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Halophilic enzymes: proteins with a grain of salt ☆

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Abstract

Halophilic enzymes, while performing identical enzymatic functions as their non-halophilic counterparts, have been shown to exhibit substantially different properties, among them the requirement for high salt concentrations, in the 1–4 M range, for activity and stability, and a high excess of acidic over basic amino residues. The following communication reviews the functional and structural properties of two proteins isolated from the extremely halophilic archaeon *Haloarcula marismortui*: the enzyme malate-dehydrogenase (hMDH) and the 2Fe–2S protein ferredoxin. It is argued that the high negative surface charge of halophilic proteins makes them more soluble and renders them more flexible at high salt concentrations, conditions under which non-halophilic proteins tend to aggregate and become rigid. This high surface charge is neutralized mainly by tightly bound water dipoles. The requirement of high salt concentration for the stabilization of halophilic enzymes, on the other hand, is due to a low affinity binding of the salt to specific sites on the surface of the folded polypeptide, thus stabilizing the active conformation of the protein.

Keywords: Halophilic enzymes; Halobacteria; Malate-dehydrogenase; Ferredoxin

☆ Dedicated to Heini Eisenberg.

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1. Introduction

The title of the Ph.D. thesis of the late Professor Benjamin Elazari-Volcani, submitted to the Hebrew University in Jerusalem in 1940, is: '*Studies on the Microflora of the Dead Sea*'. This pioneering work completely shattered the generations-old myths of total sterility of the Dead Sea by showing that this unusual lake, located at the lowest point of terrestrial earth, is the breeding ground for numerous prokaryotic and unicellular eukaryotic species. The most abundant organisms in the Dead Sea are the extremely halophilic archaea, generally called halobacteria, whose cell density can reach 10^7 cells/ml. Halobacteria which are obligatory halophiles, can only grow in media containing salt concentrations of 1.5 M or higher. Besides the Dead Sea, these archae also flourish in natural salt lakes such as the Great Salt Lake in Utah, in artificial sea water pools made for the purpose of salt extraction, in spoiled salted fish and surprisingly enough, embedded in rocks in salt mines [1].

In order to overcome the extreme osmotic pressure of these hypersaline environments, halophilic bacteria and eukaryotes accumulate mostly neutral organic compatible solutes and exclude most of the inorganic salts. In contrast, the halophilic archaea balance the external high salt concentration by intracellular accumulation of inorganic ions to concentrations that exceed that of the medium. Therefore, all the cellular components of the halophilic archaea must be adapted to function at the extremely high intracellular salt concentration.

The effect of salt concentration on biopolymers has been reviewed recently [2,3]. In general, the final conformation of a biopolymer depends on the intramolecular interactions between surfaces of the polymer, on intermolecular interactions between surfaces of the polymer with surfaces of other polymers, and the interactions of the surface of the biopolymer with water and solutes. These interactions include those that are hydrophobic in nature, as well as hydrogen bonds and electrostatic attraction and repulsion. At low salt concentrations (< 0.1 M), only the conformations of highly charged biopolymers are affected. At high salt concentrations (> 0.1 M), salts affect the conformation of biopolymers by reducing the effective concentration of water and by interacting specifically with the surface of the biopolymer. Some ions are excluded from the biopolymer surface and their effect is mainly through reducing the activity of water. The nature of the interactions of different ions with proteins is defined by the 'Hoffneister Series'. Some ions, such as sulfate, phosphate and ammonium, promote the folding of proteins, intermolecular association and even aggregation while some ions, such as iodide and guanidinium, promote dissociation of protein complexes and aggregates and the unfolding of protein chains.

The dependence of the stability and the catalytic properties of halobacterial proteins on salt concentration has been the subject of studies for many years (for reviews see Lanyi [4] and Eisenberg et al. [5]). In the following communication, we shall review the structural and biochemical properties of two halobacterial proteins whose three-dimensional structures have been determined by X-ray crystallography: (1) the enzyme malate dehydrogenase (hMDH) from *Haloarcula marismortui*; and (2) the 2S–2Fe ferredoxin (HmFd) of the same archaeon. Early studies on these proteins were performed in the laboratory of Professor Eisenberg, whose 17 years of service on the editorial board of Biophysical Chemistry is recognized by the special edition of this issue.

2. The malate dehydrogenase of *Haloarcula marismortui*

2.1. Historical perspective

Halophilic enzymes are usually very unstable in low salt concentrations. Since some of the important fractionation methods in protein chemistry, such as, electrophoresis and ion exchange chromatography, cannot be applied at high salt concentrations, the available fractionation methods for halobacterial proteins are rather limited. Malate dehydrogenase was the first halobacterial enzyme to be purified [6]. In this early purification protocol the salt concentration was reduced at

the very beginning, and methods such as ion exchange chromatography on DEAE-cellulose and electrophoresis were applied. The enzyme was, then, recovered after reactivation by dialysis against 25% (w/v) NaCl. The yield of this procedure was very poor ($\sim 0.5\%$) and a much more efficient purification protocol was needed in order to obtain the high amounts of pure enzyme that are required for biophysical characterization.

