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Ions from the Hofmeister series and osmolytes: effects on proteins in solution and in the crystallization process

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Abstract

Sephadex G-10 gel sieving chromatography, Jones-Dole viscosity B coefficients, and solution neutron and X-ray diffraction are used to show that small ions of high charge density (e.g., sulfate, phosphate, the carboxylate, sodium, and fluoride) are strongly hydrated (kosmotropes) whereas large monovalent ions of low charge density (e.g., ammonium, chloride, potassium, and the positively charged amino acid side chains) are weakly hydrated (chaotropes). The heats of solution of the crystalline alkali halides are then used to show that only oppositely charged ions of equal water affinity spontaneously form inner sphere ion pairs, and that this controls ion binding to proteins. The net charge on a protein is a major determinant of its solubility. Finally, the surface potential difference and surface tension at an air–salt solution interface are used to generate a simple model for how ions affect protein stability and solubility through indirect interactions at the protein–solution interface. A few comments about small neutral osmolytes are also included.
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1. Properties of ions

1.1. Introduction to ions and osmolytes

Early work with purified *Escherichia coli* dihydroorotase, a homodimer of subunit molecular weight 49,300, revealed that this enzyme was modified by atmospheric oxygen and additionally that it spontaneously inactivated at concentrations below about 100 μg/ml. The oxygen sensitivity proved to result from the exposure of a sulfhydryl ligand after loss of a weakly bound zinc (II), and was eliminated by removal of peroxides and other contaminants from the ethylene glycol used as a stabilizer [1], careful stripping of trace copper and iron from all solutions using chelating Sepharose, acid washing of glassware with 2 M HCl, deoxygenation of buffers using water aspiration, avoidance of added reductants, and argon overlays of storage solutions [2,3]. The dilutional inactivation was caused by the dissociation of dimers to monomers and subsequent irreversible unfolding of the monomers, processes associated with an increase in the solvent accessible surface area of the protein. A group of small molecules and salts were found empirically to stabilize the enzyme against dilutional inactivation, while a different group of small molecules and salts were found to increase the dilutional inactivation of the enzyme [4]. The rule for membership in each of these two classes of small molecules and salts has been established and the mechanism of action of both groups clarified [4–6]. Since crystallization is also associated with a decrease in solvent accessible surface area [7,8], the mechanism by which small molecules and salts stabilize proteins is directly applicable to the crystallization process. Furthermore, the mechanism by which small molecules and salts destabilize proteins is relevant to the mechanism for suppression of aggregation, another important aspect of protein crystallization. While proteins are large complex molecules, their interaction with ions and small solutes can be related with surprising directness to simple model systems (for example, an air–water interface) [5,6], and

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the purpose of this article is to apply the lessons learned from these simple model systems to the problem of protein crystallization.

1.2. Characteristics of small molecules and ions

Hofmeister [9] showed that neutral salts varied in their effect on the solubility of proteins. One group of salts could be ranked according to their efficiency at *precipitating* proteins, while a second group of salts could be ranked according to their efficiency at *solubilizing* proteins. Essentially this same total ordering of ions, with the same sign change between the two groups, can be generated by measuring their effect on protein stability or from many different physical measurements of aqueous salt solutions, such as the surface potential difference (at an air–water interface), the water activity coefficient, water proton nuclear magnetic longitudinal relaxation rates, infrared spectroscopy, behavior on Sephadex G-10 (gel sieving chromatography), Jones-Dole viscosity B coefficients, and solution neutron diffraction with isotopic substitution (NDIS) [5,10–12]. In this article, we shall discuss these last three characterizations of salt solutions because they can effectively produce ion-specific measures and are particularly informative about the origins of the Hofmeister series.

1.2.1. Sephadex G-10 gel sieving chromatography [4,13,14]

Sephadex G-10 is dextran highly crosslinked with epichlorohydrin. This produces beads with a nonpolar surface (effectively, substituted 1,4-dioxane) and small pore size. Small molecules penetrate the beads and elute from the column late, while large molecules are excluded from the beads and elute from the column early. Only neutral salts (i.e., ion pairs) penetrate the beads; nonetheless, using reasonable assumptions it is possible to extract ion-specific properties from the column. The chromatography of salts on Sephadex G-10 is dominated by the anions, and Fig. 1 shows the behavior on Sephadex G-10 of a number of salts which affect the stability of *E. coli* dihydroorotase in dilute solution, which is also dominated by the anions. [The effect of salts on the solubility of proteins is also dominated by the anions, as in other cases probably mostly because the anions are larger than the cations.] First, the Sephadex G-10 column cleanly separates the protein stabilizing anions [sulfate, phosphate, fluoride (and formate, which is not shown)] as a group below the calibration line, from the protein destabilizing ions which are above the calibration line. Second, the sequence of elution from the column of specific ions within each group correlates well with their tendency to stabilize or destabilize proteins; the behavior of the spherical halides suggests that surface charge density of the ions is the controlling property. Third, the elution of the stabilizing anions from the column is concentration

