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# Malate Dehydrogenase from the Extreme Halophilic Archaebacterium *Halobacterium marismortui*. Reconstitution of the Enzyme after Denaturation and Dissociation in Various Denaturants†

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*Received December 13, 1988; Revised Manuscript Received March 6, 1989*

ABSTRACT: Malate dehydrogenase from the extreme halophilic archaebacterium *Halobacterium marismortui* is a homodimer of 84 000 molecular weight. As taken from ultracentrifugal analysis, it does not show concentration-dependent dissociation down to enzyme concentrations as low as 10 ng/mL. There is no change in specific activity at concentrations between 1 and 500 ng/mL. The enzyme undergoes pH-dependent and ionic strength dependent dissociation, denaturation, and deactivation below pH 4.8 ± 0.3 and $I \leq 1.8$ M, respectively. In the range of the salt-dependent equilibrium transition (e.g., at $I \approx 0.8$ M), "structured monomers" are formed in a slow reaction. Reconstitution of this species is multiphasic with fast reassembly of "structured monomers" and subsequent folding/association to form the active dimer. At low salt, as well as at high concentrations of guanidine hydrochloride at high salt, the enzyme is fully unfolded. Therefore, reconstitution comprises both unimolecular folding and subsequent bimolecular association. In the case of the guanidine-dependent unfolding transition, salt effects and chaotropic effects are superimposed such that at low salt concentrations increased guanidine concentration leads to an apparent activation of the enzyme. Deactivation is determined by the total ionic strength: keeping the salt concentration over the whole transition range beyond 4 M, the equilibrium transition occurs at 2.3 M Gdn·HCl (2.4 M NaCl). Reactivation after guanidine denaturation follows the same mechanism as described for the reconstitution after denaturation at low salt concentration. Low urea concentrations (≤2 M), as well as short incubation at low salt, lead to an increase in the yield of reconstitution due to incomplete dissociation under unfolding conditions.

*Halobacterium marismortui* is an obligate halophile which requires 10–20% NaCl for optimal growth; incubation at low salt concentrations causes lysis (Larsen, 1967; Kushner, 1968; Brown, 1964). As was first reported by Ginzburg et al. (1970), *Halobacterium* from the Dead Sea possesses an intracellular salt concentration of about 4 M KCl and 2 M NaCl. The cellular inventory of the organism is adapted to these conditions. As a consequence, halophilic cytoplasmic proteins have been reported to be denatured below 1 M NaCl (Lanyi, 1974).

Native halophilic malate dehydrogenase (h-MDH)[1] from *Halobacterium marismortui* was isolated first by Mevarech et al. (1976, 1977). To avoid denaturation of the enzyme, high salt conditions were maintained during all stages of purification. As shown by ultracentrifugal analysis and SDS gel electrophoresis, h-MDH is a dimer with a molecular weight of 84 000. Its amino acid composition shows a characteristic excess of acidic over basic amino acids causing anomalous hydration (0.87 g of $H_2O$/g of protein) and salt binding (0.35 g/g of protein) (Eisenberg et al., 1977; Reich et al., 1982; Zaccai et al., 1986). At low salt concentration (<1 M NaCl), dissociation of the dimer, accompanied by deactivation and denaturation, was observed.

In the present study, the reconstitution behavior of h-MDH from *Halobacterium marismortui* after previous unfolding of the enzyme in various denaturants is described. The effect of low salt concentrations is compared with acid and guanidine denaturation. Under all conditions, denaturation is shown to

† This work was supported by grants from the Deutsche Forschungsgemeinschaft (SFB 4 and 43) and the Fonds der Chemischen Industrie.
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[1] Abbreviations: BSA, bovine serum albumin; CD, circular dichroism; Gdn·HCl, (GdmCl in figures), guanidine hydrochloride; h-MDH and m-MDH, halophilic and mitochondrial malate dehydrogenase, respectively; OA, oxalacetic acid.

0006-2960/89/0428-4979$01.50/0 

be partially reversible; under optimum conditions, the yield of reconstituted enzyme reaches 95%. The product of reconstitution is found to be indistinguishable from the native enzyme; the kinetic mechanism comprises rate-determining consecutive folding and association steps.

## Materials and Methods

### *Materials*

*Chemicals.* The isolation and purification of h-MDH were performed as described by Pundak et al. (1981). *Halobacterium marismortui* was a gift from Professor H. Eisenberg, Rehovot. Large-scale preparations made use of supernatants from ribosome preparations obtained from Professor H. G. Wittmann, Berlin. Stock solutions of the enzyme (≈1.3 mg/mL) in 4 M NaCl/50 mM sodium phosphate buffer, pH 7.5 (plus 0.3 mM NADH), were stored at 4 °C; specific activity under standard conditions of the enzymatic assay was 645 IU/mg.

NADH (grade II) and oxalacetic acid were purchased from Boehringer (Mannheim), BSA was from Serva (Heidelberg), and Gdn·HCl (ultrapure) was from Schwarz/Mann (Orangeburg, NY). Salts for buffers were analytical-grade substances from Merck (Darmstadt); quartz bidistilled water was used throughout.

### *Methods*

*Determination of enzyme concentration* in a Cary 118 C spectrophotometer made use of an absorption coefficient, $A^{0.1\%}_{280\,nm} = 0.802\ cm^2 \cdot mg^{-1}$ (Mevarech & Neumann, 1977; Pundak & Eisenberg, 1981); estimates for routine measurements were obtained from the specific activity of the enzyme under standard assay conditions.