In order to improve the yields of pure hMDH, a new approach was adopted in which the hMDH was kept at a high salt concentration throughout the purification process [7]. The purification procedure included fractionation on Sepharose 4B using decreasing ammonium sulfate concentration gradient, gel filtration at 4.3 M NaCl, hydroxylapatite chromatography at 4.3 M NaCl and affinity chromatography on 8-(6-aminohexyl)-amino—NAD^+-Sepharose. Using this protocol, milligram quantities of pure enzyme were obtained with yields of $\sim 50\%$.

Early determination of the molar mass of native hMDH at NaCl concentrations of 4–5 M, using analytical ultracentrifugation and light scattering measurements gave values of 84 000–90 000 g mol^{-1} [8,9]. These measurements required accurate determination of the density of the enzyme and the refractive index increments as a function of protein concentration at the high salt concentrations used in the ultracentrifuge and light scattering experiments. Very similar values for the molecular mass were also obtained later by a small-angle X-ray scattering study [10]. It was, therefore, believed that similarly to many MDHs, the native hMDH at a high salt concentration is a dimer composed of two identical subunits of molecular mass of 42 000–45 000 g mol^{-1}. This assumption was supported by the observation that after dissociation of the enzyme to its subunits at a low salt concentration, the reactivation reaction at high salt concentration exhibited a second order kinetic response [11].

Subsequent isolation of the gene encoding *H. marismortui* hMDH [12] and determination of its nucleotide sequence enabled the precise calculation of the subunit molecular mass to be 32 638 g mol^{-1}, and thus the calculated dimer molecular mass was significantly lower than previously determined. X-Ray data from crystals of hMDH obtained at 0.8 M NaCl and 58% methylpentanediol (MPD) showed two interacting dimers related by a crystallographic twofold axis [13], and cross-linking experiments [14] revealed that at high salt concentrations the native hMDH is a tetramer. These observations have lead to a thorough revision of the subunit structure of the enzyme in solution and to reevaluation of the solvent interactions of the native enzyme as determined by various biophysical methods [15]. New measurements of the values of the partial specific volume and the refractive index increment of hMDH at several salt concentrations and recalculations of the ultracentrifugation and light scattering measurements resulted in a molecular mass of 115 000 g mol^{-1}, much closer to that expected for a tetramer.

2.2. Structural properties of the tetrameric hMDH

Malate dehyrogenases and L-lactate dehydrogenases are members of a homologous class of 2-ketoacid NADH-dependent dehydrogenases. Although L-LDHs may exist as dimers, they are active only in the tetrameric form. On the other hand, some MDHs are active as dimers whereas others are active as tetramers. Sequence alignments and the construction of a phylogenetic tree of MDHs and L-LDHs with hMDH showed that the halophilic MDH was structurally more closely related to lactate dehydrogenases than malate dehydrogenases [12]. The crystal structure of the hMDH–NADH binary complex was solved first at 3.2 Å resolution [13] by the molecular replacement method using the dogfish L-LDH (dfLDH) as a model.

The structures of hMDH and dfLDH are very similar consisting of four identical subunits (a dimer of dimers). The major structural difference between these enzymes is the large excess of acidic over basic residues in the halophilic enzyme. Calculations showed that the surface of hMDH is comprised of twice as many acidic as basic residues, whereas the surface of dfLDH contains approximately equal numbers of the two residue types. The contact surface between the two dimers of dfLDH is composed primarily of

hydrophobic interactions. Most of these interactions are missing in hMDH, where the dimer–dimer contacts are less intimate, resulting in a less compact tetrameric structure. The major interactions in the hMDH dimer–dimer interface are instead electrostatic in nature [13].

Each hMDH subunit contains 15 arginine residues, three of which are conserved among all MDHs and are involved in catalysis and substrate binding. The remaining 12 arginine side chains are in close contact with at least one oxygen of an acidic residue, forming clusters of salt-bridges. Fourteen such salt-bridges make intra-subunit interactions between secondary structural elements, and eight salt-bridges span the subunit interfaces. In the contact region between the two dimers, two complex salt bridge clusters are located at the opposite extremes of the interface and appear to be the only inter-dimer interactions stabilizing the tetramer.

