Relative Role of Anions and Cations in the Stabilization of Halophilic Malate Dehydrogenase

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ABSTRACT: Halophilic malate dehydrogenase unfolds at low salt, and increasing the salt concentration stabilizes, first, the folded form and then, in some cases, destabilizes it. From inactivation and fluorescence measurements performed on the protein after its incubation in the presence of various salts in a large range of concentrations, the apparent effects of anions and cations were found to superimpose. A large range of ions was examined, including conditions that are in general not of physiological relevance, to explore the physical chemistry driving adaptation to extreme environments. The order of efficiency of cations and anions to maintain the folded form is, for the low-salt transition, $Ca^{2+} \approx Mg^{2+} > Li^{+} \approx NH_4^{+} \approx Na^{+} > K^{+} > Rb^{+} > Cs^{+}$, and $SO_4^{2-} \approx OAc^{-} \approx F^{-} > Cl^{-}$, and for the high-salt transition, $NH_4^{+} \approx Na^{+} \approx K^{+} \approx Cs^{+} > Li^{+} > Mg^{2+} > Ca^{2+}$, and $SO_4^{2-} \approx OAc^{-} \approx F^{-} > Cl^{-} > Br^{-} > I^{-}$. If a cation or anion is very stabilizing, the effect of the salt ion of opposite charge is limited. Anions of high charge density are always the most efficient to stabilize the folded form, in accordance with the order found in the Hofmeister series, while cations of high charge density are the most efficient only at the lower salt concentrations and tend to denature the protein at higher salt concentrations. The stabilizing efficiency of cations and anions can be related in a minor way to their effect on the surface tension of the solution, but the interaction of ions with sites only present in the folded protein has also to be taken into account. Unfolding at high salt concentrations corresponds to interactions of anions of low charge density and cations of high charge density with the peptide bond, as found for nonhalophilic proteins.

One of the main characteristics of proteins extracted from extreme halophilic *archeae* is their instability at low salt, since in general they unfold under 2−3 M NaCl or KCl (*1*−*3*). The amino acid content of soluble halophilic enzymes shows distinguishing features, in particular, a high content in acidic residues associated with a lower content in basic ones, and in a less pronounced way a decrease in large hydrophobic residues in favor of smaller ones (*4*, *5*). A homology-based modeling on an extremely halophilic glutamate dehydrogenase revealed also a significant reduction in surface-exposed lysine (*6*). The crystallographic structure of two halophilic proteins, halophilic malate dehydrogenase (*7*) and halophilic 2Fe-2S ferredoxin (*8*), are available. They show high densities of acidic residues at the protein surface. Repulsive electrostatic interactions which are screened at high salt concentrations would contribute to low salt instability. Poisson−Boltzmann calculations, using the crystal structure of the moderately halophilic ferredoxin (*9*), predict its stabilization at low salt when lowering the pH, and this tendency is observed experimentally (*10*). In the 1.9 Å resolution structure of ferredoxin, water molecules are found to be tightly bound, suggesting to the authors that halo-adaptation involves an increased water−protein binding capacity. A recent 2.6 Å resolution structure of a mutant of halophilic malate dehydrogenase also shows extensive hydration (*11*). In the case of halophilic MalDH,[1] complex salt bridges that are not present in the nonhalophilic homologous proteins have also been observed. This feature also appears to contribute to the stabilization of the folded form in thermophilic enzymes (*7*).

The low-resolution solution structure of halophilic malate dehydrogenase (halophilic MalDH) in KCl and NaCl has been extensively studied since its purification and first characterization (*12*). This protein is a tetramer which deactivates, dissociates, and unfolds under about 2 M NaCl with first-order kinetics (*13*−*16*). A great effort has been devoted to determine its solvent interactions, using the thermodynamic approach developed by Eisenberg for multi-component systems (*17*, *18*), and complementary biophysical techniques (*19*−*23*). After the exact determination of the molar mass of the polypeptide chain of halophilic MalDH from the gene sequence (*24*), data were reanalyzed and complemented by Bonneté et al. (*25*). For the folded protein in a range of NaCl or KCl concentrations, the interaction parameters with solvent components, i.e., water or salt, were interpreted in terms of an invariant particle and led to values for water and salt binding of ca. 0.4 g of water/g of protein, a usual value, and 0.1 g of salt/g of protein, a quite large value, 10 times higher than that measured for a nonhalophilic protein. The values obtained from similar studies on two other halophilic proteins, polypeptide elongation factor Tu

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[1] Abbreviations: halophilic MalDH, malate dehydrogenase from *Haloarcula marismortui*; OAc and OAc^{-}, acetate.

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(*26*) and glyceraldehyde 3-phosphate dehydrogenase (*27*) were qualitatively similar: 0.4 and 0.2 g of water and 0.2 g or 0.1 g of KCl, respectively (per gram of protein). For the three proteins studied in their folded forms in either KCl or NaCl, the solvation is characterized by very large amounts of salt, so that the solvation shell is close to saturation.

The salt concentration dependence stability of halophilic MalDH is related to the nature of the salt (*28*, *22*, *16*, *29*). For example, halophilic MalDH unfolds at lower salt concentrations in ammonium sulfate when compared to sodium chloride or in sodium chloride compared to potassium chloride. Halophilic MalDH is stabilized in a limited range of $MgCl_2$ or $CaCl_2$ concentrations. The temperature dependence of the halophilic MalDH stability also depends on the salt concentration and nature and on whether normal or heavy water is used. For example, halophilic MalDH destabilization at low temperature is not observable—until −10 °C—in sodium chloride, while a maximum of stability is observed around 10 °C in ammonium sulfate. The measurements of the second virial coefficients—which are related to protein−protein interactions and protein solubility—of halophilic MalDH gave indications of slightly repulsive or null protein−protein interactions in a variety of salt and salt concentrations and were not clearly interpreted (*30*). From early data, Zaccai et al. (*22*) proposed that the mechanisms of stabilization of halophilic MalDH would be different depending on its environment. In sodium, potassium, or magnesium chloride, enthalpic terms dominate and the protein would be stabilized by a network of hydrated ions associated cooperatively in patches at the surface of the protein. In ammonium sulfate or potassium phosphate, on the other hand, stabilization is entropy driven and would involve mainly hydrophobic interactions (*16*).