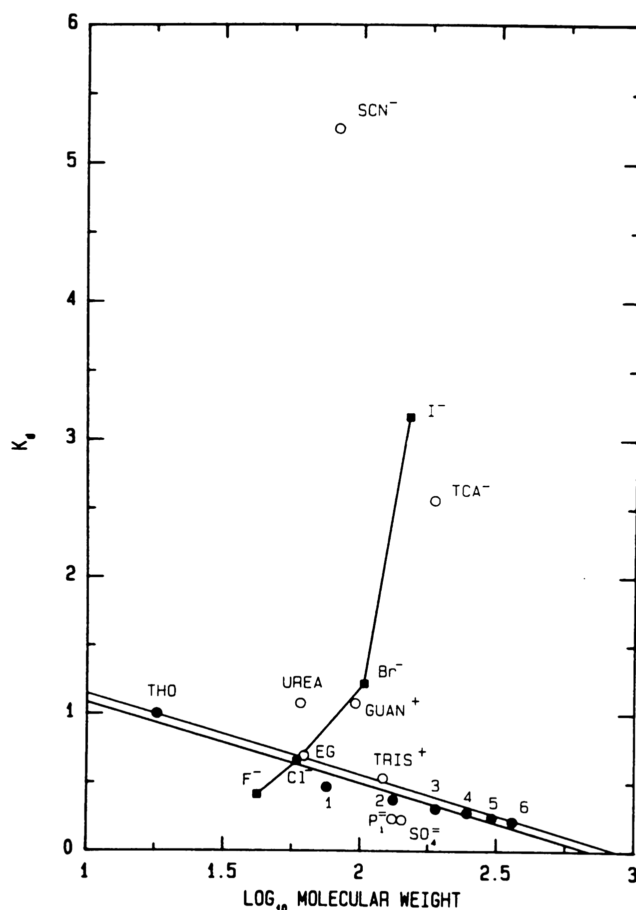

Fig. 1. Gel sieving chromatography of salts and solutes on Sephadex G-10. 1.0-ml samples containing 0.1 M solute in 0.1 M NaCl plus ~2 μCi ^3H_2O, and 0.5% dextran were chromatographed on a Sephadex G-10 column (1.5 × 85.5 cm) at 30 °C and a flow rate of 0.5 ml/min. The eluant was 0.1 M NaCl, pH 7.0. Anions were chromatographed as sodium salts; cations were chromatographed as chloride salts. The *double line* is our best estimate of ideal behavior for solutes on Sephadex G-10. The relative elution position K_d is defined as $K_d = (V_e - V_o)/(V_i - V_o)$, where V_i is the included volume (measured with ^3H_2O), V_o is the excluded volume (measured with dextran), and V_e is the elution volume for a given solute. The points labeled 1–6 represent glycine and its homopolymers through hexaglycine. EG, ethylene glycol; TCA^-, trichloroacetic acid; $GUAN^+$, guanidinium; $TRIS^+$, protonated Tris; and THO, ^3H_2O. All solutes were detected by scintillation counting or specific colorimetric assays. Data from Washabaugh and Collins [4].

and temperature independent, characteristic of gel sieving—that is, the ions do not contact the surface of the gel; the stabilizing anions have an apparent molecular weight greater than their anhydrous molecular weight because they have water molecules strongly bound to them, and the number of strongly bound waters for both cations and anions has been determined from data of this type [14] (this is one measure of the water affinity of an ion). Fourth, the elution of the destabilizing ions is concentration and temperature dependent, characteristic of adsorption to the nonpolar surface of the gel; that is, denaturing ions bind water less strongly than water binds itself in bulk solution, and denaturing ions adsorb

to nonpolar surfaces. The driving force for this adsorption is the release of weakly bound water to become more strongly interacting water in bulk solution. This pivotal observation was the first proof that the unstructured or weakly held water associated with chaotropes is immediately adjacent to the ion rather than outside of a tightly bound annulus of water. This resolved a forty-year-old controversy [5] and is important because embedded in the model of continuum electrostatics is the disproven hydration model for chaotropes.

1.2.2. Jones-Dole viscosity B coefficients [6,15]

The viscosity of a salt solution can easily be measured, for example, by determining the time required for a solution to flow through a small hole in the bottom of a tube. The results can be fitted to the following polynomial in c, the concentration of the salt, up to about 0.1 M for binary strong electrolytes:

$$\eta/\eta_o = 1 + Ac^{1/2} + Bc,$$

where η is the viscosity of a salt solution and η_o is the viscosity of pure water at the same temperature; A is an electrostatic term that is essentially 1 for moderate salt concentrations; and B is a direct measure of the strength of ion–water interactions normalized to the strength of water–water interactions in bulk solution. Table 1 presents Jones-Dole viscosity B coefficients for a series of ions of biological significance. We see first that the Jones-Dole viscosity B coefficient separates the ions into the same two groups as does Sephadex G-10 and *E. coli* dihydroorotase stability, with positive B coefficients for strongly hydrated ions and negative B coefficients for weakly hydrated ions. The point at which the Jones-Dole viscosity B coefficient changes sign represents ideal behavior as defined by the strength of water–water interactions in bulk solution (no preferential interactions). Within each group the ions are also ordered in the same manner, according to the surface charge density on the atoms to which the water molecules are attached. Second, we see that the negative charges on proteins (carboxylates) are strongly hydrated, whereas the positive charges on proteins (derivatives of ammonium) are weakly hydrated. And third, we see that the major intracellular anions (carboxylates and phosphates) are strongly hydrated whereas the major intracellular monovalent cations (K^+ and the positively charged amino acid side chains) are weakly hydrated. This mismatch in water affinity between the major intracellular anions and cations is important because it ensures that the charges on macromolecules remain free of counterions; this increases the solubility of the macromolecules (since only net neutral complexes crystallize) and functionally allows their charges to be used as binding determinants.

Table 1
Jones-Dole viscosity B coefficients

Cations	B	Anions	B
Mg^{2+}	0.385	PO_4^{3-}	0.590
Ca^{2+}	0.285	$CH_3CO_2^-$	0.250
Ba^{2+}	0.22	SO_4^{2-}	0.208
Li^+	0.150	F^-	0.10
Na^+	0.086	HCO_2^-	0.052
K^+	−0.007	Cl^-	−0.007
NH_4^+	−0.007	Br^-	−0.032
Rb^+	−0.030	NO_3^-	−0.046
Cs^+	−0.045	ClO_4^-	−0.061
		I^-	−0.068
		SCN^-	−0.103

Sources. Phosphate, formate, and perchlorate from Krestov [108]; all others from Robinson et al. [109].

High resolution X-ray crystallographic studies of protein–water interactions confirm the hydration properties of simple ions as revealed by Jones-Dole viscosity coefficients. For example, within grooves on the protein surface, it is the aspartate and glutamate side chains that are the most extensively hydrated [16]. Additionally, the amide bond has substantial zwitterionic character [17], with the anionic oxygen being strongly hydrated and the cationic nitrogen atom being weakly hydrated. A 1.2 Å crystal structure of aminopeptidase revealed that "Twice as many water molecules made hydrogen bonding interactions with main chain and side chain CO groups than with NH groups. Additionally, the average hydrogen bonding distance to the main chain and side chain nitrogen atoms (3.05 Å) was longer than that to the main chain and side chain oxygen atoms (2.95 Å) [18]. While the van der Waals radius of the amide oxygen is 1.41 Å and that of the amide nitrogen is larger at 1.49 Å [19], other studies have also found a preference for hydration of amide oxygen atoms over nitrogen atoms in protein structures [20–23], suggesting that the closer approach of water to the amide oxygen than to the amide NH is the result of stronger hydration of the former. Overall, the hydration properties of the peptide amide backbone group appear to be near that of an ideal solute [14].