The two arginines (R207 and R292) that are in the core of these two complex salt bridges were mutated to serines and the effect of these mutations on the quaternary structure of hMDH was studied in detail using enzymatic activity, sedimentation velocity, and small angle neutron scattering (SANS) measurements [16]. It was found that whereas the wild-type hMDH is a tetramer in NaCl concentration range of 2–4 M, the hMDH (R207S, R292S) mutant exists in this salt concentration range in a dimer–tetramer equilibrium. The equilibrium constant for the dimer–tetramer transition at 2 M NaCl is 3.2×10^2 M^{-1} and, therefore, at 2 M NaCl the hMDH (R207S, R292S) mutant is mainly a dimer. Interestingly, unlike L-LDHs, the dimeric form of the hMDH (R207S, R292S) mutant has identical specific activity to that of the tetramer.

To better observe solvent–protein interactions, higher resolution crystal structures of hMDH were required. Recently, the structures of the wild-type hMDH and an hMDH mutant (E267R) were determined to 2.9 and 2.6 Å, respectively [17]. Approximately 500 non-protein electron density peaks per tetramer were assigned as water molecules. Most of these water molecules are located in the first hydration shell and make hydrogen bonds with the protein. Many water molecules are associated in networks. The most extensive network fills the central cavity and is composed of 88 hydrogen-bonded water molecules. Since sodium ions and water cannot be distinguished by their electron number alone (having 10 electrons each), the assignment of electron density peaks to water rather than sodium ions relied on the coordination geometry of the peak. A water molecule makes 4-5 hydrogen bonds with its neighbors whereas sodium ions have six or nine nearest neighbors at distances of approximately 2.4 Å. Using these criteria only two peaks per tetramer were assigned as sodium ions. These apparently specific sodium binding sites are made by two symmetrically-related glutamic residues (E247) (see Fig. 1A) that are part of a small salt-bridge cluster located at the top of the monomer–monomer interface. The electron density also suggested that two chloride ions per tetramer interact with two lysine residues (Lys 205). These lysine residues are part of the two salt bridge-clusters in the dimer–dimer interface that also contain the Arg 207 residues mentioned above.

2.3. The effect of salt concentration on hMDH

Salt concentration has substantial effects on the catalytic properties of hMDH as well as on its structural stability. Detailed studies on the effect of NaCl concentration on the kinetic properties of hMDH [18] has shown that whereas the K_m for NADH is independent of salt concentration, the K_m for oxaloacetate increases with increasing salt concentration from 80 μM at 1 M NaCl to 0.5 mM at 4 M NaCl. This observation explains the artifactual activity maximum of hMDH at 1.2 M NaCl reported before [8] being the result of assaying the enzyme at subsaturating oxaloactate concentrations. When assayed at saturating concentrations of both substrates, the V_{max} of the enzyme rises very sharply with salt concentration until it reaches a plateau at 1 M.

The effect of salt concentration on the structure of hMDH has been the subject of very intensive study. This review will mention, however, only results obtained after the tetrameric structure of hMDH was recognized. The structural