The moderate stabilization of folded proteins compared to unfolded ones involves hundreds of intramolecular and intermolecular contacts and is the result of compensating enthalpy−entropy factors in which the solvent is involved (*31*). The structure of hydration and electrostatics of proteins is the object of numerous studies (*32*, *33*). In this work, we will attempt to discriminate the role of anions and cations in the stabilization of halophilic MalDH. With this protein, an exceptionally large range of salt concentrations could be examined. Our goal is a better understanding of the relation between stabilization and solvent interactions of the folded form. Since halophilic MalDH is more stable in certain salts at pH 8 and 4 °C, we investigated its stabilization in these conditions. Equilibrium studies are difficult to perform due to protein irreversible denaturation (*15*). Low salt inactivation of halophilic malate dehydrogenase was shown to be concomitant with dissociation and unfolding at low NaCl concentrations (*28*). Residual activity and fluorescence after 24 h of incubation in a given solvent were used as conformational probes since the former examines the conformation around the active site, and the latter is sensitive to the local environment of the eight tryptophans, which are localized between the subunits in the tetrameric structure. The apparent stability of halophilic MalDH in a given medium was determined by comparison with residual activity and fluorescence in NaCl 4 M.

Our experimental protocol does not allow the determination of the relative stabilities of the folded and unfolded forms as was done for other proteins (*34*−*36*), since we measured the rate of unfolding and considered only one single point at 24 h. However, it allows the comparison of the apparent relative stability of halophilic MalDH in a great variety of salt conditions. The sample preparation procedure leads to a residual NaCl concentration of 80 mM. Control dialysis experiments were performed for some salt conditions (KCl, $MgCl_2$, ammonium sulfate), however, which gave stability ranges in full agreement with the results presented below. Our general conclusions, therefore, are not strongly affected by the residual NaCl, even if its concentration corresponds to half the dissociation constant estimated for the binding of chloride to protein cation groups for *Escherichia coli* SSB protein (*37*).

MATERIALS AND METHODS

Sample Preparation. Halophilic MalDH was overexpressed in *E. coli* and purified according to the protocol of Cendrin et al. (*24*) as modified by Madern et al. (*5*). It was stocked in 4 M NaCl and 50 mM Tris-HCl, pH 8.2, at 4 °C. All salt solutions contained 50 mM Tris-HCl and were buffered at pH 8.2, and this will not be mentioned further. For the fluorescence and residual activity measurements, 12 μL of a stock solution of halophilic MalDH at 1.3 mg/mL was diluted in 588 μL of the desired solvent and incubated for 24 h at 4 °C. The residual NaCl concentration was thus 80 mM. Reference samples were made by dilution in NaCl 4 M.

Fluorescence Measurements. The fluorescence of halophilic MalDH was measured with an Aminco Bowman Series 2 spectrometer, where the cell temperature was kept at 4 °C. The fluorescence was excited at 280 nm and observed between 300 and 450 nm.

Residual Activity Measurements. After the fluorescence measurements, 50 μL of the protein solution was diluted in a 1.00 cm quartz cuvette in 1.6 mL of 2 M NaCl, 0.2 mM NADH, and 0.3 mM oxaloacetate freshly prepared, and the rate of oxidation of NADH was followed immediately during 20 s by the decrease in absorbance (A) at 340 nm as a function of time (t), in a Beckman DU 7400 spectrophotometer. The dA/dt values were corrected for the blank and compared to those obtained for the reference samples.

RESULTS

Determination of Protein Stability by Residual Activity and Fluorescence. Halophilic MalDH fluorescence and residual activity were measured after incubation for 24 h at 4 °C in the presence of various salts at various concentrations and compared to the values from a sample at the same protein concentration incubated in parallel in 4 M NaCl (condition in which the protein is stable) defined as unity. Figure 1A shows the residual activity measurements for three selected salts: $(NH_4)_2SO_4$, NaF, and NaBr. The residual activity is close to that of the fully active protein above 0.6 M NaF or 1.2 M ammonium sulfate. At lower salt values, the protein inactivates. In NaBr, the residual activity of the protein reaches a maximum at 3.4 M and decreases above this value. A similar bell-shaped curve was described previously in $MgCl_2$ (*21*). In NaF, the value for the maximum residual activity reaches about 1.1, whereas in $(NH_4)_2SO_4$ it decreases slightly from unity when increasing the salt concentration. This decrease can be related to systematic errors (e.g.,

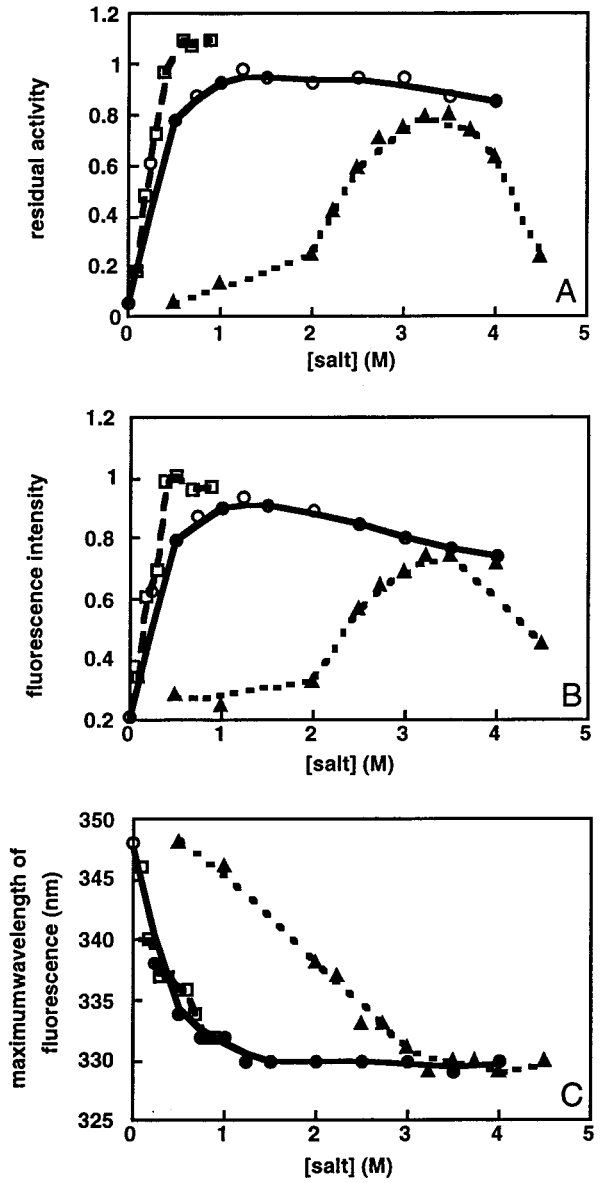

FIGURE 1: Measurement of the apparent stability curves of halophilic MalDH after 24 h incubation at 4 °C in sodium fluoride (squares), sodium bromide (triangles), and ammonium sulfate (circles). (A) Residual activity measured in standard conditions by the rate of the decrease of the absorbance of NADH at 340 nm. (B) Fluorescence intensity at 328 nm with an excitation at 280 nm. (C) Wavelength of maximum fluorescence emission; for panels A and B, data were normalized to the values obtained on the protein incubated in NaCl 4 M. The plateau regions were used to determine the percentage of native protein. Experimental details are given in the Materials and Methods.

temperature) or to the effect of the 3% residual salt concentration from the incubation medium in the buffer used for the activity measurement, since enzymatic kinetics parameters depend on the salt concentration (*13*, *5*). We considered thus the protein as fully active in the plateau region to determine the percentage of active enzyme for each salt concentration (in the case of NaBr, where there is no plateau, no correction was of course performed). We describe briefly the appearance of the residual activity curves for the other salts: $Mg(OAc)_2$ was found similar to NaF (i.e., with a negative slope in the plateau starting from above 1.0). Other salts with negative plateau slopes such as $(NH_4)_2SO_4$ were $MgSO_4$, NH_4OAc, NH_4Cl, KF, and NaOAc. Li_2SO_4, KCl, NA_2SO_4, NaCl, and CsCl reached a value of 1.0, and KOAc displayed a positive slope. A bell-shaped curve as for NaBr was found for LiCl, $CaCl_2$, and $MgCl_2$. The activity was always nul in NaI.

