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Review

Why continuum electrostatics theories cannot explain biological structure, polyelectrolytes or ionic strength effects in ion–protein interactions

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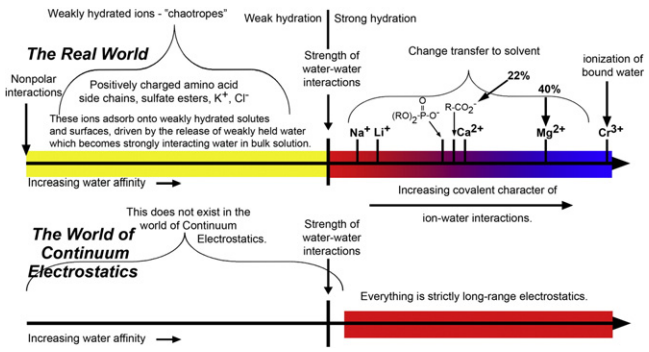
HIGHLIGHTS

- The charge density of ions controls their water affinity.
- The water affinity of ions controls their behavior.
- Chemistry is important for ions of high charge density in water.

GRAPHICAL ABSTRACT



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ABSTRACT

Continuum electrostatics models for ions in water provide apparent long range electrostatic explanations for the forces on ions. However the electro-chemical free energy of solvation of ions resides largely in the first two water layers, which control the interfacial behavior of the ions and require explicit modeling to capture their distinctive behaviors. The resulting short range forces produce such surprising charge density-dependent behaviors as ion adsorption onto nonpolar surfaces, like charge aggregation of ions, and substantial ion pairing preferences, which arise largely from the affinity of specific ions for individual water molecules. Specific ion effects controlled by the local water affinity of the ion show a diagnostic change of sign between strongly hydrated Na^+ and weakly hydrated K^+ and between strongly hydrated F^- and weakly hydrated Cl^-, in both cases marking the strength of water–water interactions in bulk solution, a critical benchmark missing from continuum electrostatics models.

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1. Introduction

1.1. Continuum electrostatics theories for ions in water fail in complex environments or concentrated solutions

A comprehensive theoretical framework for describing the behavior of ions in water was first developed in the early part of the 20th century [1,2]. These macroscopic models were cast in terms of long range electric fields in a structureless dielectric continuum, and seemed to work for dilute solutions (salt concentrations no larger than 10 mM); thus many people assumed that the models had captured the key determinants of how ions behave in water. But new experimental tools, computational capabilities, and a great deal of structural and functional information on biological systems reveal dramatic failures in the ability of the original models to explain the behavior of ions in more concentrated or complex environments

[3,4]. Among the most surprising developments of the past twenty five years are the demonstration by Washabaugh and Collins in 1986 that ions of low charge density adsorb onto weakly hydrated surfaces, driven by the release of weakly held water which becomes strongly interacting water in bulk solution [5]; the demonstration by Collins in 1997 that ion pairing preferences are controlled by the water affinity of the ions [6]; and the demonstration by Glusker and co-workers in 1994 [7] and by Merz and co-workers in 1998 [8] that ions of high charge density leak substantial amounts of charge onto the solvent. In short, the early macroscopic continuum electrostatics models fail dramatically over issues of granularity and the omission of chemistry.

Today the most sophisticated work in solution electrostatics is being done by those studying the role of charges in proteins [9] and the effects of salts on them [10,11]. These proteins typically have well defined structures whose charged amino acids residues can be selectively changed by site specific substitution, and whose solubility and stability can be altered by specific ion effects from added salts. Part of this interest in proteins is driven by the increasing use of monoclonal antibodies as therapeutic agents by the pharmaceutical industry and their requirement for stable, concentrated solutions of these antibodies [10,11]. Further undercutting the apparent initial success of the early macroscopic continuum electrostatics models is the fact that the most important current problem in protein electrostatics where fundamental understanding is missing is how to explain why electrostatic forces are so weak in these systems [[9], and other references listed below]. The answer appears to be that in the high dielectric constant regime of water the long range electric fields are fairly weak to begin with, and those generated by the positive and negative ions largely cancel out, leaving the local interfacial effects and chemistry to dominate. First of all, by examining hydration free energies *via* energetic partitioning of the potential distribution theorem, Beck has recently shown that about half of the free energy of the electro-chemical solvation of ions is manifested in the first layer of water molecules [12,13]. Since we have earlier shown that the first two water layers must be modeled explicitly to capture their distinctive behaviors [14,15], it therefore follows that the majority of the electro-chemical free energy of ion solvation is involved in the local interfacial behavior of ions, not in long range effects. And second of all, not only are biological systems moderately concentrated (the eukaryotic cytosolic K^+ concentration alone is about 160 mM [16]), but also dielectric spectroscopy indicates that the constituent ions of simple salts are usually within two water molecules of each other in moderately concentrated solutions [17]. Some have acknowledged the dominance of these microscopic short range forces by adapting hybrid models which explicitly model the first water layer adjacent to an ion while treating the far-field electrostatics with a dielectric constant [12,18–20]. Hybrid quantum mechanical/molecular dynamics approaches have also been developed in which the first hydration sphere of the ion is treated quantum mechanically and subsequent hydration layers are treated by molecular dynamics [21].

The purpose of this review is to examine the evidence for the dominant role of the water affinity of ions and of chemical processes involving ions in the control of their behavior in water. This review is organized in the following way: In Section 2 we give at least nine examples illustrating that electrostatic interactions in water are weak; in Section 3 we summarize the evidence that ion–water interactions have some covalent character for ions of high charge density, that is, that charge transfer to solvent (and thus quantum chemistry) is important for these ions; in Section 4 we describe the inability of continuum electrostatics theories to treat the following seven fundamental issues involving ions in water: weakly hydrated ions (chaotropes), chemistry, granularity in general, the strength of water–water interactions, contact ion pair formation, the stronger interaction of anions with water as compared to cations; and charge density dependent ion behaviors; in Section 5 we present the basic facts of ion charge density dependent strength of hydration; in Section 6 we describe three techniques to measure the water affinity of ions, explain how ions of matching water affinity form contact ion pairs thus lowering their solubility (with important biological implications); explain how the water affinity of an ion rather than the charge itself defines its biological role; and mention that water affinity is the origin of Hofmeister effects; in Section 7 we attempt to evaluate the role of ion polarizability; in Section 8, as an aid to the design and interpretation of experiments, we provide a current summary of the forces and processes that underlie ion-protein interactions; and in Section 9 we conclude that it will require explicit modeling of aqueous solutions with quantum chemistry to accurately represent the local forces that control the behavior of ions in water and make life possible.