*Enzymatic activity* was measured in an Eppendorf 1101M spectrophotometer equipped with a thermostated cuvette holder (40 °C). Standard assay mixture consisted of 4.5 mM oxalacetic acid and 0.4 mM NADH in 4 M NaCl and 50 mM imidazole, pH 7.5 (40 °C). The reaction was started by adding the enzyme solution; NADH oxidation was monitored at 366 nm.

$K_M$ values for NADH and oxalacetic acid were determined from Lineweaver–Burk plots.

*Deactivation of the Enzyme.* (i) In order to deactivate h-MDH at *low ionic strength* (0.2 M NaCl), the stock solution (1.3 mg/mL h-MDH in 4 M NaCl/50 mM sodium phosphate, pH 7.5) was diluted 20-fold with 50 mM Tris-HCl, pH 7.0, followed by a 30-min incubation at 30 °C. The final concentrations of salt and protein were 0.2 M and 65 μg/mL, respectively.

(ii) Deactivation at *pH 2.3 and high salt* made use of a 3-fold dilution of the stock solution with a solution of 4 M NaCl in 1 M glycine/$H_3PO_4$, pH 2.0, followed by 10-min incubation at 0 °C.

(iii) Deactivation in *5.3 M Gdn·HCl* was by 3-fold dilution with 8 M Gdn·HCl/50 mM Tris-HCl, pH 8.5, followed by 30-min incubation at 30 °C. Final salt concentrations were 5.3 M Gdn·HCl, 2.7 M NaCl, and 0.35 M Tris, pH 8.0.

(iv) Deactivation at *elevated temperature* (thermal denaturation) was applied in order to characterize the native and reconstituted enzyme. Enzyme solutions were incubated at 70 and 80 °C, respectively; at varying time intervals, the residual activity was monitored by using the standard enzyme assay at 40 °C. In order to avoid reactivation during the transfer of the enzyme into the test solution, pipets were heated to the inactivation temperature. Residual activities were tested within 1 min so that reactivation during the assay was negligible.

In all deactivation experiments, incubation times in the various denaturants were kept sufficiently long to reach the final state of denaturation. Samples were incubated in polypropylene tubes at 30 °C. In the enzyme assay, salt and denaturant concentrations were kept constant over the whole transition range.

*Reactivation of the Enzyme.* Reactivation was monitored by assaying samples of the reactivating enzyme after various time intervals under standard conditions, in the following manner:

(i) Reactivation after denaturation at *low salt* was initiated by diluting the denaturation mixture 5-fold with 5 M NaCl, 20 mM Tris, 20 mM citrate, and 20 mM imidazole, pH 8.5, plus 1 mg/mL BSA (20 °C).

(ii) Reactivation after denaturation at *pH 2.3, 4 M NaCl* was by 10-fold dilution with 4 M NaCl/0.5 M Tris-HCl, pH 10.0, plus 1 mg/mL BSA, final pH 8.5.

(iii) Reactivation after denaturation in *5.3 M Gdn·HCl* was as described previously with 30–200-fold dilution in 4 M NaCl, 50 mM Tris, and 520 mM citrate, pH 8.0, with a residual Gdn·HCl concentration of ≈25–180 mM.

*Conformation.* Conformational analysis of the enzyme made use of intrinsic fluorescence emission and circular dichroism with a Hitachi–Perkin-Elmer MPF 44A fluorescence spectrophotometer equipped with corrected spectra accessory, a Perkin-Elmer LS 5B luminescence spectrometer with an R 100A recorder (1-cm quartz cuvettes), and a Jasco J 500A CD spectrophotometer with a 500N data processor (0.01-, 0.1-, and 1-cm quartz cuvettes). Mean residue ellipticities (in degrees centimeter squared per decimole) were determined according to

$$[\theta] = \frac{\theta_{obsd}\,mrw}{10dc} \qquad (1)$$

where $\theta_{obsd}$ is the observed dichroic absorption, mrw is the mean residue weight (=110), $d$ is the path length in centimeters, and $c$ is the enzyme concentration in milligrams per milliliter.

Sedimentation measurements were performed in a Beckman Spinco Model E ultracentrifuge equipped with a high-sensitivity light source and photoelectric scanning system, using An-D and An-E rotors, fixed-partition as well as moving-partition cells, and normal double-sector cells.

## Results and Discussion

Investigations of the mechanism of self-organization of proteins require (1) the characterization of the native, denatured, and renatured states of the protein. (2) the optimization of the solvent conditions in terms of long-term stability and maximum yield, and (3) a proper analysis of the time and concentration dependence of the process of reconstitution.

In order to compare the native and renatured states, identical solvent conditions are essential. Generally, high yields of reconstitution can only be accomplished at low protein concentrations (<100 μg/mL). In this range of enzyme concentrations, measurements of the catalytic properties of the enzyme as well as fluorescence emission and far-UV circular dichroism are readily accessible. Therefore, deactivation/reactivation, as well as $K_M$ values and spectral properties, was applied to characterize the self-organization and the structure–function relationship of h-MDH.