The fluorescence spectrum of the folded protein upon excitation at 280 nm is characterized by an intense fluorescence emission at 328 nm (*12*). Upon unfolding, the quantum yield decreases by a factor of about four while the wavelength of maximum emission is shifted to about 348 nm. The ratio between the fluorescence intensities at 328 nm for the protein in its incubation buffer and in 4 M NaCl is plotted on Figure 1B for the three selected salts. The features of the variation with salt of this ratio are very similar to that observed with activity measurements, and the percentage of folded form was determined using the same correction for plateau slope. In the case of NaBr, the data were normalized considering the protein in NaCl 4 M as a reference. We describe briefly the appearance of the fluorescence intensity curves for the other salts. A negative plateau slope similar to that of NaF or $(NH_4)_2SO_4$ was found for $Mg(OAc)_2$, NH_4Cl, KOAc, and KF,NaOAc. Positive slopes were found in the presence of $MgSO_4$ and NH_4OAc. Curves reaching the intensity of fluorescence of 4 M NaCl were found in the presence of KCl, Na_2SO_4, NaCl, and CsCl. In NaI, the intensity of fluorescence was below that of the unfolded protein at low salt. Bell-shaped curves similar to NaBr, were observed in LiCl, $CaCl_2$, and $MgCl_2$.

On Figure 1C, the maximum wavelength of fluorescence emission is reported. At low salt, where the protein is unfolded the maximum of fluorescence is at 348 nm. When increasing the salt concentration, it reaches a value of 332 nm in NaF, 330 nm in NaBr or ammonium sulfate and thus close to the value of 328 nm found at high NaCl. These slight differences can be attributed to fluorescence quenching effects, since salts are present at high concentration in the solvent. In the case of NaBr at high salt concentration, the position of the wavelength maximum does not reflect the partial unfolding of the protein detected by inactivation and loss in the fluorescence intensity (Figure 1, panels A and B)—note however that, in the presence of LiCl, $CaCl_2$, $MgCl_2$, an inverse bell-shaped curve was detected. In general, because of the more efficient quenching by the salt of the fluorescence of the unfolded form, the maximum wavelength of fluorescence emission overestimates the proportion of folded form.

The calculated percentages of folded form using the two criteria of residual activity and fluorescence intensity are reported in Figure 2. Results from activity or fluorescence intensity always give similar values for the percentage of folded form, indicating that inactivation and unfolding take place together. In ammonium sulfate (Figure 2A), the two indicators give values for the percentage of folded forms in very good agreement. In NaBr (Figure 2B), the differences between the levels of the two bell curves are not significant and arise from the procedure of the normalization of the data. In NaF (Figure 2C), both activity and intensity of fluorescence are indicative of a protein completely folded above 0.4 M. The maximum of fluorescence overestimates frequently the proportion of the folded form. In NaF, however, it shows a break at 0.3 M and continues to increase until 0.9 M. This suggests the presence of a dimeric unfolding intermediate, by comparison with a dimeric mutant of

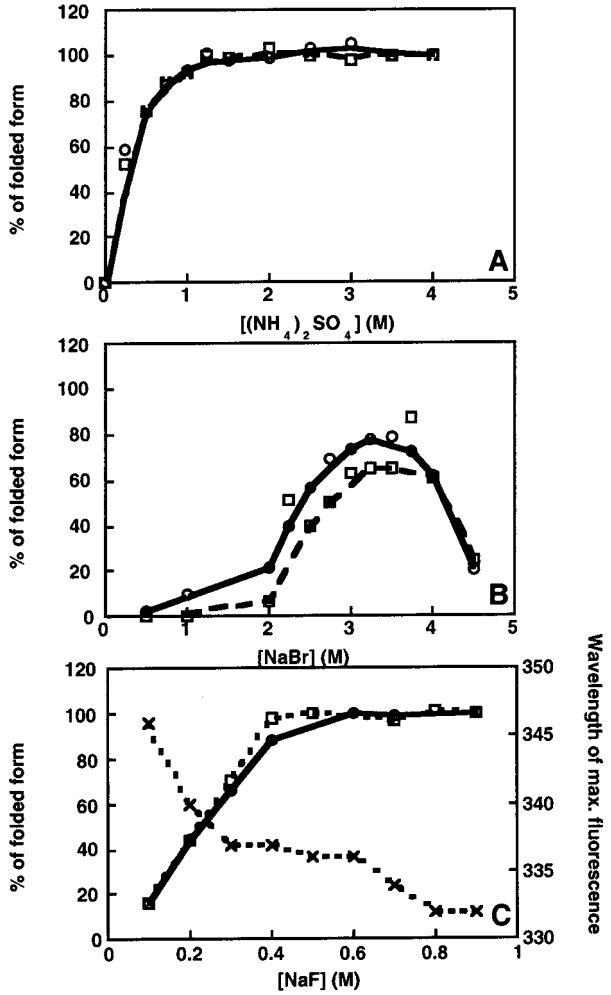

FIGURE 2: Percentage of native form for halophilic MalDH incubated for 24 h in ammonium sulfate (panel A), sodium bromide (panel B), and sodium fluoride (panel C). The percentage of residual native form was derived from two indicators, residual activity (circle), fluorescence intensity (square). On panel C are also shown the wavelengths of maximum fluorescence (X, right-hand *y*-axis). See Materials and Methods for the details and the text for interpretation.

halophilic MalDH exhibiting a maximum of fluorescence at 332 mn (Madern et al., manuscript in preparation). A similar situation is seen in Na_2SO_4 and perhaps in KF (data not shown). No unfolding intermediate was detected from our experiments in the other tested salts: $MgSO_4$, $Mg(CH_3COO)_2$, $MgCl_2$, LiCl, $(NH_4)_2SO_4$, $NH_4(CH_3COO)$, NH_4Cl, $Na(CH_3COO)$, NaCl, NaBr, NaI, $K(CH_3COO)$, KCl, $CaCl_2$, and CsCl. The folding process will not be considered further in the present paper, and we will consider below (unless specified) the apparent percentage of folded structure—tetramer or eventually dimer—as the mean value obtained using the fluorescence intensity and residual activity probes.