2. Experimental evidence that electrostatic interactions in water are weak

2.1. Crystallographic and solution evidence for "electrostatics defying" behaviors

2.1.1. Anionic phosphate binds rapidly to anionic protein sites

The controlling influence of water affinity and of short range chemical processes in the behavior of ions in water is dramatically illustrated by the approach of negatively charged inorganic phosphate to its negatively charged binding site on the *E. coli* phosphate binding protein at a near diffusion controlled rate [22]; when bound, the shortest hydrogen bond (2.4 Å) between the buried and completely dehydrated inorganic phosphate and the protein is with the carboxylate side chain of Asp56 as determined by X-ray crystallography [23]. The *M. tuberculosis* ABC phosphate transport receptor protein is almost completely covered with negative charge, and the two shortest hydrogen bonds between the bound inorganic phosphate and the protein are with the carboxylate side chains of Asp83 (at a distance of 2.52 Å) and Asp168 (at a distance of 2.54 Å) [24]. Many other instances are also known of anionic ligands binding to patches of negative charge on proteins [25].

2.1.2. Cationic arginine side chains often stack on top of each other in proteins

A particularly striking instance of water affinity effects overriding repulsive charge–charge forces is the tendency of positively charged arginine side chains to stack on top of each other in protein structures. An X-ray crystallographic dataset of 266 protein dimers is found to have about 22% of the positively charged arginine side chains mutually paired (stacked on top of each other) within subunits and an additional 17% paired (stacked on top of each other) across interfaces; that is, about 40% of the arginine side chains in proteins are stacked on top of each other [4,26]. The driving force for the formation of these stacked cationic moieties is actually three-fold: (a) a slight offset of the positively charged guanidinium groups such that partial charges of the same sign on specific atoms are not directly on top of each other; (b) the release of weakly held water upon complex formation to become strongly interacting bulk water; and (c) favorable dispersion interactions between the stacked guanidinium groups [4].

2.1.3. Anionic sulfate ions can be found inside anionic nucleic acids

Large anions are easy to see in X-ray structures whereas the small (sometimes mobile) IA cation counterions are difficult to see and to identify [151]; nonetheless, they are there, and allow the close intermingling of what are essentially neutral salts. For example, di-anions such as sulfate can bind directly to neutral amino groups inside polyanionic nucleic acids as determined by X-ray crystallography. Specifically, sulfate binds to the (A)N6 or (C)N4 in the deep (major) groove of A–U and G–C pairs, and to (G)N2 in the shallow (minor) groove of G–C pairs [27].

2.2. The surprising behavior of ions at interfaces

2.2.1. Ions of low charge density adsorb onto weakly hydrated solutes and surfaces

We demonstrated in 1985 [14], 1986 [5] and 1995 [28], that ions of low charge density adsorb to the nonpolar surface [29] of Sephadex® G-10, whereas continuum electrostatics models predict that all ions should be repelled from nonpolar surfaces *via* image forces [30–32]. We may conclude that the water molecules detected by NMR in 1964 and 1967 which have increased tumbling rates in solutions of ions with a low charge density [14,33–35] are immediately adjacent to the ions (as also directly shown by solution neutron diffraction [36]), and their release to become strongly interacting bulk water provides a driving force for forcing large monovalent ions of low charge density to the interface, as shown by the temperature dependent adsorption of these ions onto Sephadex® G-10 [5]. Therefore, the electric field coming out of these ions is not strong enough to orient the immediately adjacent water molecules, and its long range effects are probably modest. The behavior of the ions in these systems seems to be controlled more by the local properties of water affinity and perhaps polarizability than by long range electric fields.

2.2.2. Ions of low charge density adsorb to the air–water interface

Molecular dynamics simulations of the air–water interface also show that ions of low charge density adsorb to the interface [3,37,38].

2.3. More surprising characteristics of phosphate binding to proteins

2.3.1. Phosphate binds most commonly to the neutral glycine backbone

Because amide protons ($pK_a \sim 15$) are more acidic than water protons ($pK_a = 15.74$), anions of high charge density (such as F^- [39] and Cl^- [40,41], discussed in Section 8.1.1) often bind to protein amide groups. The neutral glycine residue is the most common protein binding site for negatively charged phosphate groups, and nearly a third of all protein binding sites for negatively charged phosphate do not employ ion pairing interactions [42]. Phosphate interactions with proteins (at neutral or charged sites) can be quite strong; in several cases, the interaction of the non-reacting phosphodianion group of enzyme substrates with the enzyme is responsible for about 12 kcal/mol of transition state stabilization (corresponding to an enzyme rate enhancement of about 10^9) [43].

2.3.2. Phosphate-protein binding energetics are independent of the charge on phosphate

Further illustrating the attenuated role of charge in aqueous systems, a pH dependent isothermal titration calorimetry study of the binding of phosphate and sulfate to a charged protein binding site found no dependence of the binding energetics on the charge of the anion, and concluded that "charge–charge interactions are not the dominant factor in binding" [44].

2.4. The surprising properties of protein salt bridges

2.4.1. The stability of protein salt bridges shows little dependence on charge

A dramatic illustration of the importance of chemical processes in protein stability was the systematic substitution with both alanine and glycine of all the charged residues (including salt bridges and charged hydrogen bonds) in Staphyloccal nuclease and characterization of the stability of the resulting mutants by guanidine hydrochloride denaturation using intrinsic tryptophan fluorescence to quantify the equilibrium between native and denatured states; these studies led to the conclusion that "ionizable amino acids contribute to stability predominantly through packing and bonding interactions that do not depend on their electrostatic charge" [9].

2.5. The surprising locality of electrostatic effects

2.5.1. Trivalent cations bind to strongly cationic proteins

The strongly cationic hen egg white lysozyme hydrolyzes the strongly anionic cell wall of bacteria invading the egg, illustrating the tendency of biological macromolecules to use multiple charges to harness electrostatic forces. Even so, hen egg white lysozyme, which has a net charge of about +10 at pH 4.5, easily binds the trivalent Yb^{3+} metal ion at an active site carboxylate [45,46], indicating no strong long range interaction between the Yb^{3+} ion and the many other positive charges on the protein.

2.6. Apparent protein stabilization by optimizing surface charge–charge interactions

2.6.1. Electrostatic stabilization of the redesigned protein occurs primarily in the folding transition state

Optimizing native state surface charge–charge interactions in the human protein acylphosphatase was accomplished with amino acid substitutions at 5 positions, 3 of which led to charge reversal and 2 of which introduced new charges, yielding an active protein stabilized by 10 °C or 9 kJ/mol [47]. Similar results were obtained with the Cdc42 GTPase and the Fyn SH3 domain, but examination of the kinetics of protein folding of the latter showed that it was actually stabilized primarily by an 8-fold acceleration in the folding rate [48], casting doubt on the importance of the putative moderately long-range electrostatic interactions in the native state that motivated the protein redesign.