### *Effect of Solvent Parameters on Enzyme Activity*

Enzyme assays were carried out in 4 M NaCl/50 mM imidazole, pH 7.1. As shown in Figure 1A, this pH is in the middle of the plateau of maximum catalytic activity of the enzyme. The $K_M$ values for oxalacetic acid and NADH are

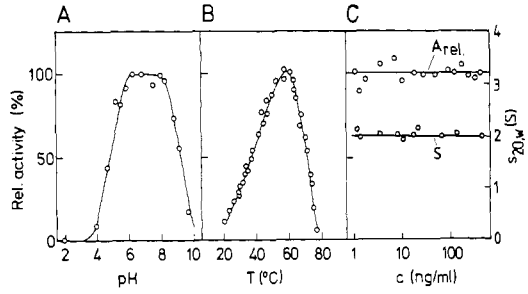


FIGURE 1: Dependence on pH, temperature, and enzyme concentration of the activity of halophilic malate dehydrogenase. (A) pH optimum of h-MDH in 4 M NaCl, 50 mM sodium phosphate, 1.5 mM oxalacetic acid, and 0.1 mM NADH, 30 °C. Assays were started by adding 10 μL of h-MDH ($c$ = 20 μg/mL) to 1.5 mL of test solution. (B) Temperature dependence of h-MDH under standard test conditions (0.1 mM NADH, 1.5 mM oxalacetic acid in 4 M NaCl, and 50 mM imidazole buffer, pH 7.1). Data were corrected for NADH autoxidation by subtracting the change in NADH absorbance in the absence of enzyme. (C) Stability of h-MDH at varying enzyme concentrations. $A_{rel}$, relative specific activity; $s$, sedimentation coefficient, as monitored by activity transport in the analytical ultracentrifuge. Relative activities were determined from the decrease/increase of enzyme activity in the upper/lower compartment of fixed-partition and moving-partition cells after 1–5-h runs at 60000 rpm (20 °C).

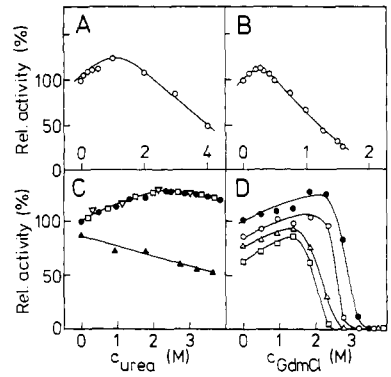


FIGURE 2: Effect of urea and guanidine hydrochloride concentration on the catalytic function of halophilic malate dehydrogenase. (A, B) Determination of h-MDH activity in the presence of varying concentrations of urea (A) and Gdn·HCl (B), respectively. Standard tests in the presence of 4 M NaCl were started by adding 10 μL of h-MDH ($c$ = 2 μg/mL) to 750 μL of assay mixture at 40 °C. (C) Stability of h-MDH against urea at varying NaCl concentration: 2 (closed symbols) and 4 M NaCl (open symbols) in 50 mM Tris-HCl, pH 7.5, 20 °C. Incubation of the enzyme ($c$ = 10 μg/mL) at the individual urea concentrations for 30 min (●, ▽) and 70 h (▲, □). Residual activity of the enzyme was determined by using the standard assay at 40 °C and constant denaturant concentration (0.2 M). (D) Stability of h-MDH against Gdn·HCl in 2 M NaCl (50 mM Tris-HCl, pH 7.0, 20 °C). Incubation of the enzyme ($c$ = 10 μg/mL) at the individual Gdn·HCl concentrations for 30 min (●), 4 h (○), 25 h (△) and 70 h (□). Determination of residual enzyme activity as in (C).

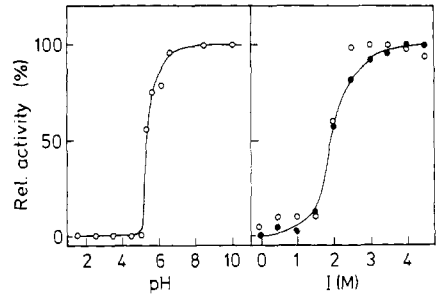


FIGURE 3: pH-dependent and ionic strength dependent deactivation of halophilic malate dehydrogenase. Residual activity after 24-h incubation. (Left) pH dependence at 2 μg/mL enzyme concentration; 4 M NaCl in 0.1 M sodium phosphate/citrate/Tris, 40 °C. Standard enzyme assay at pH 7.3 ± 0.5, 40 °C. (Right) Residual activity of h-MDH at different ionic strengths: NaCl (●) and $(NH_4)_2SO_4$ (○) (initial concentrations 4.5 and 1.5 M, respectively) in 50 mM Tris-HCl, pH 8.5, 30 °C, enzyme concentration 13 μg/mL. Standard enzyme assay at pH 7.5, 40 °C. The salt dependence for KCl parallels the profile observed for NaCl [cf. Mevarech and Neumann (1977)].

0.50 and 0.02 mM, respectively. The temperature dependence of the enzymatic activity below the range of thermal denaturation is characterized by an activation energy of 28 kJ/mol. At temperatures beyond 60 °C, heat denaturation outruns the Arrhenius rate enhancement in a dramatic way, so that deactivation is complete at ≈80 °C (Figure 1B).

There is no anomalous concentration dependence of the activity of the enzyme even at concentrations down to the nanogram range (Figure 1C); evidently, there is no concentration-dependent dissociation of the active dimer into inactive monomers down to concentrations as low as 10 ng/mL. This is clearly corroborated by sedimentation velocity experiments which yield $s_{20,4MNaCl}$ = 2.3 S over the entire concentration range, in agreement with published data (Pundak & Eisenberg, 1981).

Increasing concentrations of urea or Gdn·HCl in the enzyme assay lead to a significant activation at low denaturant concentrations. Beyond 1.2 M urea or 0.3 M Gdn·HCl, deactivation prevails. The effect points to an increase in protein flexibility which, at low urea or Gdn·HCl concentrations, enhances the catalytic efficiency of the enzyme, while in the denaturation range urea and Gdn·HCl exhibit their well-known structure-breaking effects (Figure 2A,B).