Influence of the Nature of the Ion on Protein Stability. Figures 3 and 4 show the percentage of folded form in various salts after 24 h incubation. For each panel, the cation (Figure 3) or anion (Figure 4) is fixed and the effect of a variety of co-ions is screened. The range of concentrations investigated in each salt is different because of their different solubility. Cations are fixed for the panels in Figure 3. Halides are compared with Na^+ as constant co-ion (fourth panel). In NaI, the protein is fully inactivated by 24 h of incubation whatever the salt concentration. In NaBr, a bell

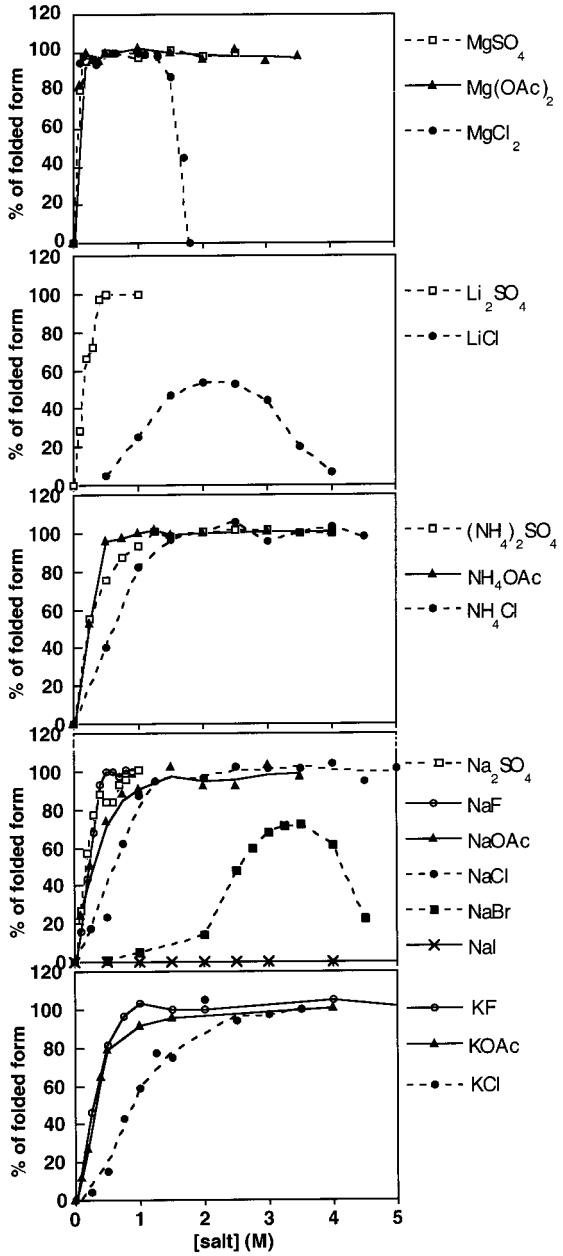

FIGURE 3: Effect of anions. Comparison of the apparent stability for halophilic MalDH incubated for 24 h at 4 °C in various salts. Apparent stability was determined from the mean percentage determined from residual activity and fluorescence intensity. Each panel corresponds to a series of salts with a common cation.

curve is observed with at least 60% of the native form being maintained between 2.7 and 4.2 M salt. In NaCl, as previously described, the curve rises to a plateau, and 50% of folded forms are found for 0.7 M NaCl at this temperature (4 °C). In NaF, the curve is shifted to lower salt, with 50% folded forms found at 0.25M NaF. The other co-ions tested with Na^+, sulfate, and acetate (OAc^-) also gave halophilic MalDH stabilization in a similar range (0.2 M salt). The precision in the percentage of remaining folded form is about 10%. The panel corresponding to the cations NH_4^+ and K^+ (third and fifth panels) displays similar comparative behavior. In the presence of K^+, the curves with F^- and OAc^- almost superimpose, and correspond to a better protein stabilization as compared to Cl^-. In the presence of NH_4^+, the same holds for OAc^- and SO_4^{2-} as compared to Cl^-. In the presence of Li^+, also, SO_4^{2-} is very stabilizing compared to Cl^-. In LiCl

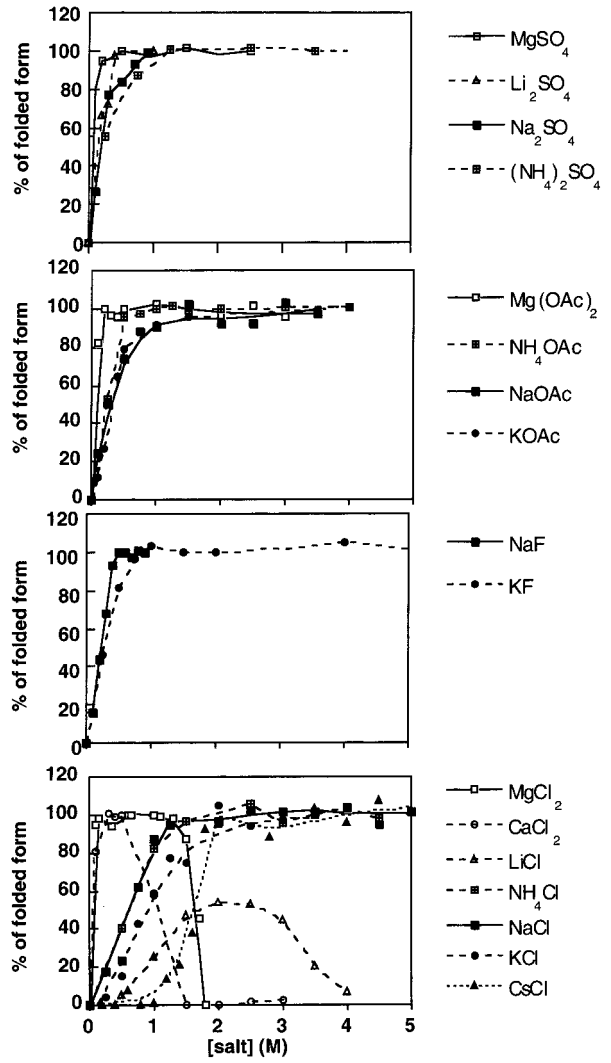

FIGURE 4: Effect of cations. Comparison of the apparent stability for halophilic MalDH incubated for 24 h at 4 °C in various salts. Apparent stability was determined from the mean percentage determined from residual activity and fluorescence intensity. Each panel corresponds to a series of salts with a common anion.