1.2.3. Neutron and X-ray diffraction by isotopic substitution of ions in aqueous solution [11,24,25]

When more than one stable isotope of an ion is available, two identical solutions that vary only in the isotope can be used as a diffracting center for neutrons or X-rays. Radial distribution functions that measure the density of the solution as a function of the distance from the isotopic ion can be generated. When neutron diffraction and deuterium oxide are used, both the oxygen and the deuteron of the solvent can be detected; this allows one to determine the orientation of nearby water molecules. These procedures were developed by Enderby and Neilson [24], and have revolutionized our understanding of ion hydration.

Fig. 2 shows the neutron and X-ray diffraction of the IA cations Li^+, Na^+, K^+, and of water. As the surface charge density of the ion decreases, the density peak of

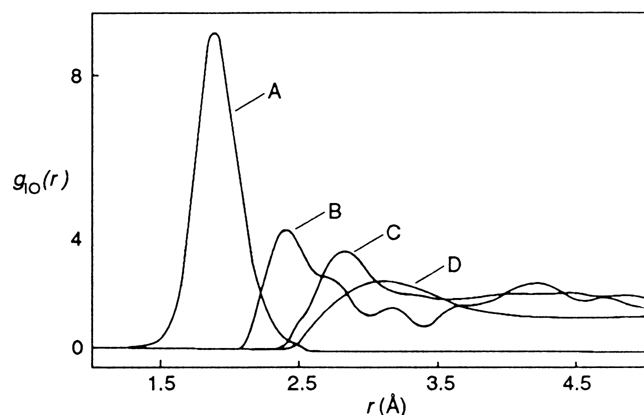

Fig. 2. The radial distribution functions $g_{10}(r)$ for Li^+ (curve A), Na^+ (curve B), water–water (curve C), and K^+ (curve D) in liquid water. These curves measure the density of the solution measured from the isotopically substituted ion, and effectively measure the distance from the monovalent cation to the nearest solvent oxygen. Curve C measures the oxygen–oxygen distance in liquid water. Reprinted from Enderby [11]. These are the data of Neilson, Enderby, and co-workers, and represent both neutron and X-ray diffraction experiments.

the nearest water oxygen is lower and further away, indicating weaker binding. The Na^+–oxygen distance is smaller than the water oxygen–oxygen distance, indicative of strong hydration for Na^+, while the K^+–oxygen distance is larger than the water oxygen–oxygen distance, indicative of weak hydration for K^+. Between Na^+ and K^+ is exactly where the Jones-Dole viscosity B coefficient changes sign. Additionally, Figs. 3–5 use neutron diffraction of deuterium oxide solutions to determine the orientation of the deuterium oxide molecules adjacent to Li^+, Ag^+ (an analog of Na^+), and K^+; strong hydration (Li^+) is associated with strong orientation of solvent, intermediate hydration (Ag^+) is associated with intermediate orientation of solvent, and weak hydration

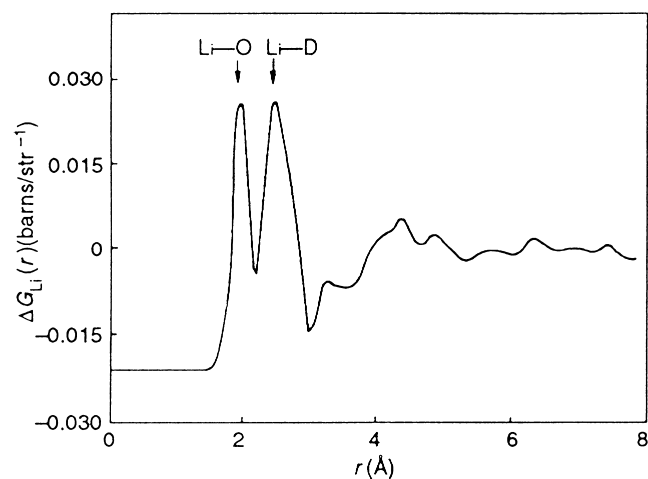

Fig. 3. The first-order difference function $\Delta G_{Li}(r)$ for Li^+ in D_2O. This curve measures the distance from the isotopically labeled Li^+ to the nearest solvent oxygen or deuteron. Reprinted from Enderby [11]. These are the neutron diffraction data of Newsome, Neilson, and Enderby.

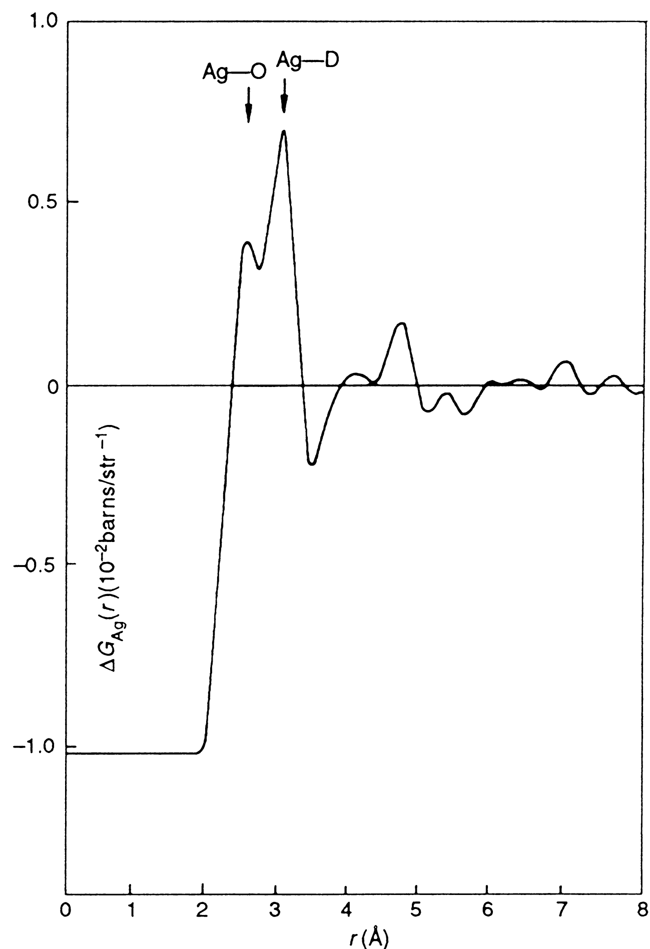

Fig. 4. The first-order difference function $\Delta G_{Ag}(r)$ for Ag^+ (an analog of Na^+) in D_2O. This curve measures the distance from the isotopically labeled Ag^+ to the nearest solvent oxygen or deuteron. Reprinted from Enderby [11]. These are the neutron diffraction data of Neilson and co-workers.