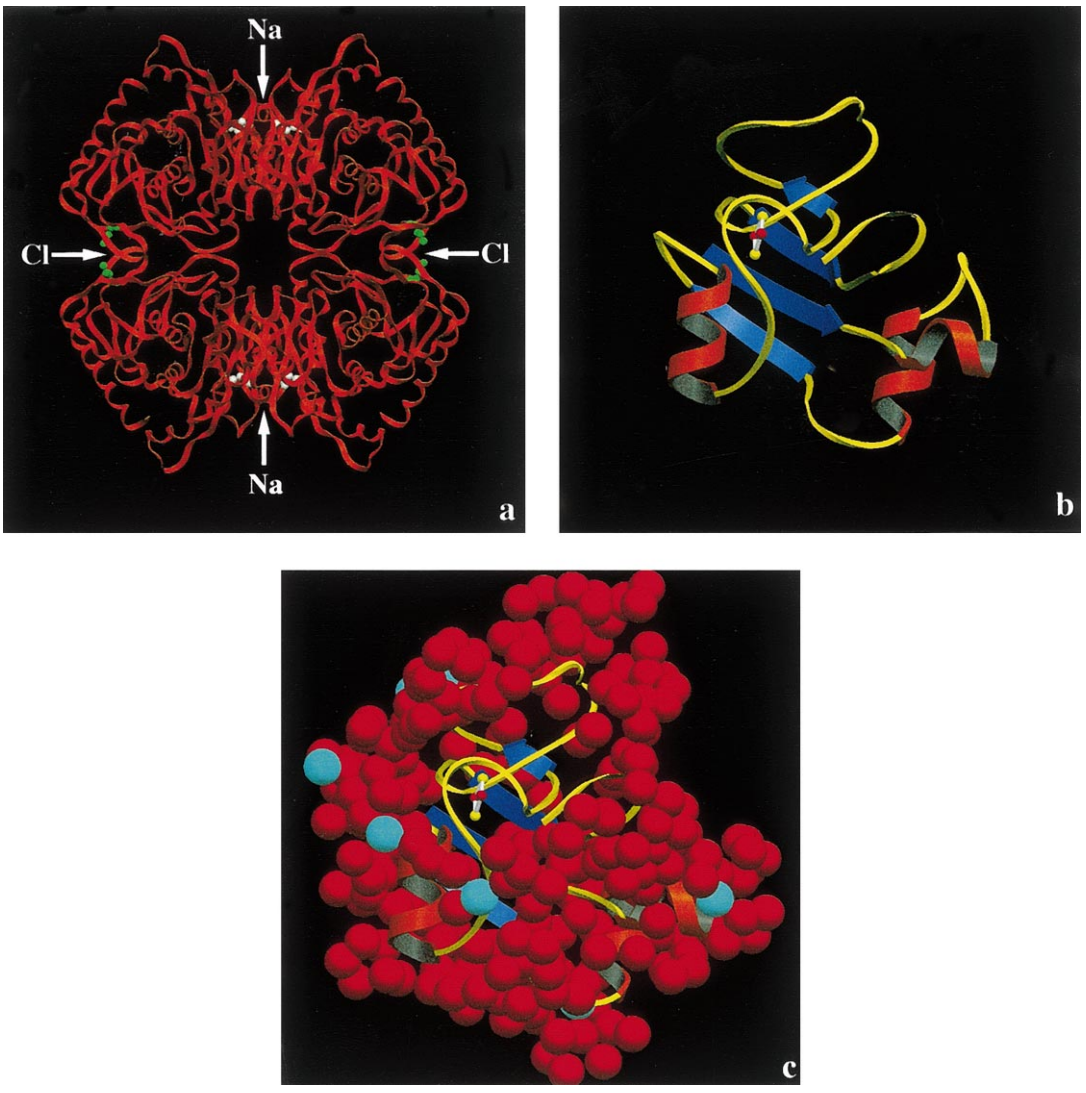

Fig. 1. (a) Ribbon representation of the tetrameric hMDH. The four glutamate residues (E247) that bind the two Na^+ ions are indicated in white at the top and bottom of the tetramer and the four lysine residues (K205) that bind the two Cl^- ions are indicated in green at the left and right sides of the tetramer. (b) Ribbon representation of the *H. marismortui* ferredoxin. The two helices on the right ($\alpha 1$ and $\alpha 2$) comprise the extra halophilic domain. (c) The tightly bound water molecules (red balls) and K^+ ions (green balls) of *H. marismortui* ferredoxin.

state of hMDH has been studied by measuring its molecular mass by ultracentrifugation, light-scattering [15,16] and neutron scattering [16] measurements. It was found that at 4 M NaCl the wild-type hMDH is a tetramer. Upon decreasing the salt concentration below 2 M, the enzyme dissociates directly into monomers as no dimer intermediates can be detected. This dissociation into monomers is accompanied by loss of the enzymatic activity and by changes in the intrinsic fluorescence and absorption properties of the enzyme. Incubation of the inactive monomers at high salt concentration leads to reconstitution of the tetramer with a concomitant reactivation of the enzyme that follows second order kinetics. When the inactive monomers are left at low salt

concentrations for extended time, the amount of the monomers that can be reactivated decreases in a first order kinetic [11].

The inactivation of hMDH upon dissociation into monomers was used to quantify the effect of salt concentration and type on the structure of hMDH. When active hMDH is exposed to low salt concentrations, activity is lost in a first-order reaction; the rate constant for this inactivation depends on the salt type but always increases as the salt concentration is decreased [8]. Thorough studies on the effects of monovalent and divalent ions [19,20], temperatures [21] and the role of anions and cations [22] in the stabilization of hMDH were performed using residual enzymatic activity measurements after long time exposure (usually 24 h) of the enzyme to the various salts and temperatures.

Not surprisingly, different salts were found to have different effects on hMDH stability. Kosmotropic salts ('salting out' salts) always stabilize hMDH. Chaotropic salts ('salting-in' salts) stabilize hMDH at low concentrations but destabilize it at higher concentrations. Intermediate salts like NaCl and KCl also stabilize the enzyme but the mode of this stabilization seems to be different from the stabilization by the kosmotropic salts. Whereas the stabilization by NaCl and KCl is enthalpy driven, the stabilization by ammonium sulfate or potassium phosphate is entropy driven [21].