(second panel), the protein never reaches full stability and a bell curve is seen with unfolding at both high and low salt. In the presence of Mg^{2+} combined with OAc^-, SO_4^{2-}, or Cl^- (first panel), folding occurs below a rather small salt concentration (0.2 M). $MgCl_2$ displays a bell curve.

The relative effect of cations on protein stabilization is seen in the panels where anions are fixed (Figure 4). The fourth panel corresponding to Cl^- is the most complete and diverse. Stability curves are either sigmoidal or bell-shaped. This last case is seen in the presence of the counterions lithium, magnesium, and calcium, for which high salt unfolding is seen above 2, 1.3, and 0.5 M, respectively. Folding by increasing the salt—the left part of the bell curves or the sigmoidal curves—occurs at very low salt—above 0.2 M—with divalent cations Mg^{2+} and Ca^{2+}. Fifty percent of the residual activity is found at about 0.7 M with both Na^+ and NH_4^+, at 0.9 M for KCl, and 1.6 M for CsCl. Note that the stabilization by RbCl was found at 25 °C to be intermediate between NaCl and CsCl (after 25 h of incubation, half of the remaining activity is found at 0.9 M NaCl, 1.8 M RbCl and 3.4 M CsCl). The counterion lithium is difficult to classify because high salt unfolding overlaps the low salt folding. The first three panels correspond to constant SO_4^{2-}, OAc^-, and F^- anions. The magnesium ion stabilizes the protein at very low salt (0.2 M) compared to the other cations tested. NaF is slightly more efficient than KF to preserve the folded form. The ammonium, sodium and lithium salts of sulfate have almost identical effects, considering the uncertainties in the determination of the percentage of active form in the upper part of the boundary as mentioned above. High salt denaturation is not observed for these anions.

The stability curve displays a rising part corresponding to the stabilization of the folded protein when increasing the salt concentration from zero to a value depending on the tested salt. The lower the salt concentration corresponding to this transition, the higher the efficiency of the salt to stabilize the folded protein. The second part of the curve, at higher salt concentration up to near saturation, is either a plateau or a decreasing part: in some salts, unfolding is observed at high salt in the available concentration range. The higher the salt concentration corresponding to this transition, the higher the efficiency of the salt to stabilize the folded form. The comparison of the stability curves at constant cation or anion allowed the classification of the anions and cations separately for both transitions since the order of efficiency of the ions of opposite charge is reproduced from panel to panel. We decided not to classify ions for the low salt transition when full stabilization is not achieved (anions Br^- and I^-, Li^+ is classified from the SO_4^{2-} panel). The order of efficiency of cations to maintain the protein folding at low salt concentration—first transition—is

$$Ca^{2+} \approx Mg^{2+} > Li^+ \approx NH_4^+ \approx Na^+ > K^+ > Rb^+ > Cs^+$$

[note that $Ca^{2+} > Mg^{2+}$ was determined at pH 7 (*29*)], and at higher salt concentrations—corresponding to high salt unfolding—the order appears to be reversed:

$$NH_4^+ \approx Na^+ \approx K^+ \approx Cs^+ > Li^+ > Mg^{2+} > Ca^{2+}$$

For the anions at the low salt concentrations, the order of efficiency appears as

$$SO_4^{2-} \approx OAc^- \approx F^- > Cl^-$$

and at higher salt, the order is similar:

$$SO_4^{2-} \approx OAc^- \approx F^- > Cl^- > Br^- > I^-$$

Both cations and anions strongly affect the stability of the protein. The order for anions is compatible with the Hofmeister series in both transitions, while the order for cation is opposite comparing the lower and higher range of salt concentrations. Note that if a cation or anion is very stabilizing the effect of the ion of opposite charge is limited.

DISCUSSION

Hofmeister Series and Nonspecific Salt Effects. Von Hippel and Schleich reviewed the effects of neutral salts on the structure and conformational stability of macromolecules in solution (*38*). It was emphasized that the effects of anions and cations in the molar range overcome the effects of the details in the composition and conformation of macromolecules. Salts, cations, or anions were classified from "salting-

Table 1: Surface Tension Increments of Various Salts[a]

	M^+					
	Li^+	Na^+	K^+	Rb^+	Cs^+	NH_4^+
M_2SO_4	2.7[b]	2.7[b]	2.9[e]			2.2[b]
MF			1.9[d]			
MCl	1.6[b]	1.6[b]	1.45[b,d]	1.4[c]	1.5[c]	1.4[b]
MBr	1.3[b]	1.3[b]	1.3[b]			1.3[b]
MI	0.8[b]	1.0[b]	0.8[b], 1.1[d]			0.7[b]

	M^{2+}	
	Ca^{2+}	Mg^{2+}
MSO_4		2.10[b]
MCl_2	3.2[b], 3.9[f]	3.04 [b], 3.9[f]

[a] The differences between the surface tensions of the solution and water in dynes/cm are reported here for a salt concentration of 1 mol/kg water, unless specified. The temperatures of the reported measurements are in the range 13−30 °C and are not specified since the surface tension increments of electrolytes are described to be nearly independent of temperature (d). [b] Ref *47*. [c] Factor et al. (1974) *Vestn. Leniongrad. University Fis. Khim. 1*, 120. [d] Hard and Johansson (1977) *J. Colloid Interface Sci. 60*, 467. [e] Ref *46*. [f] Ref *48*.

out", which promotes stabilization, auto-association, or precipitation to "salting-in", promoting denaturation, dissociation, and solubilization of the species, following the Hofmeister series. As reviewed by Collins and Washabaugh (*39*), the Hofmeister series is manifested also by a number of different measurements relative to the properties of the aqueous solvent. Effects are analyzed by these authors in terms of relative competition for hydrogen bond formation of the solutes and/or bulk water for interfacial water. The proper description of the effect of salts on equilibrium—solubility, self-assembly, or denaturation—needs to take into account that the different species interact differently with the solvent components (water and ions). The general theory of linked functions (*40*, *41*) was extensively used to understand the role of weak interactions with water and cosolvent on protein stabilization and association (*42*, *43*) and on protein−nucleic acid interactions (*44*). Among the species involved in an equilibrium or an interconversion system, those interacting in the more efficient way with the cosolvent, in our case the salt, will be stabilized by increasing the cosolvent content in the solvent.

