Extremophiles (2000) 4:91–98

MINI-REVIEW

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Halophilic adaptation of enzymes

Received: July 11, 1999 / Accepted: December 27, 1999

Abstract It is now clear that the understanding of halophilic adaptation at a molecular level requires a strategy of complementary experiments, combining molecular biology, biochemistry, and cellular approaches with physical chemistry and thermodynamics. In this review, after a discussion of the definition and composition of halophilic enzymes, the effects of salt on their activity, solubility, and stability are reviewed. We then describe how thermodynamic observations, such as parameters pertaining to solvent–protein interactions or enzyme-unfolding kinetics, depend strongly on solvent composition and reveal the important role played by water and ion binding to halophilic proteins. The three high-resolution crystal structures now available for halophilic proteins are analyzed in terms of haloadaptation, and finally cellular response to salt stress is discussed briefly.

Key words Halophilic Archaea · Halobacteria · Stabilization · Solubility · Protein–solvent interactions · Malate dehydrogenase

What is a halophilic enzyme?

Since the extensive review of Eisenberg et al. (1992), the number of publications on halophilic proteins has grown considerably. From the Greek roots *hals*, meaning salt, and *phil*, meaning loving or friendly with, halophily indicates that salt is required for function. Halophilic enzymes have been defined with respect to the halophily of the organism from which they are fractionated, or with respect to their own salt requirements for activity, stability, or solubility. In this review, we define a halophilic protein as one extracted from a halophilic microorganism that requires at least 2.5 M NaCl in the medium for optimum growth (Kushner and Kamekura 1988). Proteins from microorganisms with lower salt requirements are therefore excluded from our definition, even if they appear to require a high salt concentration for activity or stability. Three groups of halophilic microorganisms have been identified: aerobic halophilic Archaea, anaerobic halophilic methanogenic Archaea, and the halophilic bacteria (Kamekura 1998). All the well-characterized halophilic enzymes so far have been purified from the first group (*Halobacteriaceae*). These microorganisms accumulate high concentrations of KCl in their cytoplasm, approaching saturation (Christian and Waltho 1962; Ginzburg et al. 1970).

Communicated by K. Horikoshi

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The amino acid composition of halophilic proteins

Since the first amino acid composition determinations, it has become clear that halophilic enzymes present a higher proportion of acidic amino acid residues than their nonhalophilic homologues (Lanyi 1974). A statistical analysis of 26 soluble proteins confirmed the acidic nature of the halophiles and showed a significantly lower Lys content, an increase in small hydrophobic residues (Gly, Ala, Val), and a decrease in aliphatic residues (Madern et al. 1995).

To be active, stable, and soluble in high salt are major challenges facing proteins in halophilic microorganisms

High salt concentrations affect the conformational stability of proteins and, in general, salt conditions that favor solubility destabilize the folded form and vice versa (von Hippel and Schleich 1969). Halophilic proteins have, therefore, evolved specific mechanisms that allow them to be both stable and soluble in the high cytoplasmic KCl concentra-

tion. As discussed below, these adaptive mechanisms are reflected in the solvent interactions and three-dimensional structures of halophilic proteins. Stability and activity are strongly related to protein dynamics, which is itself solvent environment dependent (e.g., Lehnert et al. 1998; Cordone et al. 1999). We are currently exploring the dynamics of halophilic proteins as a function of salt conditions by neutron scattering.

Halophilic enzyme activity

It is not clear whether a general behavior pattern for the salt dependence of activity can be established for the few enzymes purified from halophilic Archaea. Systematic determinations of the catalytic efficiency (K_{cat} / K_M) as a function of salt type, concentration, and temperature remain to be performed and compared with those of nonhalophilic homologous proteins. Certain trends have, nevertheless, been observed.