*Stability of the Enzyme*

The previously mentioned solvent effects on the catalytic properties of h-MDH refer to experiments where the enzyme was exposed to the various conditions only during the time of the assay (≈4 min). In the transition range, unfolding may be exceedingly slow, so that the given data do not necessarily refer to the equilibrium state. For example, the half-time observed for the deactivation of the enzyme at 0.2 M NaCl is 2 min; the corresponding data in the presence of strong denaturants such as pH 2.3 or 5.3 M Gdn·HCl are <2 min. Thus, the time required for the standard enzyme assay is of the same order as the half-time of deactivation. Since the coenzyme has a stabilizing effect, this perturbation may become even more significant.

*Equilibrium Transitions. (A) Effects of pH and Salt.* To accomplish true equilibria, solutions were incubated for up to 70 h. Figure 3 illustrates the stability of h-MDH at varying pH and salt concentrations. At pH 7–10, the enzyme shows long-term stability (Figure 3, left panel). At pH 4.8 ± 0.3, deactivation occurs as a highly cooperative reaction. As shown by light scattering (monitored as turbidity at 340 nm), the N → D transition is accompanied by aggregation. This shows its maximum at pH 4.5, in accordance with the result of isoelectric focusing, which yields an isoelectric point close to this value. At zero net charge, precipitation is the main reason for long-term deactivation. As shown by far-UV circular dichroism and fluorescence emission, no drastic conformational changes are detectable in the transition range. At low pH values (pH 2–4), the enzyme occurs in its denatured monomeric state.

Deactivation at low salt concentrations depends on ionic strength. Figure 3 (right panel) illustrates the equilibrium transition of h-MDH under standard buffer conditions in the presence of varying concentrations of NaCl and $(NH_4)_2SO_4$. For both salts, the transition coincides at an ionic strength $I$ = 1.8 M. The scatter of the data at high and low salt concentrations does not allow a detailed discussion of the struc-

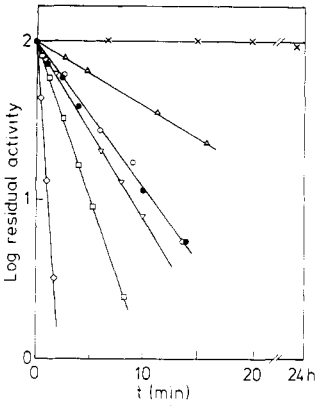


FIGURE 4: Deactivation of halophilic malate dehydrogenase at low ionic strength and high temperature. First-order plots for h-MDH ($c$ = 2 μg/mL) at 0.2 M NaCl, 30 °C, in various buffers: 10 mM sodium phosphate, pH 7.0 (◇); 10 mM sodium phosphate, pH 8.5 (△); 50 mM Tris-HCl, pH 7.0 (□); 50 mM sodium phosphate, pH 7.0 (▽); 50 mM sodium phosphate, pH 7.0, plus 5 mM NADH (×). Heat deactivation of native (○) and reconstituted h-MDH (●) at 80 °C in 4 M NaCl/20 mM Tris–citrate, pH 8.5, in the presence of 1 mg/mL BSA proving the native enzyme and the product of reconstitution to be indistinguishable. Residual activity at given times was determined by using the standard enzyme assay at 40 °C.

ture-making or structure-breaking effects of the given ions. Low concentrations of ammonium sulfate and sodium phosphate induce a stabilizing effect, while high concentrations (>1 M) seem to freeze the enzyme in a state of increased rigidity, leading to a decrease in catalytic efficiency. The effects are in agreement with the different deactivation rates of h-MDH at 0.2 M NaCl (pH ~7) where 50 mM sodium phosphate is found to cause significant stabilization (Figure 4).

The coenzyme (NADH) protects the enzyme from deactivation at low salt; on the other hand, lowering the pH leads to dramatic destabilization, as shown by the increase in the rate of deactivation (Figure 4).

*(B) Effects of Urea and Guanidine.* In applying the most efficient denaturants, urea and Gdn·HCl, to halophilic proteins, solubility problems arise. Since the native state of h-MDH in the absence of protecting ligands requires minimum salt concentrations of 3 M NaCl or 1 M $(NH_4)_2SO_4$, denaturants cannot be dissolved to sufficiently high concentrations required for protein denaturation. In standard buffer at pH 7 and 4 M NaCl, the solubility of urea at room temperature amounts to 4 M. As illustrated in Figure 2C, at this concentration transient activation is observed rather than deactivation. In 2 M NaCl where h-MDH is unstable, even in the absence of urea (cf. Figure 3, right panel), partial deactivation occurs. Since both low salt and urea contribute to the destabilizing effect, no clear-cut explanation of the deactivation profile can be given.

Gdn·HCl-dependent unfolding transitions were determined at 1 and 2 M NaCl, allowing up to 4 M Gdn·HCl to be dissolved at room temperature. Figure 2D illustrates the effect of varying Gdn·HCl concentration on the enzymatic activity. Standard enzyme assays were performed after 0.5–70-h incubation, keeping the residual denaturant concentration at 0.2 M.

As in the case of urea, at moderate guanidine concentration, the enzyme exhibits significant activation. The effect exceeds the increase in activity in the presence of 0.2 M Gdn·HCl (cf. Figure 2B). Obviously, the enhanced protein flexibility observed at moderate denaturant concentration prevails during the time of the enzymatic assay. A similar "metastable state" of the enzyme has also been reported for the N ⇌ U transition at varying salt concentration (Mevarech & Neumann, 1977).