2.4. NADH can stabilize hMDH at low salt concentrations and promote reassociation of monomers

We have seen above that salt can protect the tetrameric hMDH from dissociation into monomers. Interestingly, some halophilic enzymes, including hMDH, are stabilized by substrates or cofactors, preventing their inactivation even at very low salt concentrations. Hochstein and Dalton [23] have used 0.1 mM NADH to protect the activity of the halophilic NADH dehydrogenase in 0.35 M NaCl, enabling the use of ion exchange chromatography without a great loss of activity. The stabilization of hMDH by NADH was first reported by Pundak and Eisenberg [9] and had to be taken into consideration when the effect of salt on the stability of hMDH was studied. The effect of NADH and NaCl on the half-life ($T_{1/2}$) of inactivation of hMDH is shown in Fig. 2A. Strikingly, NADH in sub-millimolar range (the K_m for NADH is ~ 10 μM [18]) has the protecting capacity of molar concentrations of NaCl. This effect of NADH is not limited to protection against inactivation. As mentioned above, inactive hMDH monomers can be reacti-

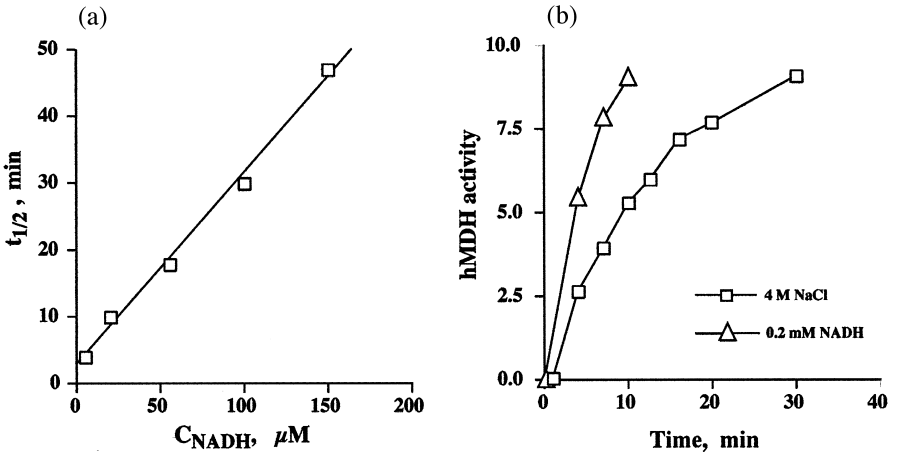

Fig. 2. (a) The dependence of the half-life ($T_{1/2}$) of hMDH at 50 mM NaCl on the concentration of NADH. (b) The kinetics of reactivation of hMDH after exposure of the monomers to 200 μM NADH (△) or 4 M NaCl (□).

vated by incubation at high salt concentrations. Fig. 2B shows the reactivation of inactive recombinant hMDH produced in *Escherichia coli* at low salt concentration at various times after incubation with 4 M NaCl or with 0.2 mM NADH in low salt concentrations. As can be seen, NADH is more effective than the salt in promoting reactivation. The significance of this observation will be discussed later.

3. The 2Fe–2S ferredoxin of *Haloarcula marismortui*

The two-iron two-sulfur ferredoxins are electron carriers in many electron transport reactions and are widely spread throughout nature. Halobacterial 2Fe–2S ferredoxin was first isolated from *Halobacterium halobium* [24] and later from *Haloarcula marismortui* [25]. Using the gene coding for *H. halobium* ferredoxin [26] as a probe, the corresponding gene from *Haloferax volcanii* was isolated and sequenced. Multiple sequence alignment shows (Fig. 3) that the three halobacterial-ferredoxins are of the plant-type but contain an additional domain of 22 residues, inserted near the N-terminus of the molecule. Plant ferredoxins are usually acidic proteins carrying a negative net charge of −12 to −18. Halobacterial ferredoxins are even more acidic, having a net negative charge of −28. These extra negative charges are located in the additional domain.

The crystal structure of the *H. marismortui* ferredoxin (HmFd) at 1.9 Å resolution has been recently published [27], and higher resolution structures have already been obtained (Frolow, unpublished data). The structure of the halobacterial ferredoxin is very similar to the plant-type structure. This ferredoxin fold consists of a four-stranded anti-parallel β-sheet flanked by α-helices (Fig. 1B). In the plant ferredoxins, the antiparallel strands β_1, and β_2 are connected by a short hairpin turn. In the halophilic ferredoxins, these two β strands are separated in sequence by the additional 22 residue domain that folds into two short helices (α_1 and α_2) that flank one side of the β-sheet, with an additional C-terminal α-helix (α_5), not found in the plant ferredoxins. The