3. Evidence for charge transfer to solvent (chemistry) in ion–water interactions

3.1. Ions of high charge density leak substantial amounts of charge onto the solvent

"Charge transfer to solvent" is associated with ions of high charge density because of the substantial chemical (covalent) character of their interaction with water. Ions of high charge density are strongly hydrated; they have positive Jones–Dole viscosity B coefficients [6] and positive apparent dynamic hydration numbers (the number of water molecules that move with the ion as it diffuses through solution) [15]. The carboxylate group has an apparent dynamic hydration number of 2.0 and leaks 22% of its charge onto the solvent [8]; the Mg^{2+} ion has an apparent dynamic hydration number of 5.8 and leaks 40% of its charge onto the 5.8 attached water molecules [7]; the Cr^{3+} ion has a dynamic hydration number of 9.6 and a pK_a of 4.3 (representing complex equilibria) [49] such that a full unit of positive charge has been dissociated as a proton into the solution at pH 7. Additionally, the quantum theory of atoms in molecules indicates that Cl^-, which structures the first shell water molecules in spite of having a water affinity slightly weaker than the strength of water–water interactions in bulk solution [50], transfers 0.2 negative elementary charges to the first shell water molecules [51], and UV resonance Raman spectroscopy detects a $Cl^- \rightarrow (H_2O)_6$ charge transfer transition in the form of a strong enhancement of the pre-resonance Raman intensity of the water bending modes [52]; this may also explain the affinity of the Cl^- for the relatively acidic protons of the asparagine side chain amide (see Section 8.1.1, below). The phosphate mono-anion, such as that in DNA, has an apparent dynamic hydration number of 1.9 (part of a cone of six water molecules hydrating the negative charge) [53] and although NMR evidence suggests charge transfer to solvent is important [54], the exact amount appears not to have been calculated. However, since the apparent dynamic hydration numbers of the carboxylate and phosphate mono-anion are similar, their charge transfer to solvent is probably also similar. While the charges on proteins tend to act as isolated charges, DNA has a partial

shell of attached negatively charged water molecules and thus behaves as a partial polyelectrolyte [[55]; see also [56]], whereas compact forms of RNA have a more complete shell of negatively charged water molecules and behave as full polyelectrolytes [55]. The isolated charges on proteins form ion pairs with their counterions according to the Law of Matching Water Affinities (see below), whereas polyelectrolytes behave as an ensemble of charges, and their interactions with their monovalent counterions are indirect (mediated by water molecules) with a preference for the monovalent cation of highest charge density (Li^+) [55]. Continuum electrostatics models attribute polyelectrolyte behavior to long range electric fields, whereas polyelectrolyte behavior shown by polymers containing strongly hydrated ions probably arises from short range chemical processes leading to charge transfer to solvent. See also Section 8.1.2, below, on ions of high charge density.

4. The limitations of continuum electrostatics theories

4.1. What is a chaotrope? (Fig. 1)

4.1.1. A weakly hydrated ion is called a chaotrope

A chaotrope is an ion (or solute) which in bulk solution binds water less strongly than water binds itself in bulk solution [5], nothing more and nothing less. People have struggled to understand chaotropes, not only because of their misleading name, but also because weakly hydrated ions simply do not exist in the world defined by continuum electrostatics theories, which does not address the strength of water–water interactions or the stoichiometric involvement of water in the association and dissociation of ions with other ions, solutes and surfaces.

Two distinctive behaviors of weakly hydrated ions are [i] their adsorption onto nonpolar surfaces [5,14] and onto weakly hydrated counterions [6] driven by the release of weakly held water which becomes strongly interacting water in bulk solution, and [ii] their "release" of adjacent water molecules to hydrate nearby solutes [5,14]. Neither behavior can be generated by continuum electrostatics models, which predict that all ions should be repelled from nonpolar surfaces by "image forces" [30–32]. Weakly hydrated ions (chaotropes) do not exist and their properties cannot be duplicated in continuum electrostatics models.

4.2. The role of chemistry in ion–water interactions (charge transfer to solvent)

4.2.1. Continuum electrostatics theories exclude chemical processes

Continuum electrostatics theories assume that everything is strictly electrostatics — that chemistry and quantum chemical calculations for ions of high charge density can be ignored. Given the facts summarized in Sections 2 and 3 above and 8.1.2 below, the resulting errors are likely to be substantial.

4.3. The problem of granularity (Fig. 2)

4.3.1. Continuum electrostatics theories exclude the water affinity of ions and other issues

In summary, many of the failings of continuum electrostatics theories can be traced to their attempt to treat water as a passive (macroscopic), featureless dielectric. That water is composed of individual (microscopic) molecules which interact with individual ions in charge density-dependent ways is known as the issue of granularity. These short range electro-chemical processes actually dominate the behavior of ions in the high dielectric constant regime of water, even though continuum electrostatics model provide only apparent long range electrostatic explanations for all behaviors. As shown in Fig. 2, continuum electrostatics theories assumes that the long range electrostatic interaction between full charges (the long arrow above the green dielectric slab) dominates the behavior of ions in water, whereas the behavior of ions in complex, moderately concentrated solutions actually correlates with the short-range charge density-dependent electro-chemical interaction of ions with the adjacent ghost water molecules or with the acidic protons or unshared electron pairs of macromolecules.

The charge density of ions and the strength of water–water interactions are critical omissions of continuum electrostatics theories. The charge density of ions determines their chemical affinity for the acidic protons or basic unshared electron pairs of water or biological macromolecules. Bulk water acts as a reservoir for the stoichiometric participation of water molecules in ion association and dissociation; the availability of this water is determined by the strength of water–water interactions. Use of the term "ionic strength" in the context of using NaCl to prevent protein aggregation often implicitly invokes intervening long range electric fields as assumed by continuum electrostatics models [1,2,15], but in reality it is almost certainly due to the binding of weakly hydrated Cl^- to weakly hydrated positive protein charges according to the Law of Matching Water Affinity (as can be seen in X-ray structures [40,58,59] and MD simulations [60] with a K_d for Cl^- of about 150 mM [61,62]. For example, weakly hydrated protein positive charges bind to the weakly hydrated sulfonate anions of Congo Red micelles [63], but added NaCl blocks the interaction, almost certainly *via* Cl^- binding to the protein positive charges. Additionally, anions *induce* the reversible oligomerization of a fusion protein in the sequence (from most effective) $I^- > Br^- > Cl^- > F^-$ (to least effective), the mechanism being the suppression of electrostatic repulsion between key arginyl and lysyl residues by direct anion binding to these protein positive charges [64]. The strongly hydrated F^- acts as an inert (non-binding) analog of the weakly hydrated Cl^- at comparable concentrations [65,66], but will bind to acidic protein hydrogen atoms at higher concentrations [11,60,67]. Since Cl^- and F^- should have very similar long range electric fields, their dramatically different behaviors with proteins must arise from different short range forces – specifically, the weaker affinity of Cl^- for water [6] and the higher affinity of F^- for acidic protons [68].