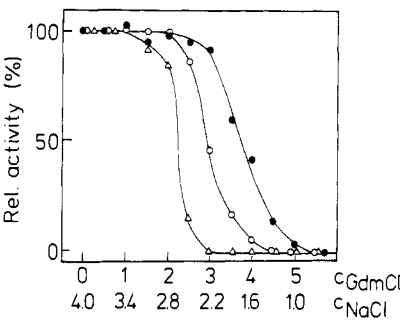


FIGURE 5: Guanidine-dependent deactivation of halophilic malate dehydrogenase at high salt concentration. Ionic strength was kept at $I \geq 4$ M, complementing $c_{Gdn\cdot HCl}$ by addition of NaCl. h-MDH ($c$ = 10 μg/mL) in 50 mM Tris-HCl, pH 7.0, was incubated at 20 °C for 5 min (●), 30 min (○), and 24 h (△), respectively. Activity was measured by using the standard assay at constant residual Gdn·HCl concentration (0.2 M).

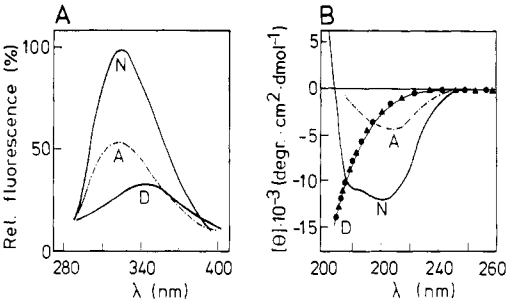


FIGURE 6: Spectral properties of native and denatured halophilic malate dehydrogenase. (A) Fluorescence emission at $c$ = 10 μg/mL; $\lambda_{exc}$ = 275 nm, 20 °C. N, native h-MDH in 4 M NaCl/10 mM Tris-HCl, pH 7.0; A, h-MDH after 60-min acid denaturation in 4 M NaCl and 50 mM glycine/$H_3PO_4$, pH 2; D, h-MDH after 60-min denaturation at low salt concentration (0.4 M NaCl/10 mM Tris-HCl, pH 7.0). (B) Far-UV circular dichroism at $c$ = 0.1 mg/mL, 20 °C. N, native h-MDH in 4 M NaCl/10 mM Tris-HCl, pH 7.5: A, h-MDH after acid denaturation in 4 M NaCl and 50 mM glycine/$H_3PO_4$, pH 2; D, h-MDH after 60-min denaturation at low salt concentration: 0.04 M NaCl/50 mM sodium phosphate, pH 7.5 (●); 6 M Gdn·HCl/50 mM sodium phosphate, pH 7.5 (▲).

At high guanidine concentration, complete unfolding of the enzyme is observed; at 2 M NaCl and 4 M Gdn·HCl, denaturation takes less than 30 min. In the experiment summarized in Figure 5, both NaCl and Gdn·HCl were complemented to high total concentration. Keeping the ionic strength beyond 4 M during all stages of guanidine denaturation, the previous optimum curves (Figure 2D) change into normal transition curves.

*Characterization of the Native and Denatured States.* Deactivation of h-MDH at low ion concentrations or low pH, as well as at high concentrations of Gdn·HCl, is accompanied by dissociation of the native dimer to the denatured monomer. Dissociation can be clearly deduced from bimodal sedimentation boundaries at salt concentrations below 2 M NaCl. A detailed sedimentation analysis has been reported previously (Pundak et al., 1981). Further evidence proving subunit dissociation is gained from reconstitution kinetics [see below; cf. Jaenicke (1987)].

The final states of denaturation in the various denaturants differ widely. As suggested by fluorescence emission spectra, denaturation at low salt leads to drastic conformational changes, while acid denaturation (4 M NaCl, pH 2.3) seems to preserve part of the native secondary and tertiary structure [Figure 6A; for comparison, see Jaenicke et al. (1979), Rudolph et al. (1986), and Hecht et al. (1986)]. Circular dichroism spectra clearly confirm these conclusions. As shown in Figure 6B, low salt and high Gdn·HCl concentrations lead

to the fully denatured state; in both cases, the far-UV spectrum shows the smooth curve characteristic for the random coil. Upon acid denaturation, a decrease in helicity from ≈30% (characteristic for the native state) to <10% is observed.

*Reconstitution*

Results given in the foregoing paragraphs clearly show that deactivation of dimeric h-MDH is accompanied by unfolding and subunit dissociation. The reverse reaction may be applied to elucidate the structure–function relationship in proceeding from the unfolded polypeptide chain to the complete native quaternary structure.

*Optimization of Reconstitution Conditions after Denaturation at Low Salt.* In order to accomplish maximum recovery of native h-MDH after deactivation in various denaturants, all relevant solvent parameters were optimized; maximum yields under optimum conditions were 95%.