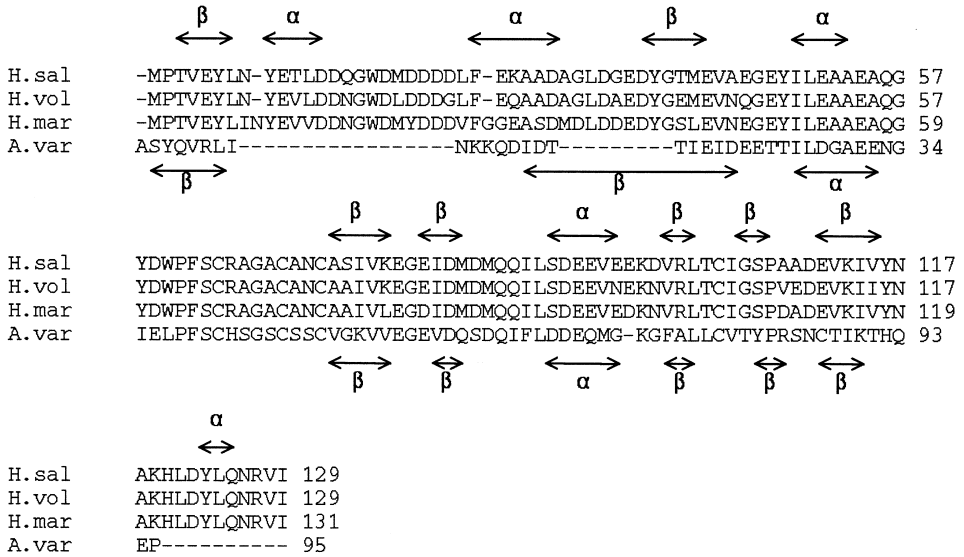

Fig. 3. Multiple amino acid sequence alignment of three halophilic ferredoxins (*H. sal, Halobacterium salinarum*; *H. vol, Haloferax volcanii; H. mar*, *Haloarcula marismortui*) and a non-halophilic one (*A. var*, *Anabaena variabilis).* The arrows above the alignment represent the structural elements of the halophilic ferredoxins as determined from the three-dimensional structure of *H. marismortui* (PDB entry 1FXA). The arrows below the alignment represent the structural elements of the non-halophilic ferredoxin (PDB entry IFRD).

other side of the β-sheet is flanked by two α-helices (α_3, α_4), as seen in the plant proteins.

The most striking feature of the HmFd structure is the preponderance of acidic residues located on the surface of the protein; this protein has the highest negative charge density of any protein structure in the Protein Data Bank. As HmFd was crystallized from 3.8 M sodium–potassium phosphate, it was expected that the highly charged surface of the protein would be covered with bound counterions. It was surprising, therefore, to find only six bound K^+ ions (see Fig. 1C); two of these ions are coordinated between side chains from symmetry-related protein molecules across the crystal interface and, therefore, might not occupy the same sites in solution. The main interactions of the other four K^+ ions with the protein are with α-carbonyl oxygens and not with the acidic side chains.

If the side chain carboxylates do not bind counterions, how are these charges neutralized? Close examination of the 1.9-Å resolution reveals a tightly bound water layer in the first hydration shell that interacts through hydrogen bonds and dipole moments with the side chain carboxylates (Fig. 1B). Higher resolution structures reveal up to five organized water layers that extend into the bulk solution. It seems that the immobile first water layer causes the organization of water far beyond the surface of the protein.

4. What can we learn from these two proteins about the halophilic adaptations of enzymes?

Before asking what makes an enzyme halophilic, we should ask what are the requirements that should be satisfied by any enzyme. The first requirement is proper folding of its polypeptide chain into a stable conformation that is soluble in its physiological environment. Being a catalyst, the enzyme must bind substrates and cofactors efficiently and enhance the conversion of the substrates into products. Such catalytic properties are very often associated with a second requirement — a certain degree of flexibility of the polypeptide chain that permits the relative movement of the various domains of the enzyme during different stages of catalysis.

Most enzymes are adapted to function in a rather limited set of physiological conditions: chemical and ionic composition of the medium, pH, temperature and pressure. Under these conditions, the polypeptides chains fold properly and their final native conformation reaches a thermodynamic minimum that is a balance between stability and flexibility. The nature of this balancing act of stability and flexibility is highlighted by a comparison of enzymes from organisms that grow at very different temperatures. For example, crystal structures of a psychrophilic MDH reveals that the polypeptide chain of this psychrophilic enzyme is flexible at low temperatures [28] while at elevated temperatures, it is denatured. In contrast, the polypeptide chain of a thermophilic MDH is rigid at the lower temperatures at which the enzyme is inactive. Thermophilic enzymes are flexible and active only at elevated temperatures.

