., Cammack, R., and Hall, D. O. (1976), *Eur. J. Biochem. 71,* 101.
Kim, E. K., and Fitt, P. S. (1977), *Biochem. J. 161,* 33.
Koppel, D. E. (1972), *J. Chem. Phys. 57,* 4874.
Lanyi, J. K. (1974), *Bacteriol. Rev. 38,* 272.
Lanyi, J. K., and Stevenson, J. (1970), *J. Biol. Chem. 245,* 4074.
Larsen, H. (1973), *Antonie von Leeuwenhoek 39,* 383.
Leicht, W. (1978), *Eur. J. Biochem. 84,* 133.
Maizel, J. (1971), *Methods Virol. 5,* 176.
Mattice, W. L., Riser, J. M., and Clark, D. S. (1976), *Biochemistry 15,* 2383.
Meredith, M. J., and Wilkins, J. (1977), *Fed. Proc., Fed. Am. Soc. Exp. Biol. 36,* 724.
Mevarech, M., and Neumann, E. (1977), *Biochemistry 16,* 3786.
Mevarech, M., Leicht, W., and Werber, M. M. (1976), *Biochemistry 15,* 2383.
Mevarech, M., Eisenberg, H., and Neumann, E. (1977), *Biochemistry 16,* 3781.
Murdock, A. L., and Koeppe, O. J. (1964), *J. Biol. Chem. 239,* 1983.
Oesterhelt, D., and Stoeckenius, W. (1971), *Nature (London), New Biol. 233,* 149.
Pahlich, E., and Joy, K. W. (1970), *Can. J. Biochem. 49,* 127.
Reeck, G. (1970), in Handbook of Biochemistry, 2nd ed, Sober, H. A. Ed., The Chemical Rubber Co., Cleveland, Ohio, p C-281.
Schaffner, W., and Weissmann, C. (1973), *Anal. Biochem. 56,* 502.
Smith, E. L., Austen, B. M., Blumenthal, K. M., and Nyc, J. F. (1975), *Enzymes, 3rd Ed. 11,* 293.
Spencer, R. L., and Wold, F. (1969), *Anal. Biochem. 32,* 185.
Sund, H., Markau, K., and Koberstein, R. (1975), *Biol. Macromol. 7c,* 225–287.
Werber, M. M., and Mevarech, M. (1978), *Arch. Biochem. Biophys. 187,* 447.
Wulff, K., Hubbard, J. S., and Miller, A. B. (1972), *Arch. Biochem. Biophys. 148,* 318.
Yphantis, D. A. (1964), *Biochemistry 3,* 297.
Zuber, H., Ed. (1976), Enzymes and Proteins from Thermophilic Organisms, Birkauser Verlag, Basel.