Enzyme activity decreases with increasing salt concentration in the molar range for malate dehydrogenase from *Haloarcula marismortui* (*Hm* MalDH) (Mevarech et al. 1977), peptidyl-prolyl *cis-trans* isomerase from *Halobacterium cutirubrum* (Nagashima et al. 1994), 1-phosphofructokinase from *Haloarcula valismortis* (Rangaswamy and Altekar 1994), catabolic ornithine transcarbamylase of *Halobium salinarium* (Ruepp et al. 1995), ribulosebisphosphate carboxylase and glucose dehydrogenase from *Haloferax mediterranei* (Rajagopalan and Altekar 1994; Bonete et al. 1996), dihydrolipoamide dehydrogenase from *Haloferax volcanii* (Jolley et al. 1996), and for seryl-tRNA synthetase from *Haloarcula marismortui* (Taupin et al. 1997). It was observed at first that the enzymatic activity of *Hm* MalDH reaches its maximum at a concentration at which the enzyme is unstable (Mevarech et al. 1977). This observation was in fact biased by the strong salt-dependent K_M variation for oxaloacetate, which increases as the salt concentration increases. At saturating concentration of the substrate for each salt concentration, the enzymatic activity of *Hm* MalDH remains constant over the NaCl concentration range where the enzyme is stable (Hecht et al. 1989). The salt-dependent K_M of substrate for other enzymes has not been reported.

In contrast to the enzymes discussed, the activity of β-galactosidase from *Haloferax alicantei* increases with salt concentration to a maximum at 4 M NaCl (Holmes et al. 1997). More strikingly, the two activities of catalase peroxidase from *Halobacterium salinarum* have a different dependence on NaCl concentration (Brown-Peterson and Salin 1993). The uncoupling of the two activities also appeared in the same enzyme from *Haloarcula marismortui* as a function of pH at constant NaCl concentration (Cendrin et al. 1994).

Enzyme activity depends on the nature of the salt. For some halophilic enzymes, activity in KCl is significantly higher than in NaCl. A complex salt dependence has been observed for 3-hydroxy-3-methylglutaryl-coenzyme A reductase from *Haloferax volcanii*. Its activity increases with increasing KCl concentration and decreases when NaCl concentration increases (Bischoff and Rodwell 1996).

Halophilic enzyme solubility

Halophilic enzymes are very soluble in highly concentrated NaCl solutions, making the use of standard chromatographic prodecures difficult. A first, efficient, chromatographic step including high ammonium sulfate (AS) concentration to fix the proteins on a Sepharose 4B matrix, followed by their elution using a decreasing gradient of ammonium sulfate concentration (Mevarech et al. 1976), is now routinely used for the purification of various halophilic proteins. In the case of halophilic proteins expressed in *E. coli*, the first fractionation steps in molar NaCl and AS precipitate most of the host proteins, while the halophilic protein remains in solution (Cendrin et al. 1993). Protein concentrations of 200 mg/ml of *Hm* MalDH in 4 M NaCl are easily obtained. For biophysical experiments requiring high protein concentration, we have prepared samples of 100 mg/ml in 3 M KCl, 80 mg/ml in 0.5–1.3 M $MgCl_2$, and 60 mg/ml in 2 M AS.

The weak protein–protein interactions between *Hm* MalDH molecules in various salt solutions have been characterized by small-angle neutron and X-ray scattering (SAXS and SANS) (Ebel et al. 1999a). The second virial coefficient A_2 is calculated from the scattering curves as a function of protein concentration. A positive value of A_2 indicates repulsion between the macromolecules, favoring solubility; a negative value indicates attraction, favoring aggregation (George and Wilson 1994; Ducruix et al. 1996). Positive values were found, for example, for *Hm* MalDH in 2–5 M NaCl; a value close to zero was found in 3 M AS, where the enzyme precipitates, and a negative value was determined in 1 M NaCl, 30% 2-methylpentane-2,4-diol (MPD), crystallizing conditions for the protein (Richard et al. 1995). The halophilic protein distributions found by SAXS and SANS in various salt solutions are currently being modelled using intermolecular potentials (Costenaro and Ebel, personal communication).

Halophilic enzyme stability under different solvent conditions

Since the pioneering studies of Hofmeister more than 100 years ago, salt effects on nonhalophilic proteins have been studied extensively. Some salts increase protein stability and, at high concentration, favor precipitation. These "salting-out" salts are frequently used by biochemists for fractionation and protein crystallographers to obtain crystals. In contrast, "salting-in" salts favor solubility but unfold the protein at high concentration (von Hippel and Schleich 1969; Schellman 1987; Timasheff 1992).