When we consider the de novo synthesis of halophilic proteins we have to remember that in the newly synthesized unfolded protein, many hydrophobic amino acid side chains, that are usually buried in the interior of the native enzyme, are exposed to high salt concentrations. The nonspecific, intermolecular aggregation of these side chains, accentuated by the high salt, is a reaction that could compete strongly with the proper intramolecular burial of these residues during productive folding. Yet, the proteins from the halobacteria are able to fold as efficiently as many nonhalophilic proteins in their respective physiological environments. It seems, therefore, that halophilic proteins have specific structural elements that enable proper folding at high salt concentrations. Unfortunately, this aspect of halophilic adaptation has not been addressed yet experimentally.

Once folded properly, the polypeptide chain of the halophilic enzymes must assume a balance between being flexible enough to enable catalytic activity and rigid enough to avoid unfolding. Since all soluble halophilic enzymes have highly negative surface charge densities, their flexibility may be achieved by repulsion forces between close

charges. Non-halophilic enzymes, on the other hand, are usually inactive at high salt concentrations due to 'freezing' of their hydrophobic core, and therefore elimination of the dynamic motions that have been shown to be required for efficient catalysis [29,30].

The instability caused by the high surface charge density should be somehow balanced otherwise the polypeptide chain will unfold. It was long believed that one of the roles of the high salt concentration was to shield this high surface charge. Indeed, classical electrostatic calculations using Poisson–Boltzmann equation [31] suggested that at pH 7 the stability of halophilic proteins is decreased by 18.2 kcal/mol on lowering the salt concentration from 5 to 0.1 M.

This explanation for the role of salt in halophilic protein stabilization is challenged by two experimental results. First, the high resolution three-dimensional structure of HmFd, described above, demonstrates very clearly that although the protein was crystallized from 3.8 M sodium–potassium phosphate, very few counterions were found to be bound to the protein and when bound, they interact with the main-chain α-carbonyl oxygen and not with the side-chain carboxylates. Second, sub-millimolar concentrations of NADH can effectively replace the requirement for molar quantities of salt in the stabilization of hMDH. Therefore, neutralization of surface charge by salt is not required for protein stability.

Using thermodynamic theories to analyze various biophysical measurements, Bonnete et al. [15] have calculated that, in its native state at 4 M NaCl, hMDH binds approximately 200 molecules of salt and almost 3000 molecules of water. These values are significantly higher than those measured for non-halophilic proteins under the same conditions and also higher than the number of salt and water molecules bound in low salt solutions in which the halophilic enzyme is unfolded. These findings were the basis for the 'halophilic stabilization model' for solutions of NaCl, KCl and $MgCl_2$ [21]. According to this model the tertiary and quaternary structures of native halophilic proteins co-ordinate hydrated salt ions on their surface at higher local concentrations than in the surrounding solution by specific interactions with the surface carboxyl groups. Through the binding of the hydrated salt ions, water molecules would be associated with the protein structure with different local salt concentrations depending on the hydrated interactions of the particular salt. When the bulk salt concentration is reduced, salt will diffuse from the 'quasi-crystalline' protein-associated layer into the bulk, destabilizing the protein surface and causing dissociation of the enzyme into its subunits and unfolding of the polypeptide chain. According to this model, the stabilization is enthalpy driven; the entropic penalty derived from the organization of the hydrated salt is compensated by the enthalpy of binding the hydrated salt to the surface carboxyl groups.

The stabilization of halophilic proteins by kosmotropic salts, such as potassium phosphate, is by the classical salting-out mechanism. Namely, the hydrophobicity of the protein core in high concentrations of potassium phosphate is sufficient to stabilize the active enzyme, and the outer domains in its structure participate in normal hydration interactions. This stabilization is entropy driven and therefore, as shown experimentally, the enzyme is inactivated at low temperatures [19].

Although this model explains many experimental observations, no structural evidence exists for the existence of hydrated salt complexes on the surface of halophilic proteins. High-resolution structures of HmFd, in the presence of potassium phosphate, show that only a few K^+ ions are bound to the main polypeptide chain. However, according to the model described above, hydrated complexes are not expected in kosmotropic salts. In the recently published 2.6-Å structure of hMDH in the presence of sodium chloride [17], only two Na^+ and two Cl^- ions were detected per tetramer. It is possible that a resolution above 2.6 Å is required in order to observe the hydrated salt structures.

We would like to conclude this communication by presenting a modified explanation for the basis of halophilic adaptation. As mentioned above, all halophilic proteins are highly negatively charged.