The large differences in the effects of specific ions on lysozyme crystallization correlate with the water affinity of the added ions as measured by their Jones–Dole viscosity B coefficients; the most weakly hydrated anions bind most strongly to the enzyme and crystallize the enzyme at the lowest added salt concentration [69–71]. The visualization of the bound anions in the resulting X-ray structures [40,72–75] establishes beyond a reasonable doubt that these anions act by a direct binding mechanism as opposed to "ionic strength" (screening) effects mediated by long range electric fields.

4.4. Additional assumptions of continuum electrostatics theories

4.4.1. Continuum electrostatics theories do not contain the strength of water–water interactions, a critical benchmark for ion behavior

There are a large number of specific ion effects in water which change sign between F^- and Cl^- (or more crudely, between F^- and I^-) for anions or between Na^+ and K^+ for cations. Examples include Jones–Dole viscosity B coefficients [6], the surface potential difference at the air–water interface [76], Hofmeister effects on protein stability [77], the apparent size of ions on Sephadex® G-10 [too large (F^-, Na^+) or too small (I^-, K^+)] [5,28], and molecular dynamics simulations of the behavior of ions at the air water interface (F^- repelled from the interface and I^- adsorbed to the interface) [3]. The change in sign corresponds to the strength of water–water interactions in bulk solution (something that does not exist in continuum electrostatics models) and implies that the short range phenomenon of water affinity is the source of the specific ion effects rather than long range electric fields.

In addition to the assumptions already mentioned, continuum electrostatics theories assume cations and anions of the same size to

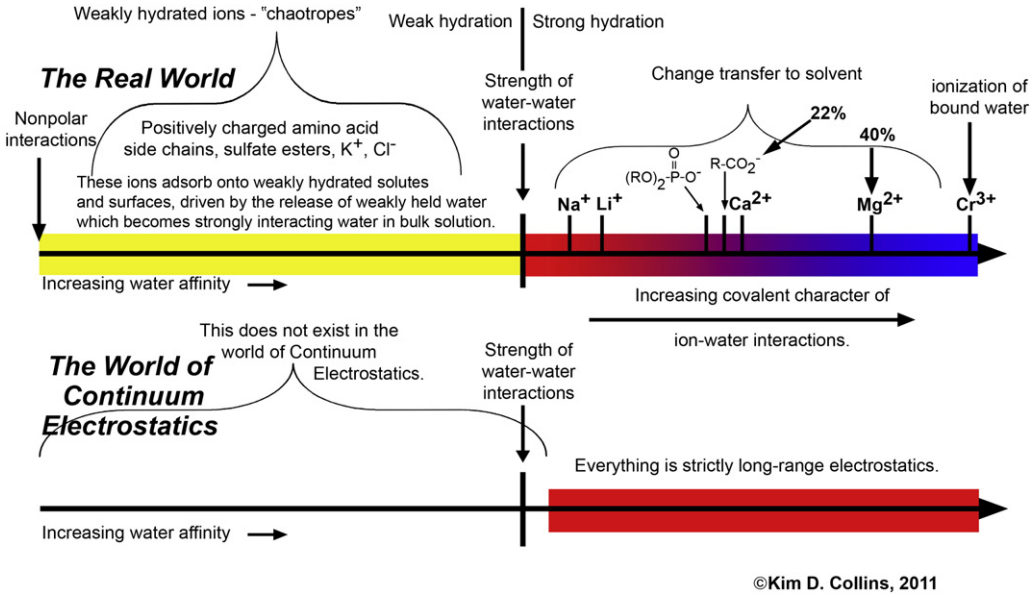

Fig. 1. Limitations of continuum electrostatics theories.Continuum electrostatics theories omit weakly hydrated ions (chaotropes), chemical processes (including charge transfer to solvent), the charge density of ions, and the strength of water–water interactions in bulk solution; they impose long range (macroscopic) electrostatic explanations for the forces on ions, even when these forces arise largely from short range (microscopic) electro-chemical interactions with the acidic protons and unshared electron pairs of molecules (which may be net neutral) such as water.

have the same enthalpy of hydration, whereas anions of a given size are actually more strongly hydrated than cations (Fig. 3) [6]. There are at least two reasons for the stronger hydration of the anions. First, quantum mechanical calculations indicate that the anions, which interact with the hydrogen atom of water, allow intra-shell hydrogen bonding of the solvating waters, whereas cations, which interact with the oxygen atom of water, do not [79]. Second, charge transfer between ion and solvent characterizes strong hydration, and water accepts negative charge from anions more efficiently than it gives up negative charge to cations.

Continuum electrostatics theories deal mostly with point charges, either positive or negative. Thus all monovalent cations are considered equivalent and all monovalent anions are considered equivalent. In the real world, monovalent anions vary from the small, strongly hydrated and usually protein stabilizing (F^-) to the large, very weakly hydrated and usually protein denaturing (SCN^-). A range of effects also exists for monovalent cations. Since the physical basis for ion specific effects is mostly the changing charge density-dependent water affinity (which is not in the model) relative to the strength of water–water interactions in bulk solution (which is also not in the model), simply drawing a circle around a point charge to correct for its actual size in a dielectric continuum does not address the fundamental issue. For example, in a recent continuum electrostatics treatment of the partitioning of ions at a water–oil interface, "the information indicating which ion is a kosmotrope and which one is a chaotrope is taken as an external input and is based on the ionic viscosity B coefficient that is measured experimentally" (this number is a measure of the water affinity of the ion), and then the behavior of the ions within each class was *assumed* to be determined by polarizability, hydrophobic and dispersion interactions rather than by the differences in the water affinity of the ions [80].

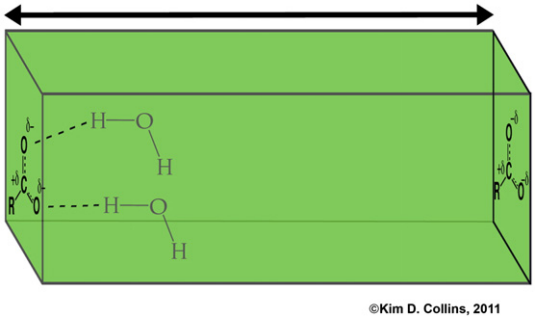

Fig. 2. What are the controlling forces on ions in water?Continuum electrostatics theories assume that the controlling forces on ions in the high dielectric constant regime of water arise strictly from long range electrostatic interactions between formal charges (indicated by the long double-headed arrow at the top of the green, featureless dielectric slab) whereas the behavior of ions in biological systems is actually controlled largely by the short range electrostatic and chemical interactions between ions and the adjacent water molecules (the carboxylate and the ghost water molecules shown in the figure), as measured by Jones–Dole viscosity B coefficients, solution neutron diffraction, gel sieving chromatography on Sephadex® G-10, and other physical techniques. The charge distribution on the atoms of the carboxylate are taken to be those for the glutamate side chain in Ref. [57]; *i.e.*, −0.82 for the each of the oxygens and +0.81 for the carbon.