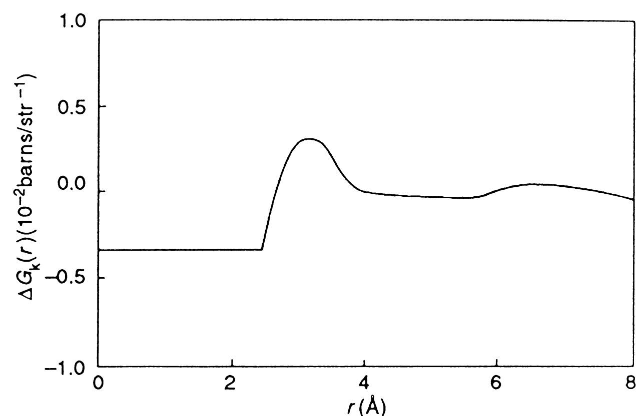

Fig. 5. The first-order difference function $\Delta G_K(r)$ for K^+ in D_2O. This curve measures the distance from the isotopically labeled K^+ to the nearest solvent oxygen or deuteron. Reprinted from Enderby [11]. These are the neutron diffraction data of Neilson and Skipper.

(K^+) is associated with no orientation of solvent. Thus, a chaotrope (such as K^+) is weakly hydrated: the immediately adjacent water is far away and not oriented. In fact,

nuclear magnetic resonance can be used to show that the water immediately adjacent to a chaotrope is actually tumbling more rapidly than is water in bulk solution [26]. Neutron diffraction has recently been used to characterize the strong denaturants guanidinium and thiocyanate in solution, verifying the weakly hydrated character of chaotropes [25]. Gel sieving experiments on Sephadex G-10 have been used to establish that Li^+ has 0.6 tightly bound water molecules, Na^+ has 0.25 tightly bound water molecules, and K^+ has no tightly bound water [13,14]. Clearly the hydration behavior of an ion is strongly dependent upon the surface charge density of the ion; ions do not behave as point charges.

"The expected oxygen distances for Na–O and K–O are approximately 2.4 and 2.7 Å, respectively, for small molecule structures," [18,27–31] and high resolution protein crystal structures (1.2–1.33 Å) are adequate to distinguish a Na^+ from a K^+ atom by the length of the metal–oxygen bonds [18,32].

1.2.4. The law of matching water affinities: oppositely charged ions in free solution form inner sphere ion pairs spontaneously only when they have equal water affinities [6]

The four techniques described above, protein stability, Sephadex G-10 gel sieving chromatography, Jones-Dole viscosity B coefficients, and neutron diffraction with isotopic substitution (NDIS) give a uniform view of ion hydration as being strongly dependent upon ion surface charge density and progressing from strong hydration for small ions of high charge density (kosmotropes) to weak hydration for large monovalent ions of low charge density (chaotropes). It is useful to consider an ion to be a sphere with a point charge at the center. As the sphere become larger (as one proceeds down the periodic table), the water molecules at the surface of the sphere becomes further from the point charge at the center of the sphere. When the water molecules at the surface of the sphere are so far from the point charge at the center that water–ion interactions are weaker than water–water interactions in bulk solution, the ion is a chaotrope. This is illustrated in Fig. 6, where the horizontal line between Na^+ and K^+ for the cations and Cl^- and F^- for the anions represents the strength of water–water interactions (ideal behavior, no preferential interactions). It is also convenient for these considerations to consider water to be a zwitterion with a cationic portion of the (interpolated) ideal cation size and an anionic portion of the (interpolated) ideal anion size at the horizontal line, also illustrated in Fig. 6. In the ensuing discussion, we shall refer to the strongly hydrated ions above the line in Fig. 6 as "small," to the weakly hydrated ions below the line as "large," and to water (at the line) as a "medium-size" zwitterion.

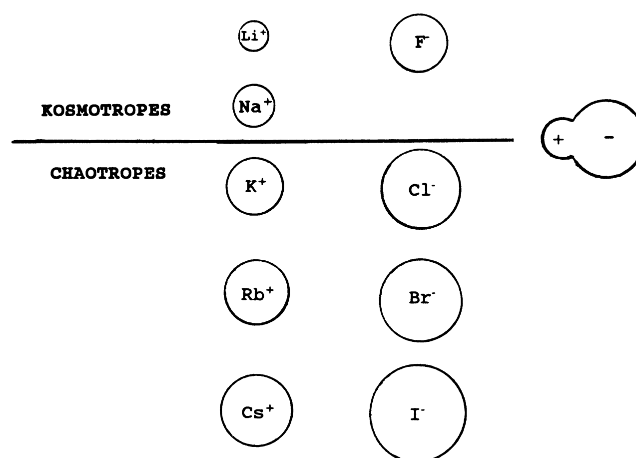

Fig. 6. Division of the group IA cations and the VIIA halide anions into [strongly hydrated] kosmotropes (water structure makers) and [weakly hydrated] chaotropes (water structure breakers). The ions are drawn approximately to scale. A virtual water molecule is represented by a zwitterion of radius 1.78 Å for the anionic portion and 1.06 Å for the cationic portion. In aqueous solution, Li^+ has 0.6 tightly attached water molecules, Na^+ has 0.25 tightly attached water molecules, F^- has 5.0 tightly attached water molecules, and the remaining ions have no tightly attached water [14].