In rigorous terms, the conformational stability of a protein is given by the free energy difference between the folded and unfolded states. To our knowledge, measurements of this difference have never been performed for halophilic proteins. Instead, the stability of an enzyme is usually assayed by residual activity measurements following incubation for a given length of time in given conditions (e.g., salt type, concentration, temperature). The activity measurement is performed by dilution into standard conditions, where the enzyme is stable. Refolding during the activity measurement is negligible, and the fraction of enzymatic activity detected is proportional to the amount of native (folded) enzyme remaining after incubation. For *Hm* MalDH in most salt conditions, intrinsic fluorescence, circular dichroism, and residual activity variations were found to superimpose, demonstrating that deactivation and unfolding are concomitant (Pundak et al. 1981; Ebel et al. 1999b). Bonneté et al. (1994) showed that the residual activity measurement can be a measure of unfolding kinetics.

Most of the halophilic enzymes studied are inactivated when the NaCl or KCl concentration of the solution decreases to less than 2 M. In early work on *Hm* MalDH, the salt concentration-dependent deactivation was shown to be of first-order kinetics, with a lower rate of unfolding in NaCl compared to KCl (Pundak et al. 1981). NaCl or KCl can be replaced by other ions or solutes; salting-out conditions such as high concentrations of sulfate or phosphate salts, but also $MgCl_2$ or $CaCl_2$, which are usually considered to be salting-in (Madern and Zaccai 1997; Taupin et al. 1997).

The temperature dependence of deactivation kinetics led to the concept that the halophilic protein "adapts" to different solvent environments through compensating entropy-driven and enthalpy-driven mechanisms (Bonneté et al. 1994). Thermal inactivation of *Hm* MalDH was investigated in various salt conditions (Zaccai et al. 1989; Bonneté et al. 1994; Madern and Zaccai 1997). In KCl or NaCl, a positive activation enthalpy term ($\Delta H\#$) governs the activation free energy of unfolding $\Delta G\#$, whereas in salting-out conditions such as molar phosphate, sulfate, or D_2O solutions, $\Delta G\#$ values result from compensation between activation entropy ($T\Delta S\#$) and $\Delta H\#$ terms. This result led to the observation of decreasing stability as the temperature is lowered, or cold unfolding, a phenomenon difficult to see in nonhalophilic proteins (Privalov et al. 1986), which was predicted from studies on protein stability (Pace 1990).

A bell-shaped stabilization curve is observed in the divalent cation solutions, the protein being deactivated at high as well as low salt concentration. The temperature dependence of the low-concentration and high-concentration parts of the residual stability curves in $MgCl_2$ and $CaCl_2$ is strikingly different. On the low-concentration side of maximum stability, the temperature profiles are like those observed in NaCl and KCl, suggesting similar (activation enthalpy-dominated) mechanisms. On the high-concentration side, the residual activity value decreases with increasing salt concentration and the temperature profile shows cold destabilization, as expected in salting-in conditions such as guanidinium hydrochloride for nonhalophilic proteins.

The thermal deactivation curves for halophilic GAPDH in KCl and potassium phosphate, respectively, also show distinctive enthalpic and entropic dominance (Krishnan and Altekar 1993), suggesting that observations from *Hm* MalDH might well be valid for other halophilic proteins.

In a systematic study of ion effects, it was found that the stabilization of *Hm* MalDH results from a subtle equilibrium between the respective influence of anions and cations (Ebel et al. 1999b). When a cation or anion is very stabilizing, the effect of the salt ion of opposite charge is limited. Anions of high charge density (e.g., SO_4^{2-}, acetate^{2-}, F^-) are the most efficient to stabilize the folded form, in accordance with the Hofmeister series, whereas cations of high charge density (e.g., Mg^{2+}, Ca^{2+}, Li^+) are stabilizing only at low concentrations and tend to denature *Hm* MalDH at high concentrations. These effects are only marginally related to the surface tension of the solution and were attributed mainly to the presence of strong or weak ion-binding sites on the folded form. Specific interactions at high concentration of anions of low charge density and cations of high charge density with the peptide bond promotes unfolding of *Hm* MalDH, as observed for nonhalophilic proteins (Ebel et al. 1999b).

Halophilic enzymes can also be stabilized by significantly lower concentrations of NaCl or KCl, in the presence of heavy water (D_2O), or in certain organic solutes, such as glycerol or members of the betaine family, which can therefore be considered as salting-out. This result was shown for *Hm* MalDH (Bonneté et al. 1994; Vuillard et al. 1995), β-galactosidase from *Haloferax alicantei* (Holmes et al. 1997), and glutamate dehydrogenase from *Halobium salinarium* (Cadenas and Engel 1994). We note that these are stabilizing conditions also for nonhalophilic proteins.