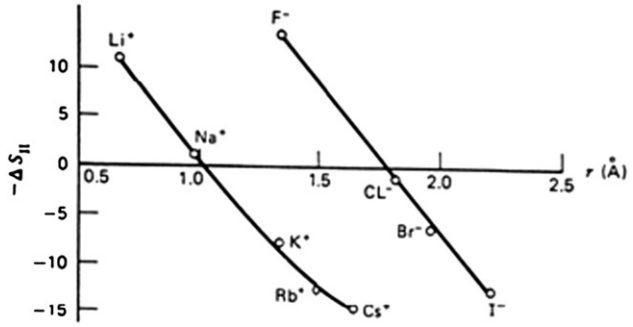

Fig. 3. Charge density dependent strength of hydration.The difference between the partial molar entropy of the ion and that for water in water in cal $°K^{-1}$ mol^{-1} is plotted on the ordinate; thus, ΔS_{II} is the local entropy change for turning a water molecule into an ion [12]. The crystal radii of the ions in angstroms are plotted along the abscissa. Positive values of ΔS_{II} (*lower portion of figure*) indicate water that is more mobile than bulk water. Negative values of ΔS_{II} (*upper portion of figure*) indicate water that is less mobile than bulk water. Kosmotropes are in the upper portion of the figure; chaotropes are in the lower portion of the figure. Adaption of data of G.A. Krestov as presented in Ref. [78].

5. Ion charge density dependent strength of hydration

5.1. The charge density of ions controls their water affinity; the water affinity of ions controls their behavior

Fig. 3 shows the difference between the partial molar entropy of the ion and that for water in water (y-axis) *vs.* the crystal radius of the ion in angstroms (x-axis); thus, ΔS_{II} is the local entropy change for turning a water molecule into an ion [13]. Small ions of high charge density (kosmotropes, shown above the line) bind adjacent water molecules tightly, thus immobilizing them, whereas large monovalent ions of low charge density (chaotropes, shown below the line) actually "free up" adjacent water molecules, allowing more rapid motion than in bulk solution. F^- and K^+ are approximately the same size, but F^- is strongly hydrated whereas K^+ is weakly hydrated for the reasons described above. Fig. 4 illustrates "how to think about ions." The horizontal line represents the strength of water–water interactions, with the small, strongly hydrated ions above the line and the large, weakly hydrated ions below the line. The zwitterionic "virtual water molecule" on the right contains a positive portion with a radius of 1.06 Å which does not alter the tumbling rate of pure water and a negative portion with a radius of 1.78 Å which also does not alter the tumbling rate of pure water.

6. The importance of water affinity

6.1. Measuring water affinity

The water affinity of a solute has traditionally been measured by various colligative methods, for example, by the solvent vapor pressure deviation of solutions from the mole fraction of the solvent (*i.e.*, from Raoult's Law). We have found gel sieving chromatography on Sephadex® G-10 [5,15,28], Jones–Dole viscosity B coefficients [6] and solution neutron diffraction [36,81] to be the three most useful ways to characterize the water affinity of ions.

6.1.1. Sephadex® G-10 counts the attached water molecules that move with an ion of high charge density (i.e., determines the dynamic hydration number of the ion)

Sephadex® G-10 is most useful for characterizing strongly hydrated ions, which are separated by gel sieving and yield the number of attached water molecules that flow through the column with the ion; weakly hydrated ions are separated by adsorption to the surface of the column, and the results are harder to interpret because of the possible involvement of ion polarizability. Sephadex® G-10 may be thought of as nonpolar [29] beads containing pores of uniform size. Polyglutamic acid (MW 13,700) is excluded from the pores, flowing around the beads and thus taking a short path through the column, eluting early: this is the excluded volume. H_2O^{18} penetrates the pores, flowing through the beads and taking a long path through the column, eluting late: this is the included volume. The gel sieving relative elution position varies from 0 (the excluded volume) to 1 (the included volume). Polymers of glycine (n = 2–6) appear not to show preferential interactions with water (except for a small deviation arising from the C-terminal carboxylate) and are thus used to calibrate the column according to molecular weight; these polymers tend to have an extended conformation [82]. The observed molecular weight is that of a neutral salt plus any attached water, and is expressed as the number of water molecules associated with an ion as it diffuses through the column (the Apparent Dynamic Hydration Number, or ADHN). Since the ADHN of a salt is the sum of the ADHN's of the constituent ions, the ADHN of an ion can be determined by chromatographing the ion of interest as a Cl^- or K^+ salt, ions which have ADHN = 0 while not interacting significantly with the column. The data are plotted as $\log_{10}$ molecular weight (x-axis) *vs* the relative elution position (the y-axis); the $\log_{10}$ is required to convert from a hydrodynamic radius to a volume or molecular weight. The experiments are conducted at 30 °C, and the flow rate through the 1 m × 1.6 cm diameter column is 1.2 ml/min; each experiment takes from 2.5 to 12 h. The sample is added to the column as 0.6 ml of a 0.1 M solution. Only ion-specific detection methods are used to characterize the column effluent: radioisotopes are used when available; otherwise specific colorimetric methods are used. The most informative points of comparison between gel sieving chromatography and other techniques are where natural discontinuities occur: the change from weak to strong hydration between K^+ and Na^+ and between F^- and Cl^- as determined by Jones–Dole viscosity B-coefficients [6]; the change from weak to strong second-shell hydration between Mg^{2+} by [as shown by solution X-ray diffraction [83]] and Be^{2+} [as shown by *ab initio* molecular orbital calculations [84] and solution neutron diffraction studies [85] or Cr^{3+} [as shown by solution neutron [86] and X-ray diffraction [87] and the change from an inner sphere coordination number of six for Mg^{2+} [83] to four for Be^{2+} [84,85]. The Apparent Dynamic Hydration Numbers (Table 1) determined by gel sieving chromatography on Sephadex® G-10 are in complete agreement with these calibration points from other techniques.