Nonspecific effects do not depend on the chemical nature of the macromolecule. As an example of a nonspecific effect, polar salts increase the surface tension of water (*45*), increasing the positive free-energy change needed to form a cavity in the solvent to accommodate the protein. In Table 1 are reported molal surface tension increments for some salts. Surface tension increments have been described to be little dependent on salt concentration (*46*). It is not the case, however, for acetate salts (*47*, *48*), which are not reported in the table: for example, the surface tension of $Mg(OAc)_2$ solutions was found to increase with the addition of 0.45 M salt and to decrease above this value with a slope of −1.2 dynes/cm for 1 mol of salt/kg of water added (*47*). An increase of the surface tension corresponds to a relative increase of the water concentration or ionic depletion at the interface. This can be explained at low salt by electrostatic theory (*49*), is consistent with measurements of interfacial tension considering the theory of dispersion forces (*50*), and can be explained by the fact that strongly hydrated ions have to dehydrate to accommodate to the surface (*48*, *51*). Therefore, the folded or associated forms are favored over the unfolded or dissociated species by the presence of solute increasing surface tension (*52*−*56*), in connection with measured hydration parameters greater for native than for the denatured form (*57*, *58*). However, the surface tension argument is inadequate to describe the complexity of the effects of salts. The predominance of the effect of the anions in the Hofmeister series was related by Collins and Washabaugh (*39*) to the relative ease of the water oxygen to accept partial negative charge. Concerning cations, the correlation between their water-structuring properties and ability to increase the surface tension (*48*) and the stabilizing potencies of the salts is obviously not evident (*38*). Another nonspecific effect has been described: the steric exclusion of large cosolutes, like poly(ethylene glycol) leads to an increased interaction of the macromolecule with water and explains the relative stabilization of species with reduced surface exposed to the solvent (*59*).

The binding interactions between salts or cosolutes and chemical groups of macromolecular species will stabilize that species. These interactions, even if weak, can concern chemical groups common to large families of macromolecules and, thus, appear as not very specific. The solubilization of membrane proteins by surfactants and the denaturing effects of sodium dodecyl sulfate, guanidinium hydrochloride, or urea are well-known to be related to large amounts of these molecules being associated to proteins (*60*−*65*). The salting-in effect on the peptide group by NaI, $NaClO_4$, or NaSCN, $CaCl_2$ (*66*) explains the denaturing power of these salts. Lithium and calcium ions may complex directly the amide moieties (*67*). In agreement with this view, the measurements of the interactions of salts with proteins (*68*) were interpreted by the binding of divalent cations overcoming the salt exclusion due to the surface tension increase. As an other aspecific effect, the adsorption to nonpolar surfaces of large chaotropic ions described by Collins (*69*) could explain a part of their salting-in properties. More specifically, and even if a very few sites are concerned and with relatively low affinity constants, the binding of some ions strongly affects stability or intermolecular associations. Ribonuclease is stabilized by anion and cation binding: as an example, the binding of two Na^+ with binding constants in the molar range explains the increase of 20° in the protein melting temperature in the presence of 2 M NaCl (*70*). Formation of ion pairs on the surface of basic proteins can explain their special solubility properties not predictable by the Hofmeister series (*71*−*72*).

Stabilization of Halophilic Malate Dehydrogenase. The order found for the increasing stabilization effect of the anions corresponds well to the Hofmeister series in the two transitions. One striking feature is that sulfate, fluoride, and acetate have a great stabilizing power which masks the effects of the cations in both transitions: nearly all the low salt transitions occur with these anions at the same salt concentration, whatever the nature of the cations; no tendency of high salt unfolding is seen for the protein in the presence of the highest tested concentrations of magnesium sulfate (2.5 M), magnesium acetate (3.5 M), or lithium sulfate (1 M). For the cations, the order found is reversed for the two transitions. Looking at the high salt unfolding transition, the order of increased stabilization of the protein is that usually found for nonhalophilic proteins and is related to the capacity of the destabilizing ions to interact with the peptide bond.

Looking at the low salt unfolding transition, the halophilic MalDH folding transition at lower salt, the order of the cations for stabilization is opposite the previous one (see the position of calcium, magnesium, and lithium ions in the observed series).

Both anions and cations with the highest charge density (water-structuring ions, those displaying the highest tendency to increase the surface tension) stabilize the halophilic MalDH at the lowest salt concentration. If salts of magnesium, calcium, sulfate, or fluoride are very efficient to increase the surface tension, see Table 1, the effect of the salts on the low salt transition cannot be explained only with surface tension arguments. If it were the case, we would find very similar values for the surface tension of the solutions for the salt concentration corresponding to the folding transition. However, we calculate values which are very different for the increase of the surface tension of the solution with respect to pure water at the salt concentrations corresponding to the low salt folding transition, from very low values in $MgCl_2$ or $CaCl_2$ to a high value in CsCl: 0.2 dynes/cm for $MgCl_2$ or $CaCl_2$ 0.05 M, 1 dynes/cm for NH_4Cl 0.7 M, 1.2 dynes/cm for NaCl 0.7 M, 1.5 dynes/cm for KCl 0.9 M, and 2.4 dynes/cm for CsCl 1.6 M (values are taken from ref *48*).

The effect of the cations and anions with high charge densities to stabilize the folded form of halophilic MalDH can be the indication of alternative or simultaneous salt interactions with the folded protein. Halophilic proteins were described to be solvated in sodium or potassium chloride by large amounts of salts (*25*). The ion binding capability would be related to their specific affinity for the folded protein. The formation of specific binding sites or polyelectrolyte properties has to be invoked, since the affinity has to be larger for the folded than for the unfolded protein to account for salt stabilization. It was shown that mutations of surface acidic residues on halophilic MalDH noticeably decreases the protein stability (*5*). The polyelectrolyte property has been put forward to explain the very high concentrations of counterions generated in the vicinity of nucleic acids—condensation of mobile hydrated ions—due to their high charge densities (*73*−*75*). The requirement of a specific spatial arrangement of the carboxylates to form binding sites for ions at the surface of the folded halophilic malate dehydrogenase is demonstrated by a recent study of the effect of salts and pH on the transition of a negatively charged 30-residue peptide from a random coil monomer to a coiled coil dimer (leucine zipper), interacting through an hydrophobic interface, surrounded by four ranges of glutamates (*76*). At alkaline pH, Na_2SO_4, $(NH_4)_2SO_4$, and KF promotes the folding at a salt concentration of 2.5 M, while LiCl, NaCl, KCl, $MgCl_2$, and $NaClO_4$ were inefficient even at 4 or 5 M. For this highly negatively charged system and in contrast to the halophilic MalDH system, the nature of the cation is negligible for the stabilization of the folded associated form, and strengthening hydrophobic interactions occurs only via the effects of the anions. Mechanisms for ion pair selectivity have been proposed by Collins (*77*), which explain preferential chaotrope−chaotrope or kosmotrope−kosmotrope ion−ion interactions, while, in an association between chaotrope and kosmotrope, ions tend to remain separated in aqueous solution (kosmotrope ions structure surrounding water, and chaotrope ones destructure it). Collins pointed out that carboxylates—like phosphates—are kosmotrope and thus tend to form ion pairs with kosmotrope highly charged cations. It is the balance of the electrostatic and hydration forces that determines the selectivity of the cation binding, which is found to differ for various compounds containing carboxylates: it was shown that tubulin polymerization like the coil−helix transition in polyglutamic acid were promoted by cations in the order $Na^+ > K^+ > Li^+ > Cs^+$ (*78*, *79*). On the other hand, the order $Li^+ > Na^+ > K^+$ was found for the selectivity of carboxylate ion-exchange resin, consistent with a strong fixed field (*80*). We recall that the order for the stabilization of halophilic MalDH is $Li^+ \approx Na^+ > K^+ > Cs^+$. In the halophilic folded form of MalDH, the surface is covered with acidic amino acids and sites involving more than one carboxylate could reinforce the interaction with kosmotrope cations. The fact that the same order of cation efficiency is found for protein unfolding at higher salt concentration is the indication that the same properties of the involved ions are needed for the interaction with the chemical groups—most probably the peptide bonds—which are exposed upon protein unfolding.