The stabilizing effect of D_2O can be understood in terms of strengthening both the hydrophobic effect (i.e., apolar groups are even less soluble in D_2O than in H_2O) and the hydrogen bond (the D-bond is stronger than the H-bond). The deactivation at high salt concentrations of both $MgCl_2$ and $CaCl_2$, however, was not sensitive to the water isotopic effect, showing that D_2O does not compensate for the salting-in effect of these conditions (Madern and Zaccai 1997).

Halophilic enzyme stability has therefore been studied in a large range of salt conditions, in some of which it behaves similarly to the stability of nonhalophilic proteins. Specific halophilic behavior appears in molar KCl or NaCl and on the low-concentration side of stabilization curves in $MgCl_2$ or $CaCl_2$, where deactivation barriers are enthalpy dominated.

The stabilization studies on *Hm* MalDH were performed on the apo-protein (without the bound coenzyme). In millimolar NADH, the stability of the enzyme is shifted toward significantly lower salt concentrations (Pundak et al. 1981; Madern and Zaccai 1997). The potential protective effect of ligand binding against low-salt deactivation was not tested for other halophilic enzymes that require coenzyme.

Determination of molar mass and solvent interactions for halophilic enzymes

Folding and stabilization processes of the protein native state cannot be elucidated without an understanding of protein–solvent interactions (Dill 1990). The role of thermodynamics in the determination of biological macromolecule molar mass and solvent interactions has been reviewed by Eisenberg (1995). Molar mass measurements are not straightforward. Despite manufacturers' claims, for example, gel-exclusion chromatography provides a measure of diffusion coefficient and not of molar mass. The determination of molar mass by rigorous biophysical methods (analytical ultracentrifugation, AUC, SAXS, and SANS) also provides information on solvent–macromolecule interactions. By measurements of interaction parameters it is possible to probe the amounts of salt and water associated with a protein in a given salt solution (Eisenberg 1994). In this way, Timasheff (1992) and coworkers found that solvent interactions of the folded and unfolded forms of certain nonhalophilic proteins were correlated with stability. Solvent interactions were found to be particularly strong in halophilic proteins, with significant water and salt binding to the native state of *Hm* MalDH in NaCl or KCl solvent (Pundak and Eisenberg 1981; Calmettes et al. 1987; Bonneté et al. 1993). This result is in contrast to nonhalophilic proteins, for which there is no salt binding to the native state, in NaCl solvents, and salt binding to the unfolded state is observed only in the case of salting-in conditions such as guanidinium hydrochloride solvents.

For many years after its first purification, *Hm* MalDH was thought to be a dimer in solution. Because of the excess negative charge, its migration on SDS-PAGE is anomalous, suggesting a significantly heavier protomeric polypeptide than in reality (this is the case for many halophilic proteins, for which the apparent molecular weight on the gel could be overestimated by as much as 50%). After the precise molar mass of the *Hm* MalDH polypeptide was calculated from its gene and confirmed by mass spectroscopy (Cendrin et al. 1993), the tetrameric structure was firmly established by complementary measurements of molar mass and solvent interactions with SANS, AUC, and densimetry (Bonneté et al. 1993). It was confirmed that significant salt and water binding was associated with the native state of the enzyme in molar NaCl or KCl solutions. Biophysical studies of two other halophilic proteins, elongation factor Ef1α (or EFTu) and glyceraldehyde-3-phosphate dehydrogenase, revealed that their native state also binds significant amounts of salt in concentrated NaCl or KCl solution (Ebel et al. 1992, 1995).

Solvent interaction parameters were determined for *Hm* MalDH for various salt conditions. In salt concentration ranges where the enzyme is stable, these parameters are strongly dependent on the nature of the salt. The numbers of salt and water molecules in the solvation shell are different for $MgCl_2$ (on the low-concentration side of the stability curve) and for NaCl or KCl, but in the three cases correspond to a salt concentration in the molal range that is similar to or larger than that in the bulk solvent. A strong inverse correlation was found between the hydration of the salt ions and the minimum salt concentration required for stability, in the order $CaCl_2$, $MgCl_2$, NaCl, and KCl (Madern and Zaccai 1997). Salt binding in close-to-physiological conditions, therefore, appeared to be a specific characteristic of molecular adaptation to halophilic conditions. In high concentrations of potassium phosphate or ammonium sulfate, on the other hand, salt exclusion (negative binding) was determined, as for nonhalophilic proteins in salting-out conditions (Zaccai et al. 1986a,b, 1989; Ebel et al. 1998; C. Ebel, personal communication).