As suggested from the determination of long-term stability, h-MDH requires high salt concentrations for proper reconstitution. In going from 2 to 4 M NaCl, a steady increase in reactivation from 0 to 20% is observed (enzyme concentration 10 μg/mL in 50 mM Tris-HCl, pH 8.5, 20 °C). In the case of ammonium sulfate under the same buffer conditions, maximum reconstitution (40%) is obtained at 2 M $(NH_4)_2SO_4$. Additives such as 0.1 mM oxalacetic acid, 5 mM EDTA, or 80 mM $MgSO_4$ did not improve the yield significantly; 1 mM NADH and 1 mg/mL BSA increased the yield to ≈30 and 60%, respectively. Reconstitution reaches its maximum value at pH 9, 4 M NaCl (in 20 mM imidazole/citrate/phosphate buffer). At neutral pH, the yield drops to ≈30%. The optimum temperature range is 15 ± 5 °C. Beyond 20 °C, a steady decrease in reconstitution is observed until, at 50 °C, denaturation becomes irreversible. Although there is no concentration-dependent dissociation of the dimeric enzyme, low enzyme concentrations strongly diminish the yield of reconstitution. This effect has also been observed with other oligomeric enzymes [cf. Rudolph and Jaenicke (1986)]. In contrast to mitochondrial MDH and other dehydrogenases, where reactivation reaches a plateau value (or even decreases) at protein concentrations beyond 10 μg/mL, h-MDH shows a steady increase in yield beyond this range. This may be caused by a decrease in relative adsorption or by an increase in stability at elevated protein concentrations. In summary, optimum conditions of reconstitution were found to be ionic strength ≥ 4 M [e.g., 2 M $(NH_4)_2SO_4$], 0.1 M imidazole, pH 8–9, enzyme concentration > 10 μg/mL, temperature ≤ 20 °C, 1 mg/mL BSA, residual guanidine concentrations below 0.7 M.

*Comparison of the Reconstituted and Native States after Deactivation at Low Salt.* The significance of in vitro reconstitution experiments depends on the identity of the enzyme after reassociation and reactivation with the native enzyme. As taken from fixed-partition experiments in the analytical ultracentrifuge, both the renatured enzyme and the native enzyme show identical sedimentation coefficients characteristic for a dimer of ≈80 000 molecular mass: $s_{20,4MNaCl} = 2.3$ S; $s_{20,w} = 5.6 \pm 0.3$ S.

To compare the gross conformation of the reconstituted and native states, the thermal stability at 70 °C and heat deactivation at 80 °C were measured. Both the reactivated enzyme and the native enzyme show identical behavior: at 70 °C, there is no deactivation within 4 h, while at 80 °C both are inactivated with a half-time of $t_{1/2} = 210 \pm 10$ s.

Michaelis constants were used to monitor the functional integrity of the reconstituted enzyme. The respective figures for native and reconstituted h-MDH do not differ significantly; the maximum deviation amounts to 10%: $K_{M,oxalacetate} = 500$ μM and $K_{M,NADH} = 20$ μM (±10%). In conclusion, in vitro reconstitution after denaturation of h-MDH at low salt yields a product which, by the above criteria, is indistinguishable from the enzyme in its native state. The same holds for the enzyme after acid or guanidine denaturation: $K_{M,OA} = 500$ μM, $K_{M,NADH} = 23$ μM (±10%).

*Kinetics of Reconstitution.* The reconstitution of the dimeric enzyme must include first-order transconformation reactions and the second-order association of the folded monomers to the native enzyme according to

$$2M' \xrightarrow{k_1} 2M \xrightarrow{k_2} M_2 \xrightarrow{k_1} D \qquad (2)$$

where M′ and M refer to the monomer in different conformational states and D refers to the native dimer. It cannot be predicted which of the consecutive steps is actually rate determining. Porcine mitochondrial MDH and cytoplasmic MDH exhibit widely differing reconstitution mechanisms (Jaenicke & Rudolph, 1979, 1983; Rudolph et al., 1986). However, the kinetic mechanism is not affected by the mode of denaturation. As shown for lactate dehydrogenase and glyceraldehyde-3-phosphate dehydrogenase, this observation cannot be generalized (Zettlmeissl et al., 1981; Hermann et al., 1981; Jaenicke & Rudolph, 1983).

h-MDH reconstitution was investigated after denaturation at low salt concentration, low pH, and high Gdn·HCl concentration. All three modes of denaturation yield different conformational states or different states of solvation. From the above equilibrium data, it is evident that the reconstitution of the enzyme at 4 M NaCl takes place under essentially irreversible conditions. The recovery of enzyme activity is found to be determined by folding and subsequent subunit association. Determination of the reaction order suggests a complex mechanism involving uni- and bimolecular processes. Intermediates on the pathway of folding and association are unstable so that protecting agents are highly efficient in improving the yield of reconstitution. For example, 1 mg/mL BSA practically eliminates long-term instability of the native enzyme and its folding intermediates, thus increasing the yield of reactivation by a factor of 3 without affecting the kinetic mechanism. Altering the solvent conditions by dialysis doubles the reactivation yield (also in the absence of BSA). However, in this case, no sound kinetic analysis is possible because of the ill-defined switch from low to high salt concentration which does not allow the zero time of renaturation to be quantified.

*(A) Reconstitution after Denaturation at Low Salt Concentration.* The denaturation/renaturation cycle of h-MDH requires a sequence of mixing steps which cause an overall dilution of the enzyme by a factor of at least 100. Full deactivation is accomplished at $c_{NaCl} \leq 0.2$ M (1:20 dilution starting from 4 M NaCl). Maximum solubility of NaCl in the given buffer at room temperature is 5 M; thus, a 5-fold dilution is required to arrive again at optimum reconstitution conditions. The concentration of the enzyme was altered in the range between 0.2 and 20 μg/mL. At such low concentrations, long-term stability of the enzyme can only be maintained in the presence of 1 mg/mL BSA. As a consequence, neither fluorescence nor circular dichroism could be applied to monitor renaturation.

Figure 7 illustrates the effect of enzyme concentration, pH, temperature, and the coenzyme on the reactivation of h-MDH. Normalizing the regain of activity to the final yield (taken as 100%), reactivation is found to depend on enzyme concentration, thus proving the dimerization of the enzyme to be a necessary requirement for full catalytic activity (Figure 7A).