The existence of ion-binding sites on halophilic proteins is more and more documented. For halophilic ferredoxin, the 1.9 Å resolution structure allowed the distinguishing of six potassium ions at the surface (*8*). For a mutant of halophilic MalDH, the 2.6 Å resolution structure revealed sodium- and chloride-binding sites (*11*). The stabilization of the dimeric form of halophilic MalDH, suggested for some salt conditions in the present work by the displacement of the maximum fluorescence emission, could be related to anion binding. On halophilic dihydrolipoamide dehydrogenase, a K^+-binding site was identified from homology modeling, and its importance for halophilism recognized from site-directed mutagenesis (*81*). For the TATA box binding protein from *Pyrococcus woesei*, whose cytoplasm contains 0.8 M salt, the binding of cations in the interface between negatively charged lobes on protein and the negatively charged DNA corresponding region was inferred from the increase of the equilibrium association constant when increasing the salt concentration (*82*).

As a conclusion, due to the fact that halophilic proteins are unstable at low salt, the study of their stability at various salt concentrations allows the evaluation of the role of the ions from "low" salt concentration, where increasing the concentration of most of the salts is stabilizing, to higher salt concentration, where opposite destabilizing effects eventually occur. These effects were analyzed with the simple argument that the form—folded or unfolded—interacting more efficiently with the salt or less efficiently with water will be stabilized when increasing the salt concentration. At very low salt, the unfolded form is the favored one. Increasing salt concentration in the medium leads to the stabilization of the folded form. We found for this transition that both cations and anions of high charge density, able to structure water in their vicinity and thus kosmotropic, are the most efficient. The increase of the surface tension, a nonspecific effect—equivalent to an interaction with water proportional to the exposed surface—favors the folded form but does not allow to explain by itself the stabilizing power of the salts. The formation of ionic binding sites specific to the folded protein, related to the spatial arrangement of the chemical groups and corresponding to increased binding capacities for

the same ions—of high charge densities—has also to be taken into account. At higher concentration of either chaotrope anions or kosmotrope cations, their interactions with the peptide bond lead to protein denaturation. The relative importance of, on one hand, mediated through water nonspecific effects and direct binding interactions of anions and cations, on the other, cannot be quantified from the comparison of the inactivation experiments presented here. The evaluation of salt and water binding of halophilic MalDH in various salts from neutron scattering forward intensity and density measurements is under way and will be presented soon.