The solvation-stabilization model for *Hm* MalDH

The extensive data on *Hm* MalDH have shown that in strongly salting-out (e.g., molar ammonium sulfate) or salting-in (e.g., molar $CaCl_2$) conditions, solvation and thermal stabilization are similar to those of nonhalophilic proteins. However, the solvation parameters of the halophilic enzyme in molar NaCl and KCl solutions showing significant salt binding, correlated with enthalpy-dominated activation free energy of unfolding, appeared to reflect specific halophile behavior. An attempt to explain these observations led to a solvation-stabilization model (Zaccai et al. 1989; Zaccai and Eisenberg 1990). The model for *Hm* MalDH is based on three key observations:

1. Enthalpic mechanisms dominate the kinetic deactivation in molar KCl, NaCl, and on the low-concentration side of the stability curve in $MgCl_2$.
2. The folded protein binds relatively large amounts of salt and water in KCl, NaCl, or $MgCl_2$ solvents.
3. The excess of acidic amino acids in the protein composition could provide favored sites for specific water and ion binding to the tertiary or quaternary structure.

In the model, the protein forms a particle stabilized by cooperative hydrated ion networks, organized by carboxyl groups in the tertiary or quaternary structure. Why does the binding have to be cooperative? Carboxyl groups are known to be strongly hydrated whether in the folded or unfolded form of a polypeptide (Kuntz 1971). Ion and water binding would contribute to the stabilization of the folded form (as suggested by the activation enthalpy), however, only if it were enhanced by the folded structure, with respect to the usual solvation of carboxyl groups in the unfolded state. A prediction of the model that could be explored by site-directed mutagenesis and crystallographic studies was, therefore, that halophilic adaptation resulted in a specific arrangement of carboxyl groups in the folded structure, and not only in an excess of negative charge in the protein composition.

Site-directed mutagenesis

We concentrate here on haloadaptation, and site-directed mutagenesis to study enzyme function in halophilic proteins

is not discussed. In the framework of the solvation-stabilization model, it was expected that a modification of the protein–solvent interactions would have a strong impact on stability under halophilic conditions. Before the crystal structure was solved, a mutant of *Hm* MalDH was designed in which a glutamic acid residue predicted to be in contact with the solvation network was replaced by arginine (the residue present at the equivalent position in nonhalophilic homologues of the enzyme). This mutant was characterized and shown to require higher concentrations of NaCl, or a lower temperature, for equivalent stability with the wild type. The kinetic parameters for enzymatic activity and spectroscopic properties were not affected by the mutation, suggesting that the modified behavior was not caused by a change in protein structure (Madern et al. 1995). The crystallographic structures and of this mutant and of the wild-type *Hm* MalDH discussed next (Dym et al. 1995; Richard et al. 2000) guided further explorations of protein stability and provided further information on the role of protein–solvent interactions.

A mutant in which arginines involved in salt bridge clusters of *Hm* MalDH located at the dimer-dimer was designed and characterized (Madern et al. 2000). Surprinsingly, even though in the crystal structure of Dym et al. (1995) these salt bridges appeared as the only link between two dimers that make up the tetramer, the resulting protein was still a tetramer above 2 M NaCl. At lower salt concentrations, it dissociates toward an active dimer. The analysis of the higher-resolution structure of Richard et al. (2000) provided a rational explanation for these observations. In fact, other salt-dependent protein–solvent interactions are involved in dimer–dimer stabilization and are sufficient to maintain the tetramer assembly at high salt, even when the arginines involved in the salt bridge clusters are mutated.

Stability mutants were also studied in another halophilic enzyme. In a model-building study of *Haloferax volcanii* dihydrolipoamide dehydrogenase, a potential K^+-binding site was located at the dimer interface of the enzyme. Disruptive mutations of one of the four acidic amino acids involved in the site (E423) modified the salt-dependent activity without any effects on the thermal deactivation in 2 M KCl. In contrast, a conservative mutation E→D strongly alters the thermal stability without affecting enzyme activity, which remains close to that measured for the wild type (Jolley et al. 1997). The authors warned, however, that caution must be exercised in the interpretation of results based on model building rather than crystallographic data.