Several observations contributed to the realization that a simple law controlled the tendency of ions of opposite charge to form inner sphere ion pairs. These observations were (a) the striking systematic dependence of the heats of solution of the simple alkali halides on the water affinity of the individual ions (absolute free energies of hydration); (b) the dependence of the solubilities of the alkali halides on ion size; (c) the fact that Cl^- competes with DNA for positively charged (basic) binding proteins but glutamate (the major intracellular anion in *E. coli*) does not; and (d) the fact that basic proteins are crystallized most effectively by chaotropic anions [6]. We shall argue here from the heats of solution of the alkali halides. In Fig. 7, the enthalpy (heat) of solution is plotted on the vertical axis: those salts clearly above the line at 0 produce cold solutions upon dissolution; those salts clearly below the line at 0 produce hot solutions upon dissolution. Plotted on the horizontal axis is the difference in absolute free energies of hydration (water affinity) of the constituent ions of the salt. We see that when the constituent ions of a salt are matched in water affinity (kosmotrope–kosmotrope and chaotrope–chaotrope salts), cold solutions are produced, suggesting that no strong interactions with water have occurred (which would release heat) and that the oppositely charged ions of the dissolved salt tend to stay together. This is to be expected: the point charge at the center of a (small) kosmotropic ion can get closer to the point charge at the center of an oppositely charged (small) kosmotropic ion than it can to the point charge at the center of the oppositely charged portion of a medium size zwitterion (water molecule); and, the point charges at the centers of the two charges on the medium size zwitterions (water molecules) can get closer to the charges on other water molecules than it can to the point charge at the center of a

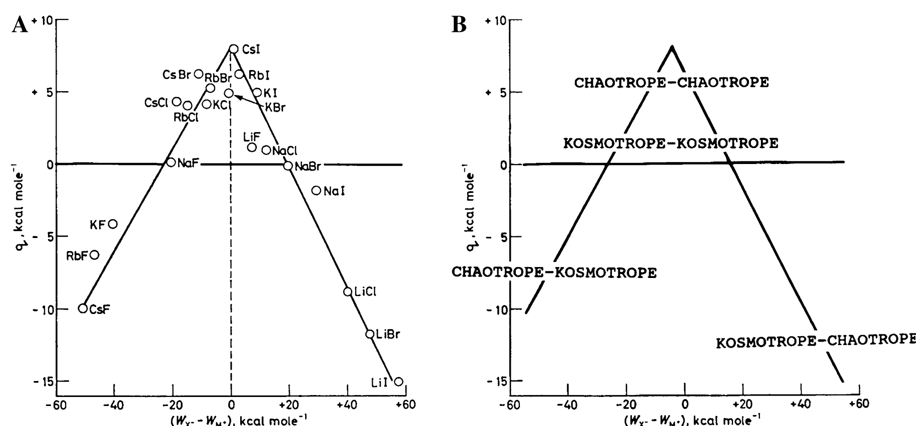

Fig. 7. (A) Relationship between the standard heat of solution of a crystalline alkali halide (at infinite dilution) in kcal mol^{-1} and the difference between the absolute heats of hydration of the corresponding gaseous anion and cation, also in kcal mol^{-1}. Source: Morris [33]. (B) Identification of ions as chaotropes (weakly hydrated) or kosmotropes (strongly hydrated). The enthalpy of solution of chaotrope–chaotrope and kosmotrope–kosmotrope salts is positive (takes up heat), whereas the enthalpy of solution of chaotrope–kosmotrope and kosmotrope–chaotrope salts is either negative (gives off heat) or positive (takes up heat).

(large) chaotrope. In contrast, when the constituent ions are mismatched in water affinity (kosmotrope–chaotrope and chaotrope–kosmotrope salts), hot solutions are often produced, suggesting that a strong interaction of the small ion with water has occurred and that the oppositely charged ions of the dissolved salt have separated. This is also to be expected, since the point charge at the center of a (small) kosmotropic ion can get closer to the point charge at the center of the oppositely charged portion of a medium size zwitterion (water molecule) than to the point charge at the center of the oppositely charged (large) chaotrope. The requirement of a chaotrope–kosmotrope or kosmotrope–chaotrope salt for an exothermic heat of solution is a necessary but not sufficient condition since when such a salt is dissolved the kosmotropic ion will generate heat as it goes from a (large) chaotropic partner to a (medium size zwitterionic) water molecule, and the chaotropic ion will take up heat as it goes from a (small) kosmotropic partner to a (medium size zwitterionic) water molecule. The net effect can be exothermic or endothermic. Thus, the chaotrope–kosmotrope salt ammonium sulfate is endothermic and generates cold solutions upon dissolution.

Fig. 8 contains much the same information. Small ions of opposite charge will tend to come together because the point charges at their centers can get closer to each other than with the point charges at the centers of the medium size zwitterions (water molecules). Large ions of opposite charge will come together because the released water molecules can form stronger medium–medium interactions. And (small) kosmotropic ions will not spontaneously dehydrate to form an inner sphere ion pair with an oppositely charged (large) chaotropic ion because the point charge at the center of the kosmotropic ion can get closer to the point charge at the center of the oppositely charged portion of a medium size zwitterion (water molecule) than to the point charge at the

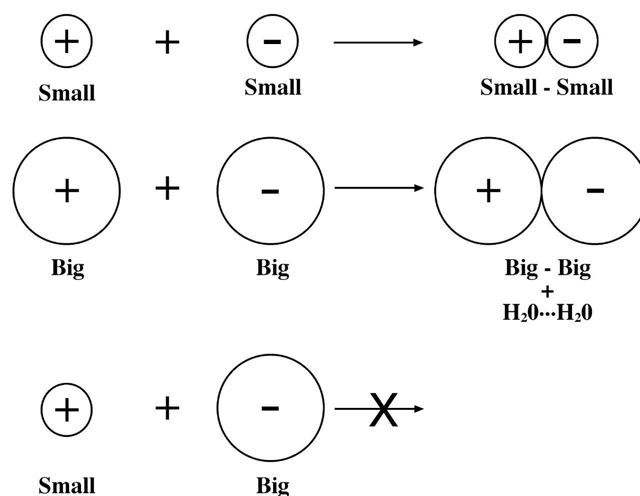

Fig. 8. Ion size controls the tendency of oppositely charged ions to form inner sphere ion pairs. Small ions of opposite sign spontaneously form inner sphere ion pairs in aqueous solution; large ions of opposite sign spontaneously form inner sphere ion pairs in aqueous solution; and mismatched ions of opposite sign do not spontaneously form inner sphere ion pairs in aqueous solution. A large monovalent cation has a radius larger than 1.06 Å; a large monovalent anion has a radius larger than 1.78 Å.

center of an oppositely charged (large) chaotrope. Thus, we conclude that oppositely charged ions in free solution spontaneously form inner sphere ion pairs only when they have equal water affinities.