The ADHN for H^+ is 1.9–2 at 0.1 M, indicating the dihydrate (Zundel proton) [88]. Photoelectron spectroscopy experiments combined with electronic structure calculations [89] performed at 3–4 m H^+ concentration favor the Eigen core (H_3O^+) form of the proton. A solution neutron diffraction study of 6 M HCl also found an H_3O^+ core, participating in three short and strong hydrogen bonds to produce the (hydrated) Eigen proton $(H_9O_4)^+$ [90]. X-ray absorption experiments extending from 0.1 M to 6 M H^+ combined with molecular dynamics calculations [91] also find evidence favoring the Eigen core (H_3O^+) at high H^+ concentrations "while the proton is less localized to a specific water under

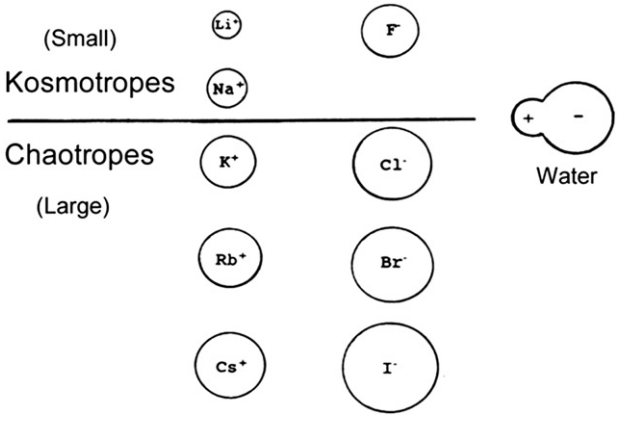

Fig. 4. How to think about ions in water.Division of the group IA cations and the VIIA halide anions into (strongly hydrated) kosmotropes and (weakly hydrated) chaotropes. The ions are drawn approximately to scale. A virtual water molecule is represented by a zwitterion of radius 1.78 Å for the anionic 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 [15].

Table 1
Apparent dynamic hydration numbers.

Cations	ADHN	Anions	ADHN
Cr^{3+}	9.6	PO_4^{3-}	5.1
Mg^{2+}	5.9	HPO_4^{2-}	4.0
Ca^{2+}	2.1	$H_2PO_4^-$	1.9
H^+	1.9 (Zundel)	HCO_2^-	2.0
Li^+	0.6	F^-	5.0
Na^+	0.22	HO^-	2.8 (trihydrate)
K^+	0	Cl^-	0

Source: Ref. [15].

less acidic conditions". The results from these different techniques are internally consistent: the hydrated form of H^+ appears to vary with concentration, changing from the dihydrate at 0.1 M to the tetrahydrate at 3–4 m and above, where no bulk water exists. The ADHN for HO^- is 2.8 (*i.e.*, the trihydrate), consistent with experimental and theoretical results [92]. The coordination number of F^- is about 5 [67], and thus matches the number of tightly bound waters (the ADHN). Of the ions shown in Table 1, only Cr^{+3} has tightly bound water in the second hydration layer.

6.1.2. The Jones–Dole viscosity B coefficient is a scaling factor that measures the water affinity of an ion relative to the strength of water–water interactions

The Jones–Dole viscosity B coefficients have the advantage of giving the same quantitative measure for both strongly and weakly hydrated ions, and of avoiding the involvement of interfaces. 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. Since the B coefficient of a salt is the sum of the B coefficients of the constituent ions, extracting ion-specific B coefficients is straight-forward. Table 2 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, 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 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 2
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 Ref. [93]; all others from Ref. [94].

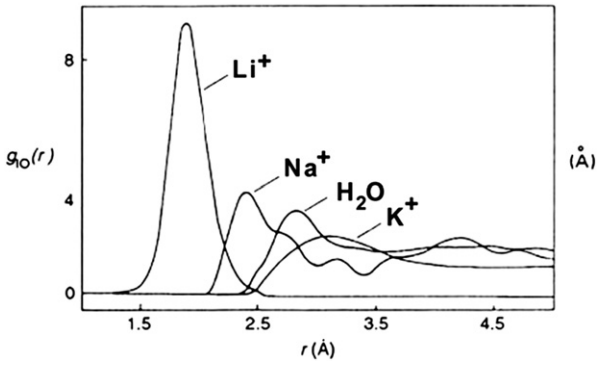

Fig. 5. The radial distribution functions $g_{10}(r)$ for Li^+, Na^+, water, and K^+ in liquid water. These curves measure the density of the solution as a function of the distance from the isotopically substituted ion, and effectively measure the distance from the monovalent cation to the nearest solvent oxygen. The curve labeled "H_2O" measures the oxygen-oxygen distance in liquid water. Both neutron and X-ray diffractions were used to generate these data. Reprinted from [36]. The radial distribution for Li^+ is drawn assuming a coordination number of six; subsequent experiments suggest a number closer to four, with no major effect on the results presented here.

6.1.3. Solution neutron diffraction "sees" the orientation of water (D_2O) strongly attached to the reference ion in defined shells

Solution neutron diffraction, as developed by John Enderby and George Neilson, has proven to be an enormously informative procedure for characterizing ion–water interactions [36,81]. "Simple ions in water generate long range electric fields which can be detected by various resonance techniques, such as fluorescence resonance energy transfer, over distances of 30 Å (about 11 water diameters) or more [95]. It has often been assumed that the long range electric fields generated by simple ions in water are strong enough to orient water dipoles over long distances. But solution neutron and X-ray diffraction techniques developed in the 1970s and applied to various ions in water in the years since have produced a very different picture.

When more than one stable isotope of an ion is available, two identical aqueous salt solutions that vary only in that isotope can be used to study the spherically averaged structure around that ion by neutron diffraction [81,96]. In particular, radial distribution functions that measure the density of the solution as a function of the distance from the isotopic ion can be calculated. When neutron diffraction experiments are carried out on aqueous solutions of a salt in heavy water, correlations between the isotopically substituted ion and both the oxygen and the deuteron of the solvent can be detected; this allows one to determine, quantitatively, nearest neighbor distances, dynamic hydration numbers, and from the viewpoint of this review the most probable orientation of nearby water molecules and a qualitative understanding of the strength of the ion–water correlations.