Crystallographic structures of halophilic proteins

Only three crystallographic structures of halophilic proteins have been solved so far. The extensive biochemical and biophysical results discussed earlier established that haloadaptation cannot be considered without taking protein–solvent interactions into account. In this context, what information has been extracted from the analysis of the crystal structures of two monomeric and one tetrameric halophilic proteins?

In contrast, to other halophilic enzymes, the amino acid composition of dihydrofolate reductase from *H. volcanii* (*Hv* DHFR) is not significantly different from its nonhalophilic homologues, especially with relation to charged residues (Böhm and Jaenicke 1994a). The hydrogen-bonding pattern and the surface area and volume of *Hv* DHFR found in the crystal structure are similar to those in other nonhalophilic DHFR (Pieper et al. 1998). Clusters of acidic residues and two facing glutamic residues could lead to low-salt instability. The salt-dependent stability of *Hv* DHFR, however, has not been fully characterized. Solvent interactions depend strongly on the nature of the salt. In the salting-out phosphate buffer (2.4 M) used for crystallization, salt exclusion and water binding are expected. Three phosphate ions and 80 water molecules were found, but the resolution limit of the crystal structure did not allow a complete analysis of the solvent.

Ferredoxin from *Haloarcula marsimortui* (*Hm* Fd) was solved to 1.9 Å, the highest resolution achieved for a halophilic protein, making possible an analysis of the protein–solvent interactions in the crystallization conditions (3.8 M phosphate) (Frolow et al. 1996). There are 34 acidic amino acid residues on the *Hm* Fd surface. The number of water molecules around carboxylates varies between 2 and 6, significantly higher than the average of 1.9 per residue of *Hm* Fd. Compared to other ferredoxins, *Hm* Fd has an N-terminal extension containing 15 negative charges. This "halophilic addition" was proposed to play a major role in the behavior of *Hm* Fd by providing a higher solvent-accessible surface area. There are no unfavorable interactions between facing carboxylate groups such as described in *Hv* DHFR. The number of water molecules observed in a crystal structure depends strongly on the quality and resolution of the data as well as on crystal conditions (Bon et al. 1999); 237 water molecules were identified in the *Hm* Fd structure. Most of the negative charges on the protein surface are surrounded by water molecules, and bound surface water molecules show extensive hydrogen bonding. Based on this observation, the authors suggested that haloadaptation involves better water-binding capacity. Note, however, that the high-phosphate crystallization conditions are typically salting-out conditions in which water binding and salt exclusion are expected. Four K^+ were located at the protein surface and two others were found between adjacent protein molecules in the crystal.

Tetrameric *Hm* MalDH in its holo form (with bound NAD) was the first halophilic enzyme structure to be solved (Dym et al. 1995) and the only oligomeric one so far. It was crystallized from a 1.8 M NaCl solution by using MPD as the precipitating agent. The crystallization phase diagram has been explored systematically by Richard et al. (1995). Because of aggregation, however, it has not been possible to determine protein–solvent interactions in NaCl/MPD solvents. The hope is that it is the particle made up of protein, bound NaCl, and water molecules which is crystallized. This first crystal study was at 3.2 Å resolution and contained no information on protein–solvent interactions.

Nevertheless, other important structural features were described that could play an important role in the halophilic as well as the thermoadaptation of *Hm* MalDH; these include acidic residues at the N-terminus of alpha helices and an increase in their Ala content. More salt bridges were found in the *Hm* MalDH structure than in the dogfish lactate dehydrogenase that had been chosen as the homologous protein for molecular replacement. At the dimer–dimer interface in the tetramer, two clusters of complex salt bridges were found in which an Arg interacts with more than one acidic residue. Since this work, crystal structures of many hyperthermophilic proteins have been published in which an increase of intersubunit salt bridge networks is frequently found, compared to mesophilic proteins (Hennig et al. 1995; Aguilar et al. 1997; Villeret et al. 1998). Analysis of these data suggested that oligomerization acts as a stabilization mechanism that would also be observed in other proteins isolated from extremophiles.