1.3. Forces affecting protein behavior in solution

1.3.1. Preferential interactions

The association of any two moieties in aqueous solution involves at least a partial dehydration, and since only moieties with matching water affinities (absolute free energies of hydration) spontaneously form inner sphere ion pairs, it is possible to predict the behavior of a

small molecule or ion by knowing its water affinity, which can be measured or calculated in several different ways, some of which were described above. The "preferential interaction" of a solute or ion with water is a measure of its water affinity normalized to the strength of water–water interactions in bulk solution. The preferential interaction of a protein with a small molecule or ion can also be measured directly, usually by dialysis [34], ultracentrifugation [35], or vapor pressure measurements [36]; it results from the relative water affinity of the exposed moieties of the protein and the ion or small molecule, and indicates whether the interaction of the small molecule or ion with the protein is largely direct or indirect (mediated through intervening water molecules). Another form of preferential interaction important in protein crystallization is that between protein molecules (the potential of mean force). It is usually measured by the second osmotic virial coefficient as determined by light scattering [37] although other measurement procedures have been developed [38] and indicates the tendency of the protein molecules to associate [39–42]. The "crystallization slot" is a range of the second osmotic virial coefficient such that the attractive force between protein molecules is strong enough to favor crystallization, but not so strong as to cause nonspecific aggregation. The cloud point temperature has been used to measure the effects of salts on the liquid–liquid phase separation of lysozyme [43].

1.3.2. Excluded volume

The effect of neutral solutes on protein solubility and interactions has been concluded to involve a significant excluded volume component [44,45].

1.3.3. Surface potential difference

The surface potential difference at an air–salt solution interface generates the Hofmeister series with a sign inversion in the center of the sequence [46], mimicking the effects of the same salts on protein stability and solubility. The molecular mechanism underlying the surface potential difference appears to be the same as that underlying the *indirect* (mediated through intervening water molecules) effect of ions and neutral solutes on the *polar* portion of the protein surface, including the backbone amide residues [47] exposed to the solvent upon transient partial unfolding events ("breathing" motions). This surface potential difference effect is probably the dominant mechanism energetically by which strongly hydrated solutes such as sulfate and malonate stabilize proteins and decrease their solubility.

The mechanism by which ions (or other solutes) modulate the ability of an aqueous solution to solvate a polar surface is illustrated in Fig. 9. We shall conceptually divide the interfacial region near a test solute such as a protein molecule into three layers, each layer being one water molecule thick. The first water layer immediately adjacent to the protein surface is designated the solvation layer; the second water layer is designated the transition layer; and the third layer is designated the bulk surface [5]. The test solute determines the behavior of the solvation layer; the bulk solution determines the behavior of the bulk surface; and the solvation layer and bulk surface both compete for hydrogen bonding interactions with the transition layer. Ions (or other solutes) inserted into the third water layer modulate the ability of the second water layer to "help out" the first water layer solvate the protein surface, as shown schematically in Fig. 9 by the arbitrarily chosen number of hydrogen bonds between the first and second water layers. Highly directional polar hydrogen bonding interactions dominate in aqueous solution, and a water molecule cannot achieve the maximum pairwise enthalpy of interaction with each of its immediate neighbors simultaneously; it will therefore "choose" to interact most strongly with the neighbor for which it has the most favorable pairwise enthalpy of interaction. In the absence of ions (or other small solutes), the third water layer (bulk surface)

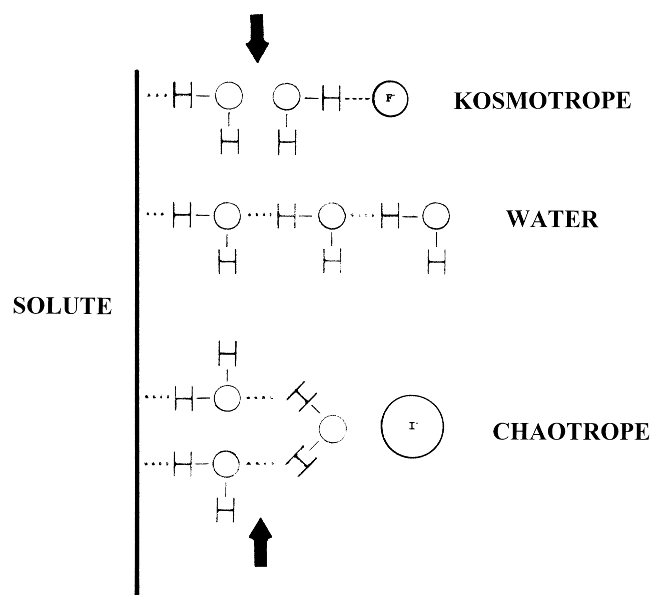

Fig. 9. Interfacial water near the polar surface of a test solute (protein molecule). Ions inserted into the third interfacial water layer modulate the interaction of the second interfacial water layer with the first interfacial water layer (arrows). While the number of hydrogen bonds between the second and first interfacial water layers must increase from the top of the figure to the bottom, the actual number shown is arbitrary. A [strongly hydrated] kosmotrope inserted into the third interfacial water layer makes the bulk solution a poorer solvent, and causes the protein molecule to minimize its solvent accessible surface area both by becoming more compact and also by forming crystal contacts (i.e., it decreases the solubility of the protein). A [weakly hydrated] chaotrope inserted into the third interfacial water layer makes the bulk solution a better solvent, and causes the protein molecule to maximize its solvent accessible surface area (i.e., it increases the solubility of the protein); however, the dominant mechanism by which chaotropes increase protein solubility is by interacting directly with the weakly hydrated portions of the protein. See the text for more details.

interferes somewhat with the ability of the second water to help out the first water layer in solvating the protein surface (middle of Fig. 9). When a strongly hydrated anion or neutral solute (kosmotrope) is inserted into the third water layer, the second water layer is "busy" solvating the kosmotrope and cannot help the first layer solvate the protein surface (top of Fig. 9); the solution thus becomes a poorer solvent and the protein attempts to minimize its solvent exposed surface area by becoming more compact (and rigid) [4,48–52] and forming protein crystal contacts [7,8]. In contrast, when a weakly hydrated anion (chaotrope) is inserted into the third water layer, the second water layer is freed up to help out the first water layer solvate the protein surface more effectively (bottom of Fig. 9); the solution thus becomes a better solvent and the protein attempts to maximize its solvent exposed surface area by unfolding. [In fact, the dominant mechanism by which chaotropes unfold proteins is to adsorb directly to the weakly hydrated portions of the protein [53].