Fig. 5 shows the neutron and X-ray diffraction of the IA cations Li^+, Na^+, K^+, and of water. As the charge density of the ion decreases from Li^+ to Na^+ to K^+, the density peak of the nearest water oxygen is lower and further away, indicating weaker binding. The Na^+-oxygen distance is smaller than the oxygen-oxygen distance of pure water, indicative of strong hydration of for Na^+, while the K^+-oxygen distance is larger than the oxygen-oxygen distance of pure water, indicative of weak hydration for K^+. A charge density between that of Na^+ and K^+ is exactly where the Jones–Dole viscosity B coefficient (a measure of water affinity) changes sign, marking the strength of water–water interactions. Additionally, Figs. 6, 7 and 8 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

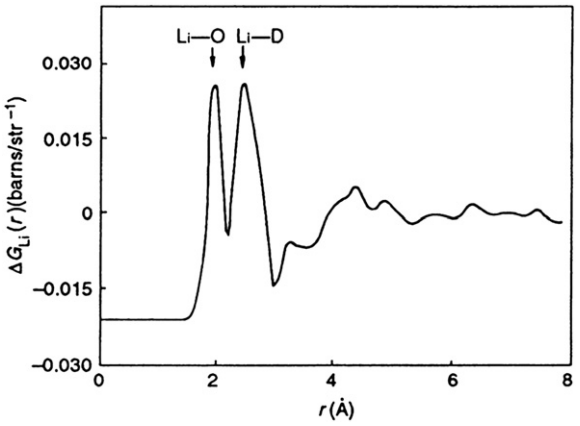

Fig. 6. Radial distribution function for Li^+ in D_2O.The first-order difference function $G_{Li}(r)$ for Li^+ in D_2O. This curve measures the distance from the isotopically labeled Li^+ to the nearest solvent oxygen or deuteron. These are neutron diffraction data from Ref. [97].

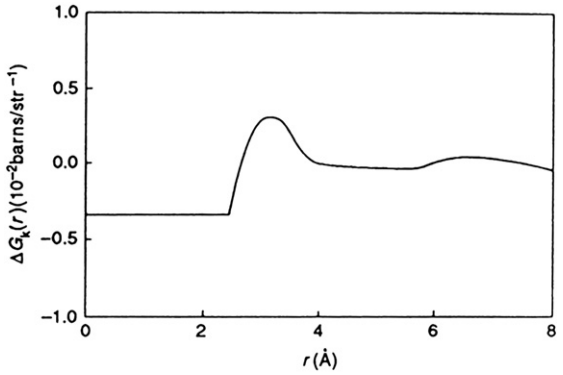

Fig. 8. Radial distribution function for K^+ in D_2O.The first-order difference function $G_K(r)$ for K^+ in D_2O. This curve measures the distance from the isotopically labeled K^+ to the nearest solvent oxygen or deuteron. These are neutron diffraction data from Ref. [99].

intermediate orientation of solvent, and weak hydration (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…". Neutron diffraction has [also] been used to characterize the strong denaturants guanidinium and thiocyanate in solution, verifying the weakly hydrated character of chaotropes [100,101]. The ion–water distance obtained by solution neutron diffraction and computer simulation is linearly correlated with the Jones–Dole viscosity B coefficient [102].

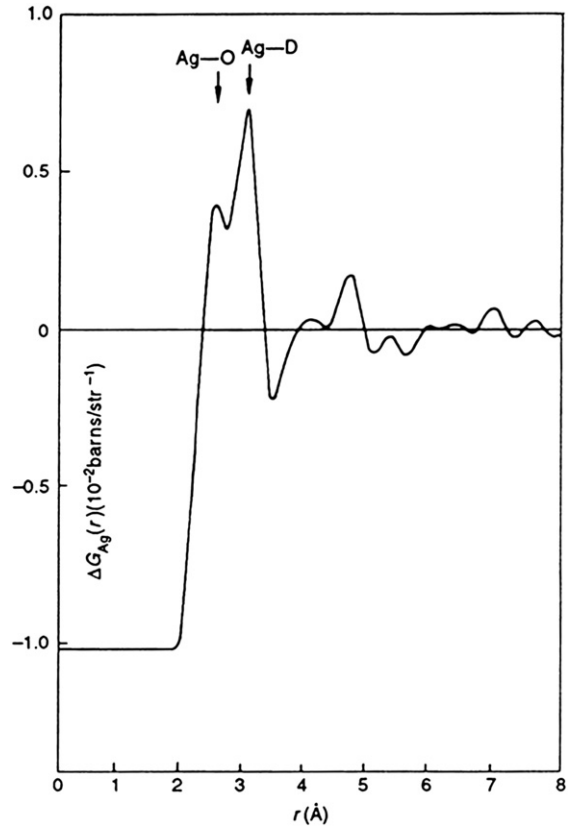

Fig. 7. Radial distribution function for Ag^+ (an analog of Na^+) in D_2O.The first-order difference function $G_{Ag}(r)$ for Ag^+ (an analog of Na^+) in D_2O. This curve measures the distance from the isotopically labeled Ag^+ to the nearest solvent oxygen or deuteron. These are neutron diffraction data from Ref. [98].

6.2. The Law of Matching Water Affinity

6.2.1. Ions of opposite charge form contact ion pairs in solution when they have matching water affinities

The Law of Matching Water Affinity states that ions of opposite charge tend to form contact ion pairs in solution when they have matching water affinities [6]. Since water affinity is a strong function of ion size (small ions of high charge density bind water strongly whereas large monovalent ions of low charge density bind water weakly), small ions tend to form contact ion pairs with each other and large ions tend to form contact ion pairs with each other, but large-small contact ion pairs tend not to form (Fig. 9). This same pattern is manifested in the solubility of the alkali halides (see below). The small ions of opposite charge form contact ion pairs because of electrostatic attraction; the large ions of opposite charge form contact ion pairs because this releases weakly hydrated water which becomes strongly interacting water in bulk solution (see below).

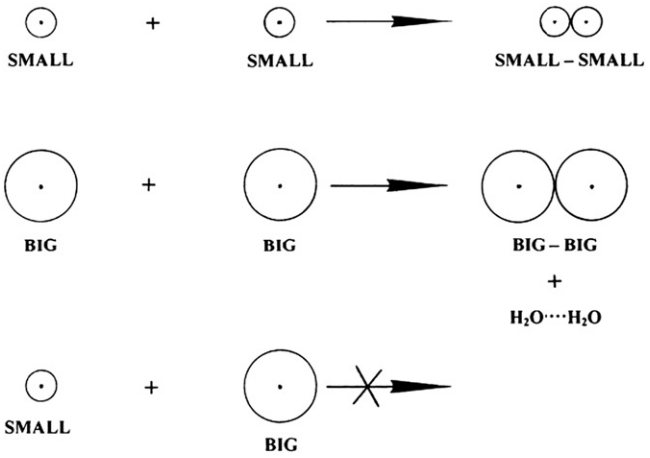

Fig. 9. The law of matching water affinity.Because the charge density of an ion controls its water affinity, ion size controls the tendency of oppositely charged ions to form inner sphere ion pairs. Small ions of opposite sign spontaneously form inner sphere ion pairs in aqueous solution; large ions 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.