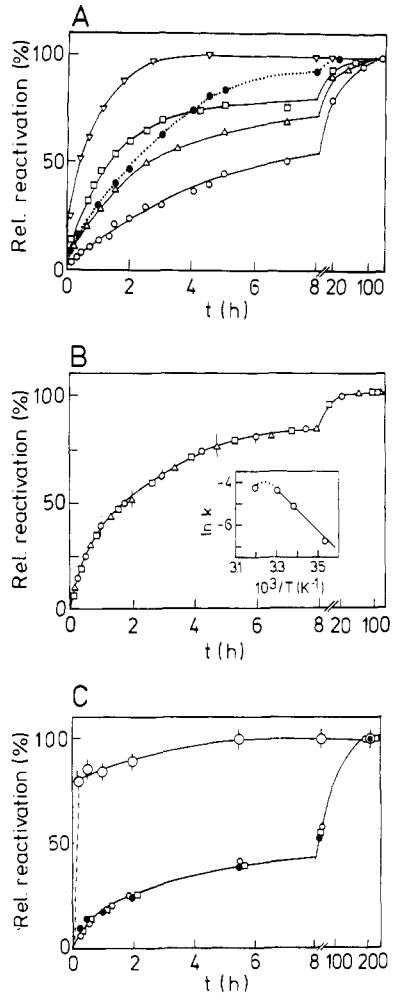


FIGURE 7: Kinetics of reactivation of halophilic malate dehydrogenase after deactivation at low salt concentration ($c_{NaCl} \leq 0.4$ M). (A) Reactivation at varying enzyme concentrations. Deactivation by dialysis against 50 mM Tris-HCl, pH 7.0; reactivation in 4 M NaCl and 20 mM imidazole/citrate, pH 8.5, plus 0.8 mg/mL BSA. $c_{h\text{-}MDH}$ varied between 40 (▽), 10 (□), 4 (△), and 2 (○) μg/mL. The dotted line (●) refers to 2 μg/mL in the presence of 1 mM NADH. (B) Reactivation at varying pH and temperature. Deactivation by 30-min incubation in 0.4 M NaCl/50 mM Tris-HCl, pH 7.0, 30 °C; reactivation at $c_{h\text{-}MDh}$ = 6 μg/mL in 4 M NaCl and 20 mM imidazole/sodium phosphate/citrate, pH 7.5 (□), pH 8.5 (△), and pH 9.5 (○), in the presence of 0.8 mg/mL BSA. Insert: Arrhenius plot for the reactivation at pH 8.5. The activation energy of the second-order reaction amounts to 84 kJ/mol. (C) Reactivation after deactivation of h-MDH at various residual salt concentrations. Deactivation by 60-min incubation in 0.8 M (◇), 0.4 M (○), 0.08 M (●), and 0.02 M (□) NaCl/50 mM Tris-HCl, pH 7.0, 30 °C; reactivation at $c_{h\text{-}MDH}$ = 6 μg/mL in 4 M NaCl and 50 mM sodium phosphate, pH 8.5, plus 0.8 mg/mL BSA. Activity was determined by using the standard enzyme assay.

The holoenzyme (at 1 mM NADH in the presence of 1 mg/mL BSA) shows a drastic increase in the rate and yield of reactivation.

The pH is found to have no effect in the range between 7.5 and 9.5 (Figure 7B). The activation energy of the reactivation reaction, determined in the temperature range between 10 and 30 °C, is exceedingly high: $E_A$ = 84 kJ/mol (insert, Figure 7B). Beyond 40 °C, folding intermediates are unstable; at ≥50 °C, no reactivation is observed although the native enzyme exhibits long-term stability under this condition.

Low NaCl concentrations (0.02–0.4 M) in the denaturation mixture affect neither the kinetics nor the yield of reactivation. At 0.8 M NaCl, deactivation is very slow (1 h in 50 mM Tris, pH 7.0). It is accompanied by the dissociation of the dimer to "structured monomers". Since in this case reconstitution does not involve folding, the overall reaction is greatly enhanced (Figure 7C). A similar behavior is observed after short incubation (1 min) at 0.2 M NaCl. Long incubation (>2 h) leads to a slow transconformation at the monomer level ($t_{1/2} \approx 3$ h) which is accompanied by a decrease in the yield of reactivation.

*(B) Reactivation after Denaturation at Acid pH (4 M NaCl).* Deactivation of the enzyme in 1 M glycine/$H_3PO_4$, pH 2.0, and 4 M NaCl (0 °C) was complete after ≤1 min. Long incubation again leads to a decrease in the yield of reactivation. After 1-min denaturation, the yield amounts to >90%; after ≈10 h, only 50% recovery is observed. Incubation up to 2 h does not alter the kinetic profiles significantly; after >12-h incubation, a slow transconformation reaction determines the rate [cf. Zettlmeissl et al. (1981)].

Recovery of enzymatic activity was measured at various final concentrations of the enzyme. Again, the concentration dependence of the reactivation reaction proves that a bimolecular process must be involved. Sigmoidicity becomes significant especially at low enzyme concentration, corresponding to a unibimolecular reaction scheme, similar to the one observed for m-MDH after acid denaturation (Jaenicke et al., 1979).