This model is based upon the following evidence:

(i) Strongly hydrated anions are excluded from the surface of Sephadex G-10 [4] and from the surface of proteins [53].
(ii) The surface potential difference at an air–salt solution interface [46] generates the same ion sequence (Hofmeister series) with the same sign change in the middle as do protein solubility [9] and protein stability [10].
(iii) The magnitude of the surface tension increment at an air/0.1 M salt solution interface indicates a separation of the ions from the interface of two water molecules [5].
(iv) Gas phase studies of K^+ solvation indicates that water is unique in that the second solvent layer makes a large contribution to the free energy of interaction of the solvent with test solutes [54].
(v) The hydrodynamic radii of polar kosmotropes are increased by chaotropes and decreased by polar kosmotropes, illustrating that kosmotropes make interfacial water molecules less available and chaotropes make them more available [4].

The "ionic strength" of a salt solution has been interpreted in terms of the hydrated size of the ions—that is, in terms of short range hydration forces [14].

1.3.4. Surface tension [55]

Dissolved solutes change the surface tension at a water–nonpolar interface such as a water–air or water–protein interface. Except for ions of very low charge density or large nonpolar species, both of which adsorb to the nonpolar surface of an interface and decrease the surface tension, dissolved solutes act indirectly to increase the surface tension [46]. This surface tension increase occurs because the dissolved solutes near the interface create a more complex geometry available to water, making it more difficult for water molecules to maximize their interactions in the interfacial region; water molecules thus prefer an interior location away from the interface, creating a force which tries to minimize the amount of interface. The increase in surface tension appears to be the major effect of strongly hydrated solutes near the *nonpolar* portions of the protein surface (typically about 57% of the total surface for soluble globular proteins [56]), and creates an increased driving force for the burial of the nonpolar surface [6,57,58,111].

1.3.5. Water activity

The water activity, or effective water concentration, contributes directly to the equilibrium constant for any process such as binding or crystallization which involves a dehydration. By measuring the distribution between free and associated protein as a function of water activity (which can be determined from the vapor pressure of the solution), the number of water molecules released upon association can be calculated [59]. Lower water activities favor protein association by suppressing the back (hydration) reaction.

1.3.6. Counterions of protein charged groups

The positive charges on proteins, being derivatives of the weakly hydrated ammonium ion, are all weakly hydrated. The guanidinium and imidazolium ions have lower charge densities than ammonium, and are therefore more weakly hydrated than ammonium. Since it is oppositely charged ions of equal water affinity which form inner sphere ion pairs, weakly hydrated anions such as chloride and thiocyanate bind directly to proteins while strongly hydrated anions such as sulfate, phosphate, and fluoride have predominantly indirect interactions with proteins (via intervening water molecules). Anions of lower charge density bind more tightly to proteins (the reverse Hofmeister series) just as they do to the nonpolar surface of Sephadex G-10 [4]; that is, thiocyanate binds more tightly than iodide, which binds more tightly than bromide, and which binds more tightly than chloride [60]. The binding of anions to proteins has been detected by equilibrium dialysis [53,61], electrophoresis [62,63], X-ray crystallography [32,64–68], nuclear magnetic resonance [69,70], and many other techniques [71]. Typical dissociation constants for the interaction of Cl^- with proteins are in the range of 30–60 mM [70], while the intracellular eukaryotic Cl^- concentration is 3 mM [72]. A wide range of dissociation constants for the binding of Cl^- to proteins have been reported: 2.5 mM [73]; 34 mM [70]; 56 mM [70]; 112.9 mM [73]; 133 mM [75]; and 150 mM [74]. In contrast to the weakly hydrated Cl^-, the strongly hydrated F^- interacts with proteins indirectly [48,75,76] except when proteins have an HO^- binding site; F^- acts as an HO^- analog in biological systems [77]. The binding of large, weakly

hydrated anions such as thiocyanate, nitrate, and iodide is characterized by a preference for interactions with the weakly hydrated positively charged side chains of arg, lys, and his as well as the weakly hydrated partial positive charge on amide NHs [32]. Because of their large size and multiple contacts with the protein, thiocyanate, nitrate, and iodide also have a tendency to bridge protein subunits [32,78]. Simple considerations of water affinity also explain the greater affinity of the weakly hydrated positive charges on proteins for the weakly hydrated anionic sulfate ester ion exchangers as compared to the strongly hydrated anionic carboxylate ion exchangers [79]. Rapidly soaked halides can also be used to phase crystallographic macromolecular structures [68].

The negative charges on proteins, which are carboxylates, are strongly hydrated: the carboxylate binds two water molecules tightly [14]. It is oppositely charged ions of equal water affinity that form inner sphere ion pairs. The monovalent cation observed to be the most effective at binding to and "neutralizing" the carboxylate is Na^+, followed by K^+, followed by Li^+, and followed by Cs^+ [80,81]. Na^+ has a dissociation constant with the carboxylate of about 40–50 mM [80].

The hydration properties of NH_4^+ are very similar to those of K^+ (Table 1). Ca^{2+} is closely matched in water affinity to the carboxylate and forms salts of low solubility with dicarboxylates [110]. Ca^{2+} binds rapidly to protein carboxylates and functions as a signaling molecule in vivo [82,83]; Ca^{2+} typically has about seven protein ligands of variable geometry [84]. Mg^{2+}, although more strongly hydrated than the carboxylate, also binds readily to proteins, strongly preferring 6 oxygen ligands in an octahedral configuration [84,85]. Both Ca^{2+} and Mg^{2+} may complex directly with amide moieties and function as denaturing salts [10,53,86–89]. Obviously, proteins can have highly specific binding sites for Na^+, K^+, Ca^{2+}, or Mg^{2+} which may control the conformation of the protein [90–92]. Weakly bound ions may also play more subtle roles in protein crystals; for example, a surface Na^+ in aminopeptidase crystals appears to mediate a crystal contact [18].