A pertinent analysis has been performed by Elcock and McCammon (1998) on the crystal structures of *Hm* Fd and *Hm* MalDH to evaluate how electrostatic interactions contribute to the stability of halophilic proteins as a function of salt concentration and pH. They used the Poisson Boltzmann equation to compare calculated pK_a values of ionizable residues for the folded and unfolded forms of *Hm* Fd and for folded monomers and the tetrameric assembly of *Hm* MalDH. At pH 7, the clustering of acidic residues on the surface of halophilic proteins results as an inherently destabilizing feature at all salt concentrations unless the residues participate in salt bridge formation. It was concluded that they were important for haloadaptation because of their contribution to solubility. Specific ion-binding sites as postulated by the solvation-stabilization model, however, could not be taken into account in the electrostatic calculations. The results, however, may contain hints as to where they may occur. For example, acidic groups in *Hm* MalDH whose pK_a values decrease on tetramer formation would form very favorable cation-binding sites.

We recently solved the crystallographic structures of a mutant of *Hm* MalDH and the wild-type protein, both in the absence of NADH (Richard et al. 2000). This work was done in parallel with a mutagenesis study of the complex intersubunit salt bridges (Madern et al. 2000). It highlighted a variety of interesting protein–solvent features involved in halophilic adaptation. A sum of such interactions appeared to contribute to the stability of the *Hm* MalDH tetramer in high salt. The enzyme can be considered as a dimer of dimers. A central cavity contains a large network of ordered water molecules linking two dimers. The contact region between dimers is made up of complex salt bridges, similar to what was observed in thermophilic proteins. In a novel feature, however, the salt bridge clusters in *Hm* MalDH are "locked" in by bound chloride ions. Other salt bridge clusters at the monomer–monomer interface bind sodium ions. The intersubunit salt bridge clusters are accessible to the solvent, which suggests that they would be destabilized by high-salt concentrations; solvent ions would compete with acidic and basic side chain charges. The specific binding of chloride and sodium ions by the salt bridge clusters provides a mechanism to favor salt bridge stability in high salt. The crystallographic analysis combined with results from salt bridge disruption experiments in *Hm* MalDH (see earlier section on site-directed mutagenesis) showed that halophilic adaptation is not aimed uniquely at "protecting" the enzyme from the extreme salt conditions, as may have been expected, but on the contrary consists of harnessing the high-salt concentration in the environment through solvent ion binding.

Structural model building

Conclusions from homology-based structural models of halophilic enzymes should be considered with caution. For example, acidic clusters modeled in *Hv* DHFR and postulated to contribute to low-salt instability through charge repulsion (Böhm and Jaenicke 1994b) were found not to be in electrostatic interaction when the crystal structure was solved (Pieper et al. 1998). A homology model of the hexameric glutamate dehydrogenase from *Halobacterium salinarum* shows a surface covered by acidic residues except in the vicinity of the active site (Britton et al. 1998). Symmetry-related salt bridge networks were modeled between subunits, similar to those observed in *Hm* MalDH. It was also calculated that a reduction in lysine residues contributes significantly to the decrease in hydrophobic character of the protein surface. We recall that the acidic character of halophilic protein primary structures results from a decrease in lysine as well as from an increase in glutamic and aspartic acid residues, compared to their nonhalophilic homologues (Madern et al. 1995).

From in vitro to in vivo

The extensive experimental data on *Hm* MalDH lead us to the inevitable conclusion that mechanisms of haloadaptation cannot be understood without taking into account the environment and its physical chemistry. A description of halophilic enzyme behavior in vivo should therefore include the cytoplasm, with respect, of course, to salt type and concentration but also to molecular crowding from high protein concentration, substrate channeling, presence of osmolyte, coenzyme binding, etc. There is also haloadaptation at the cellular level, and recent work in our laboratory has identified a soluble factor of about 55 K (P55), induced in *Haloarcula marismortui* cells under low-salt stress conditions, that appears to have molecular chaperone structures and properties (Franzetti, personal communication).

Conclusion

A halophilic enzyme cannot be considered simply as a set of well-folded polypeptide chains interacting with each other

in a high-salt environment. It is, in fact, a complex of prote in and solvation shell, which inherently includes specific interactions between salt ions, water molecules, and the polypeptides; these interactions intervene in solubility, in the stabilization of subunits, and also in intersubunit interactions. It is not easy to take into account specific protein–solvent interactions, some of which may be impossible to observe even in a crystal structure because of resolution limits or disorder. Despite these difficulties, however, they cannot be ignored because they may well represent the most essential part of haloadaptation.

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