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REFERENCES

1. Lanyi, J. K. (1974) *Bacteriol. Rev. 38*, 272−290.
2. Jaenicke, R. (1981) *Annu. Rev. Biophys. Bioeng. 10*, 1−67.
3. Eisenberg, H., Mevarech, M., and Zaccaï, G. (1992) *Adv. Protein Chem. 43*, 1−62.
4. Rao, J. K. M., and Argos, P. (1981) *Biochemistry 20*, 6536−6543.
5. Madern, D., Pfister, C., and Zaccai, G. (1995) *Eur. J. Biochem. 230*, 1088−1095.
6. Birron, K. L., Stillman T. J., Yip, K. S. P., Forterre, P., Engel, P. C., and Rice, D. W. (1998) *J. Biol. Chem. 273*, 9023−9030.
7. Dym, O., Mevarech, M., and Sussman, J. L. (1995) *Science 267*, 1344−1346.
8. Frolow, F., Harel, M., Sussman, J. L., Mevarech, M., and Shoham, M. (1996) *Nat. Struct. Biol. 3*, 452−458.
9. Pieper, U., Kapadia, G., Mevarech, M., and Herzberg, O. (1998) *Structure 6*, 75−88.
10. Elcock, A. H., and McCammon, J. A. (1998) *J. Mol. Biol. 280*, 731−748.
11. Richard, S. (1999) Doctoral Thesis, University Joseph Fourier, Grenoble, France.
12. Mevarech, M., Eisenberg, H., and Neumann, E. (1977) *Biochemistry 16*, 3781−3785.
13. Mevarech, M., and Neumann, E. (1977) *Biochemistry 16*, 3786−3792.
14. Zaccai, G., Bunick, G. J., and Eisenberg, H. (1986) *J. Mol. Biol. 192*, 155−157.
15. Hecht, K., and Jaenicke, R. (1989) *Biochemistry 28*, 4979−4985.
16. Bonneté, F., Madern, D., and Zaccai, G. (1994) *J. Mol. Biol. 244*, 436−447.
17. Eisenberg, H. (1976) *Biological Macromolecules and Polyelectrolytes in Solution*, Clarendon Press, Oxford.
18. Eisenberg, H. (1981) *Q. Rev. Biophys. 14*, 141−172.
19. Pundak, S., and Eisenberg, H. (1981) *Eur. J. Biochem 118*, 463−470.
20. Zaccai, G., Wachtel, E., and Eisenberg, H. (1986) *J. Mol. Biol. 190*, 97−106.
21. Calmettes, P., Eisenberg, H., and Zaccai, G. (1987) *Biophys. Chem. 26*, 279−290.
22. Zaccai, G., Cendrin, F., Haik, Y., Borochov, N., and Eisenberg, H. (1989) *J. Mol. Biol 208*, 491−500.
23. Ebel C. (1995) *Colloid Polym. Sci. 99*, 17−23.
24. Cendrin, F., Chroboczek, J., Zaccaï, G., Eisenberg, H., and Mevarech, M. (1993) *Biochemistry 32*, 4308−4313.
25. Bonneté, F., Ebel, C., Zaccaï, G., and Eisenberg, H. (1993) *J. Chem. Soc., Faraday Trans. 89*, 2659−2666.
26. Ebel, C., Guinet, F., Langowski, J., Urbanke, C., Gagnon, J., and Zaccai, G. (1992) *J. Mol. Biol. 223*, 361−371.
27. Ebel, C., Altekar, W., Langowski, J., Urbanke, C., Forest, E., and Zaccai, G. (1995) *Biophys. Chem. 54*, 219−227.
28. Pundak, S., Aloni, H., and Eisenberg, H. (1981) *Eur. J. Biochem. 118*, 471−477.
29. Madern, D., and Zaccai, G. (1997) *Eur. J. Biochem. 249*, 607−611.
30. Ebel, C., Faou, P., and Zaccai, G. *J. Cryst. Growth* (in press).
31. Makhatadze, G. I., and Privalov, P. L. (1995) *Adv. Protein Chem. 47*, 308−422.
32. Honig, B., and Nicholls, A. (1995) *Science 268*, 1144−1149.
33. Israelachvili, J., and Wennerström H. (1996) *Nature 379*, 219−225.
34. Tams, J. W., and Welinder, K. G. (1996) *Biochemistry 35*, 7573−7579.
35. Wang, A., and Bolen, D. W. (1997) *Biochemistry 36*, 9101−9108.
36. Kaushik, J. K., and Bhat, R. (1999) *Protein Sci. 8*, 222−233.
37. Overman, L. B., and Lohman, T. M. (1994) *J. Mol. Biol. 236*, 165−178.
38. Von Hippel, P. H., and Schleich, T. (1969) *Structure and Stability of Biological Macromolecules* (Timasheff, S. N., and Fasman, G. D., Ed.) Vol. 2, pp 417−574, Marcel Dekker, New York.
39. Collins, K. D., and Washabaugh, M. W., (1985) *Q. Rev. Biophys. 18*, 323−422.
40. Wyman, J., (1964) *Adv. Protein Chem. 19*, 224−284.
41. Tanford, C., (1969) *J. Mol. Biol. 39*, 539−544.
42. Schellman, J. A. (1987) *Biopolymers 26*, 549−559.
43. Timasheff, S. N. (1993) *Annu. Rev. Biophys. Biomol. Struct. 22*, 67−97.
44. Record, M. T., Jr., Ha, J.-H., and Fischer, M. A. (1991) *Methods Enzymology 208*, 291−343.
45. Melander, W., and Horvath, C. (1977) *Archives Biochem. Biophys. 183*, 200−215.
46. Horvath A. L. (1985) in *Handbook of acquous electrolytes solutions, Physical properties, estimation and correlation methods* (Kemp, J. P., Ed.) pp 306, Ellis Horwood, Chichester, GBR.
47. *International Critical Tables of Numerical Data, Physics, Chemistry and Technology* (1928) pp 463−466, McGraw-Hill, New York.
48. Weissenborn, P. K., and Plugh, R. J., (1996) *J. Colloid Interface Sci. 184*, 550−563.
49. Onsager, L., and Samaras, N. T. M. (1934) *J. Chem. Phys. 2*, 528−536.
50. Aveyard, R., and Saleem, S. M. (1975) *J. Chem. Soc., Faraday Trans. 172*, 1609−1617.
51. Hey, M. J., Shield, D. W., Speight, J. M., and Will, M. C. (1981) *J. Chem. Soc., Faraday Trans. 177*, 123−128.
52. Lee, J. C., and Timasheff, S. N. (1981) *J. Biol. Chem. 256*, 7193−7201.
53. Lin, T.-Y., and Timasheff, S. N. (1996) *Protein Sci. 5*, 372−381.
54. Kella, N. K. D., and Kinsella, J. E. (1988) *Int. J. Pept. Protein Res. 32*, 396−405.
55. Jensen, W. A., Amstrong, J. McD., De Giorgio, J., and Hearn, M. T. W. (1995) *Biochemistry 34*, 472−480.
56. Jensen, W. A., Amstrong, J. McD., De Giorgio, J., and Hearn, M. T. W. (1996) *Biochim. Biophys. Acta 1296*, 23−34.
57. Xie, G., and Timasheff, S. N., (1997) *Biophys. Chem. 64*, 25−43.
58. Xie, G., and Timasheff, S. N. (1997) *Protein Sci. 6*, 211−221.
59. Arakawa, T., and Timasheff, S. N. (1985) *Biochemistry 24*, 6756−6762.
60. Reynolds, J. A., and Tanford, C. (1970) *Proc. Natl. Acad. Sci. 66*, 1002−1007.
61. Ibel, K., May, R. P., Sandberg, M., Mascher, E., Greije, E, and Lundhal, P. (1994) *Biophys. Chem. 53*, 77−84.
62. Poklar, N., and Lapanje, S. (1992) *Biophys. Chem. 42*, 283−290.
63. Eisenberg, H. (1994) *Biophys. Chem. 53*, 57−68.
64. Arakawa, T., and Timasheff, S. N. (1984) *Biochemistry 23*, 5924−5929.
65. Makhatadze, G. I., and Privalov, P. L. (1992) *J. Mol. Biol. 226*, 491−505.

66. Nandy, P. K., and Robinson, D. R. (1972) *J. Am. Chem. Soc. 94*, 1299−1308.
67. Bello, J., Haas, D., and Bello, H. R (1966) *Biochemistry 5*, 2539−2548.
68. Arakawa, T., and Timasheff, S. N. (1984) *Biochemistry 23*, 5912−5923.
69. Collins, K. D., (1995) *Proc. Natl. Acad. Sci. 92*, 5553−5557.
70. Pace, C. N., and Grimsley, G. R. (1988) *Biochemistry 27*, 3242−3246.
71. Riès-Kautt, M. M., and Ducruix, A. F. (1991) *J. Cryst. Growth 110*, 20−25.
72. Riès-Kautt, M. M., and Ducruix, A. F. (1997) *Methods Enzymol. 276*, 23−59.
73. Manning, G. S. (1978) *Q. Rev. Biophys. 11*, 179−246.
74. Anderson, C. F., and Record, M. T., Jr. (1990) *Annu. Rev. Biophys. Chem. 19*, 423−465.
75. Jayaram, B., and Beveridge, D. L. (1996) *Annu. Rev. Biophys. Biomol. Struct. 25*, 367−394.
76. Jelesarov, I., Dürr, E., Thomas R. L., and Bosshard H. R. (1998) *Biochemistry 37*, 7539−7550.
77. Collins, K. D., (1997) *Biophys. J. 72*, 65−76.
78. Wolff, J., Sackett, D. L., and Knipling, L. (1996) *Protein Sci. 5*, 2020−2028.
79. Satoh, M., Komiyama, J., and Ijima, T. (1991) *Biopolymers 21*, 1927−1931.
80. Gregor, H. P., Hamilton, M. J., Oza, R. J., and Bernstein, F. (1956) *J. Phys. Chem. 60* 263−267.
81. Jolley, K. A., Russel, R. J. P., Hough, D. W., and Danson, M. J. (1997) *Eur. J. Biochem. 248*, 362−368.
82. O'Brien, R., DeDecker, B., Fleming, K. G., Sigler, P. B., and Ladbury, J. E. (1998) *J. Mol. Biol. 279*, 117−125.

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