Often one aspect of the crystallization process dominates the overall process. For proteins with an excess of weakly hydrated positive charges, the limiting aspect appears to be the binding of anions to produce a net neutral protein, which then crystallizes [60,93]. For example lysozyme, which has a net charge of about +11 at pH 5 where it is often crystallized, crystallizes readily in the presence of weakly hydrated ions such as Cl^- or thiocyanate, but only with difficulty from sulfate or phosphate. That is, this protein shows a reverse Hofmeister effect—weakly hydrated anions are the most effective precipitants [60,93]. Therefore, in this case the requirement for direct binding of a weakly hydrated anion to produce a net neutral protein seems to be more important than the indirect-acting interfacial effects of the strongly hydrated sulfate or phosphate which make the bulk solution a poorer solvent; in fact, the protein in sulfate or phosphate solutions may crystallize only when enough sulfate or phosphate binds directly to the protein to produce a net neutral species [94,95]. Sulfate and phosphate are strongly hydrated (Table 1) [6,14], and thus do not readily form inner sphere ion pairs with the weakly hydrated positive charges on proteins.

In contrast, an acidic protein with an excess of strongly hydrated negative charges near neutrality such as the *Hypoderma lineatum* collagenase, which presumably must bind a number of monovalent cations to produce a net neutral protein capable of crystallizing, shows a normal Hofmeister effect [96]. That is, strongly hydrated anions are the most effective precipitants, indicating that the (indirect acting) interfacial effects associated with dehydrating the excess of strongly hydrated negative charges on the protein are the limiting aspect of crystallization rather than the binding of monovalent cations to the protein carboxylates to produce a net neutral protein.

2. Protein stability

2.1. Ion effects

Proteins are stabilized by high concentrations of strongly hydrated anions and destabilized by high concentrations of weakly hydrated anions or strongly hydrated cations [10]. Stabilization and crystallization are both associated with a decrease in the solvent accessible surface of a protein; destabilization and solubilization are both associated with an increase in the solvent accessible surface of a protein.

Because protein salt bridges are composed of a strongly hydrated carboxylate and a weakly hydrated cationic amino acid side chains, they are only marginally stable (it costs more to dehydrate the carboxylate than one gets back by forming the salt bridge with the ammonium-based cation) [97]. Salt bridges are more stabilizing in thermophilic environments because the energetic cost of dehydrating the carboxylate is smaller at higher temperatures. Cation–π interactions, on the other hand, involve two weakly hydrated amino acid side chains; there is thus no desolvation penalty for their formation, and they are estimated to be about twice as strong as salt bridges [98,99].

2.2. Favorable and unfavorable interactions of osmolytes

Typically about 57% of a soluble globular protein surface is nonpolar [56], and the uncharged compatible solutes have been shown to have a significant affinity for nonpolar surfaces [Kiriukhin and Collins, unpublished].

Thus, the uncharged compatible solutes appear to be acting at least partially as chemical chaperones intercepting nonpolar protein surfaces and preventing nonspecific aggregation. It is likely that many crystallization solutions require soluble components such as 2-methyl-2,4-pentanediol [100] that function at least partially as weak detergents which prevent nonspecific aggregation.

3. Protein solubility

3.1. Solubility and electroneutrality

The solubility minimum of a protein is found at its isoelectric point, where the protein has no net charge in the absence of added ions [101,102]; the isoelectric point is defined experimentally as that pH at which the protein does not migrate in an electric field. Crystallization of the net neutral protein at its isoelectric point does not require that any additional ions be bound to the protein. Away from its isoelectric point, the concentration of net neutral protein decreases in the absence of added ions. Crystallization of the charged protein away from its isoelectric point requires that additional ions lose their independent freedom of motion and bind to the protein to produce a net neutral species.

3.2. Salting in and salting out

"Salting in," an increase in protein solubility upon the addition of salt, appears to occur when ions bind to proteins and increase their net charge [53,93,103]. Under these conditions, the net neutral species (which are the only ones that can crystallize) are reduced in concentration, and thus the solubility of the total protein has increased. "Salting in" effects can sometimes be dramatic: for example, an increase of 50 mM in KCl concentration produces a 20-fold increase in the solubility of T7 RNA polymerase followed by a decrease in solubility that is almost as abrupt [104]. Another example: although the solubility of thermolysin is only 1.0–1.2 mg/ml in 40 mM Tris–HCl buffer, pH 7.5, in the temperature range between 0 and 60 °C, it is increased greatly by the addition of salts. With NaCl, the solubility shows a bell-shaped behavior with increasing NaCl concentration, and the maximum solubility (10 mg/ml) is at 2.0–2.5 M NaCl. With LiCl and NaI, it increases progressively to 20–50 mg/ml with increasing salt concentration up to 5 M. The solubility observed in the presence of salts decreases with increasing temperature from 0 to 60 °C, and also with decreasing charge density of the anion [81]. A final example: the *E. coli* Ic1R-like proteins require up to 0.6 M KCl for solubility [105]. To repeat, salting in appears to be the result of *direct* binding of ions to proteins. While there probably is an *indirect* "salting in" effect in high concentrations of chaotropes analogous to the indirect "salting out" mechanism for strongly hydrated anions (kosmotropes) described above, proteins are not crystallized in high concentrations of chaotropes.

"Salting out" appears to result from interfacial effects of strongly hydrated anions *near* the surface of proteins; in contrast, strongly hydrated cations such as Ca^{2+} and Mg^{2+} in high concentration tend to interact with the polar surface of the protein (such as amide groups) and increase protein solubility. Strongly hydrated anions such as sulfate compete for water molecules in the second hydration layer of the protein, making them unavailable to "help out" the first hydration layer in solvating the polar surface of the protein [this is the phenomenon measured by the surface potential difference at an air–water interface]. The net effect is to make the bulk solution a less good solvent, encouraging the protein to minimize its solvent accessible surface area. For *nonpolar* portions of the protein surface, it is more accurate to think of the nearby strongly hydrated anions such as sulfate as raising the surface tension of the bulk solvent, again encouraging the protein to minimize its solvent accessible surface area. That is, salting out is an *indirect* effect, mediated by intervening water molecules. The logarithm of the protein solubility often decreases linearly with increasing ionic strength of ammonium sulfate solutions [106,107]. Ionic strength is an empirically observed parameter that can be interpreted in terms of the hydrated size of ions [14].

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