*(C) Reactivation after Denaturation at 5.3 M Gdn·HCl (2.7 M NaCl).* Renaturation of h-MDH after preceding denaturation at elevated concentration of Gdn·HCl (cf. Materials and Methods) can only be accomplished at a residual Gdn·HCl concentration below 0.7 M. Considering the equilibrium transition (cf. Figure 2D), this means that there exists a broad concentration range (0.7–1.5 M) where h-MDH in the presence of 2.7 M NaCl shows "hysteresis" (Mevarech & Neumann, 1977; Jaenicke, 1978). Outside this range, reactivation at $c_{Gdn\text{·}HCl}$ = 0.04 and 0.15 M yields 55 and 70%, respectively. This result is close to the limiting values obtained after denaturation at low salt concentrations. Slightly destabilizing conditions do not improve the yield (Jaenicke & Rudolph, 1988). The rates of reactivation are strongly decreased with increasing Gdn·HCl concentration, even at concentrations as low as 40 mM. Again, a similar behavior has been observed for lactate dehydrogenase (Zettlmeissl et al., 1982).

Comparing the kinetic profiles of reconstitution after Gdn·HCl denaturation with those after denaturation at low salt concentrations or low pH, it is interesting to note that no increase in sigmoidicity is observed. As taken from the far-UV CD spectra, renaturation after guanidine denaturation and denaturation at low salt concentrations start from the completely denatured state, while at pH 2 residual secondary structure is left (cf. Figure 6B). Obviously, formation of the residual structure still preserved at pH 2 is not rate determining in the recovery of the native structure of h-MDH. The overall reconstitution kinetics after complete denaturation in 5.3 M Gdn·HCl are closely similar to those given in Figure 7.

## CONCLUSIONS

The extreme halophile *Halobacterium marismortui* shows intracellular salt concentrations of ≥5 M. Thus, its cytoplasmic protein inventory must be adapted to low water activity. With the use of h-MDH as an example, the aim of the present study was to compare the salt-dependent reversible denaturation of a halophilic enzyme with its renaturation after denaturation at extremes of pH, or at high concentrations of guanidine hydrochloride.

The question of reversibility is of interest in connection with recent attempts to express halophilic enzymes in nonhalophilic microorganisms (M. Mevarech, personal communication). As

taken from the present experiments, expression should only yield inactive enzyme. The high cytoplasmic protein level, as well as the presence of the coenzyme, may provide protective effects. However, at high expression, protein denaturation and subsequent aggregation are expected to be predominant so that inactive enzyme is expected to precipitate in "inclusion bodies". As indicated, reactivation may be accompanied by Gdn·HCl solubilization and subsequent reconstitution. In the case of thermophilic proteins, the situation seems to be different. As shown by Fabry et al. (1988), thermophilic enzymes may be expressed in *Escherichia coli* at high yield and in active form.

Comparing the denaturation/renaturation behavior of h-MDH with its nonhalophilic homologues, a number of peculiarities are obvious. Low pH and salt concentration, as well as chaotropic components, promote deactivation, denaturation, and dissociation. This means that under conditions where the salt no longer competes with the enzyme for its hydration, destabilization of the native structure occurs; Gdn·HCl, due to its ionic character, can replace the salt up to a certain limiting concentration, leading to intermediary activation of the enzyme. Beyond the optimum, randomization occurs. Spectroscopically, denaturation at low salt and high Gdn·HCl concentration is complete, while at pH 2 residual structure is retained. The monomer is inactive under all conditions.

The mechanism of reconstitution after denaturation in either of the previously mentioned denaturants resembles the kinetic pattern observed for the mitochondrial enzyme (m-MDH) (Jaenicke et al., 1979). However, for m-MDH, various denaturing conditions lead to identical sigmoidal reactivation profiles, indicating that one common rate-determining folding step governs the overall mechanism. In the case of h-MDH, sigmoidicity is only observed after long-term incubation under strongly denaturing conditions where side reactions lead to a decrease in the yield of reconstitution. LDH-$M_4$ has been reported to exhibit similar properties (Zettlmeissl et al., 1981).

Ligands such as the coenzyme (NADH), or additives like BSA, improve the yield of reactivation, while residual denaturant (e.g., low concentrations of Gdn·HCl) competes with structure formation. Obviously, there are unstable folding intermediates on the pathway of reconstitution which are stabilized by specific ligands. In contrast to m-MDH where NADH only increases the yield, h-MDH shows a significant enhancement of the yield and the rate of reactivation. The effect is unexpected because NAD binding requires the domain structure of the enzyme to be more or less in its native state. A similar results has been obtained in the case of glyceraldehyde-3-phosphate dehydrogenase from yeast (Krebs et al., 1979; Jaenicke et al., 1980).

In the absence of stabilizing ligands and outside the optimum range of reconstitution, folding intermediates are unstable so that at $T > 40$ °C, pH 6.5, and at Gdn·HCl concentrations between 0.7 and 2.0 M, reconstitution can no longer be accomplished. The same result is caused by conformational rearrangements of the dissociated monomers after long-term denaturation at low salt concentrations. A simple two-step mechanism according to

$$N \rightleftharpoons U \rightarrow U' \qquad (3)$$

describes the data; here, N stands for the native enzyme, and U and U′ are the unfolded protein in two conformational states differing in their reconstitution capacity. The relative proportion of folding/association vs "irreversible denaturation" depends on the denaturation/renaturation conditions. Side reactions may be "wrong aggregation", or trapping of the tertiary structure in wrong (local) energy minima, e.g., by wrong domain interactions.

ACKNOWLEDGMENTS

We thank Drs. F. X. Schmid, R. Seckler, G. Böhm, J. Buchner, and A. Wrba for valuable discussions. *Halobacterium marismortui* and supernatants of the homogenized microorganism were gifts from Profs. H. Eisenberg and H. G. Wittmann. The technical assistance of H. Blaschek and M. Kongsbak-Reim is gratefully acknowledged.

**Registry No.** MDH, 9001-64-3.

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