These charges have two roles: (1) they provide hydrated carboxylate groups that maintain the solubility of the proteins at high salt concentrations; and (2) their destabilizing electrostatic repulsion offsets the stabilization from the salt's enhancement of hydrophobic effect, given that the solvent prefers to interact with the folded rather than with the unfolded polypeptide. The presence of molar quantities of salt would rigidify the enzyme's hydrophobic core, forcing the enzyme structure into a deep free energy minimum that would freeze out the dynamic fluctuations and flexibility of the protein necessary for catalytic activity. The electrostatic repulsion allows the enzyme to maintain the marginal stability that is the hallmark of protein structure, even in the presence of very stabilizing salts. The salt-induced stabilization of the protein can arise, in the case of kosmotropoic ions, like phosphates that are excluded from the protein surface, by ordering of the bulk solvent, but also through a direct interaction of ions with the folded protein. The requirement for high NaCl or KCl concentrations for the stabilization of hMDH can be simply explained by specific, but low affinity binding of a few ions to the folded protein. Therefore, molar amounts of salt are necessary to saturate these binding sites. The different efficacy of stabilization of different salts might reflect a combination of their position in the Hoffmeister series in enhancing the hydrophobic effect and the affinity of the specific ion for these sites. Salts that do not stabilize the folded state, or that stabilize the folded state at low salt concentrations and destabilize it at high concentrations are those that will interact with the specific stabilizing sites at low concentration but will interact preferentially with the unfolded polypeptide at high salt concentrations, shifting the equilibrium to the unfolded state.

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References

[1] W.D. Grant, R.T. Gemmell, T.J. McGenity, Extremophiles 2 (1998) 279.
[2] R.L. Baldwin, Biophys. J. 71 (1996) 2056.
[3] M.T. Record, W. Zhang, C.F. Anderson, Adv. Protein Chem. 51 (1998) 281.
[4] J.K. Lanyi, Bacteriol. Rev. 38 (1974) 272.
[5] H. Eisenberg, M. Mevarech, G. Zaccai, Adv. Protein Chem. 43 (1992) 1.
[6] P.K. Holmes, H.O. Halovorson, J. Bacteriol. 90 (1965) 312.
[7] M. Mevarech, H. Eisenberg, E. Neumann, Biochemistry 16 (1977) 3781.
[8] M. Mevarech, E. Neumann, Biochemistry 16 (1977) 3786.
[9] S. Pundak, H. Aloni, H. Eisenberg, Eur. J. Biochem. 118 (1981) 471.
[10] M.H. Reich, Z. Kam, H. Eisenberg, Biochemistry 21 (1982) 5189.
[11] M. Mevarech, Ph.D. thesis, Weizmann Institute of Science, Rehovot Israel, 1977.
[12] F. Cendrin, J. Chroboczek, G. Zaccai, H. Eisenberg, M. Mevarech, Biochemistry 32 (1993) 4308.
[13] O. Dym, M. Mevarech, J.L. Sussman, Science 267 (1995) 1344.
[14] E. Daniel, A. Azem, I. Shaked, M. Mevarech, Comp. Biochem. Physiol. 106B (1993) 401.
[15] F. Bonnete, C. Ebel, G. Zaccai, H. Eisenberg, J. Chem. Soc. Faraday Trans. 89 (1993) 2659.
[16] D. Madern, C. Ebel, A Mevarech, S.B. Richard, C. Pfister, G. Zaccai, Biochemistry 39 (2000) 1001.
[17] S. Richard, D. Madern, E. Garcin, G. Zaccai, Biochemistry 39 (2000) 992.
[18] K. Hecht, A. Wrba, R. Jaenicke, Eur. J. Biochem. 183 (1989) 69.
[19] F. Bonnete, D. Madern, G. Zaccai, J. Mol. Biol. 244 (1994) 436.
[20] D. Madern, G. Zaccai, Eur. J. Biochem. 249 (1997) 607.
[21] G. Zaccai, F. Cendrin, Y. Haik, N. Borochov, H. Eisenberg, J. Mol. Biol. 208 (1989) 491.
[22] C. Ebel, P. Faou, B. Kernel, G. Zaccai, Biochemistry 38 (1999) 9039.
[23] L.I. Hochstein, B.P. Dalton, Biochim. Biophys. Acta 302 (1973) 216.
[24] L. Kerscher, D. Oesterhelt, FEBS Lett. 67 (1976) 320.
[25] M.M. Werber, M. Mevarech, Arch. Biochem. Biophys. 187 (1978) 447.
[26] F. Pfeifer, J. Griffig, D. Oesterhelt, Mol. Gen. Genet. 239 (1993) 66.
[27] F. Frolow, M. Harel, J.L. Sussman, M. Mevarech, M. Shoham, Nat. Struct Biol. 3 (1996) 452.
[28] S.Y. Kim, K.Y. Hwang, S.H. Kim, H.C. Sung, Y.S. Han, Y. Cho, J. Biol. Chem. 274 (1999) 11761.
[29] G.P. Miller, S.J. Benkovic, Chem. Biol. 5 (1998) R105.
[30] M.R. Sawaya, I Kraut, Biochemistry 36 (1997) 586.
[31] A.H. Elcock, J.A. McCammon, J. Mol. Biol. 280 (1998) 731.