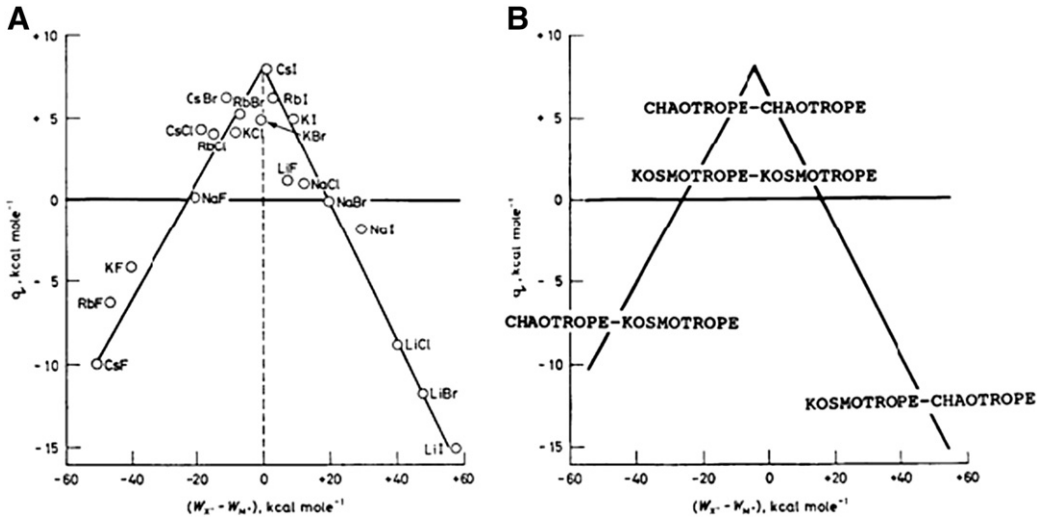

Fig. 10. Volcano plots.(A) Relationship between the standard heat of solution of a crystalline alkali halide (at infinite dilution) in kcal mol^{-1} on the y-axis and the difference between the absolute heats of hydration of the corresponding gaseous anion and cation, also in kcal mol^{-1} on the x-axis. Source: [103] © 1969 reprinted with kind permission from Springer Science & Business Media). (B) Identification of ions as chaotropes (weakly hydrated) or kosmotropes (strongly hydrated). The enthalpy of solution of chaotrope–chaotrope and kosmotrope–kosmotrope salts tends to be positive (takes up heat), whereas for the enthalpy of solution to be negative (gives off heat), the salt must have a kosmotropic and a chaotropic ion.

6.2.2. The heat of solution of the alkali halides plotted as a function of the difference in the water affinity of the constituent ions [a volcano plot] supports the Law of Matching Affinities

"We shall interpret Fig. 10 to indicate that oppositely charged ions with equal water affinity tend to come together in solution to form contact ion pairs whereas oppositely charged ions with differing water affinities tend to stay apart. We shall attribute the release of heat to the formation of strong bonds and the uptake of heat to the breaking of strong bonds, and shall assume that the strongest interactions in the system will tend to dominate the behavior of the system. In aqueous salt solutions of kosmotropes (small ions of high charge density which are strongly hydrated) and chaotropes (large ions of low charge density which are weakly hydrated), the interactions in order of decreasing strength are as follows: kosmotrope–kosmotrope > kosmotrope–water > water–water > chaotrope–water > chaotrope–chaotrope [6,62,101]. Fig. 10 shows the relationship between the standard heat of solution of a crystalline alkali halide at infinite dilution (on the vertical axis; this is a measured quantity) and the difference between the absolute heats of hydration of the constituent gaseous anion and cation (on the horizontal axis; this is a calculated quantity). [In this context, "absolute" refers to the conceptual experiment of transferring an isolated ion from the gas phase to the solution.] Fig. 10 illustrates that a necessary but not sufficient condition for the standard heat of solution of a crystalline alkali halide to be negative (exothermic) is that one of the ions be a chaotrope and the other ion to be a kosmotrope, suggesting that kosmotrope plus chaotrope neutral salts dissociate extensively upon dissolution, and that the kosmotropic ion of this salt acquires stronger interactions with water in solution than it has with chaotropes in the crystal, thus tending to release heat. In contrast, when crystalline kosmotrope–kosmotrope alkali halides dissolve in water, the kosmotrope–kosmotrope ion pairs will tend to stay together, and insofar as the constituent ions do separate, strong kosmotrope–kosmotrope interactions are broken, thus tending to take up heat. When crystalline chaotrope–chaotrope alkali halides dissolve in water, relatively strong water–water interactions will keep the chaotrope ion pairs together, and insofar as the constituent ions do separate, relatively strong water–water interactions will be broken, thus tending to take up heat. These patterns suggest that oppositely charged ions with equal water affinities will tend to form contact ion pairs in solution, whereas those with differing water affinities will tend to separate [6,62,101]. In simplest possible terms, kosmotropes pair with kosmotropes and chaotropes pair with chaotropes; that is, like likes like. Since all of the salts in the volcano plot are monovalent, the long range electric fields generated by each salt must be very similar, and the dramatic differences in their behavior must be due to the differences in the strength of their short range [electro-chemical] interactions with water. Forming a contact ion pair requires a partial dehydration of both the positive and negative ion, which occurs most readily when both ions have the same water affinity. A simple model can be used to show that the relative affinity of a monovalent ion for water closely correlates with its relative affinity for monovalent ions of opposite charge [6]. Therefore, when one ion is more strongly hydrated than its oppositely charged partner, dehydrating the more strongly hydrated ion costs more in energy than is gained by forming a contact ion pair with the more weakly hydrated ion, and thus these ions tend to stay apart. The issue of charge density-dependent microscopic hydration-dehydration is not included in the electrostatic calculation using the macroscopic dielectric constant, but energetically it actually dominates and controls the process of contact ion pair formation." [101] Evidence for contact ion pairing in water comes from protein X-ray crystallography [40,58,59] and dielectric spectroscopy [17] in addition to those techniques discussed in the context of the Law of Matching Water Affinity above and below. The Law of Matching Water Affinity is supported by molecular dynamics simulations of the alkali halides [104] and by activity coefficients [105], as well as by other techniques discussed below. Ion pairing of Na^+ with the carboxylate group is discussed in Section 8.1.1 below.

6.3. Water affinity plays a major role in ion specific effects, which can be large and important

6.3.1. Hofmeister effects on proteins correlate with the charge density and thus the water affinity of the ions

Hofmeister effects on proteins are perhaps the most dramatic example of large and important specific ion effects [14,34]. The sign of the Hofmeister effect on proteins (stabilizing or destabilizing) corresponds with the water affinity of the anions (strongly or weakly hydrated, respectively) [5].

6.3.2. The solubility of the alkali halide salts correlates with the charge density and thus the water affinity of their constituent ions

Although lattice enthalpies play a role [107,108], the solubility of simple salts appears to be controlled largely by the tendency of the constituent ions to form contact ion pairs in solution as shown by the pattern of their solubility: salts composed of ions with similar water affinities have lower solubility (Table 3). "For example, both Li^+ and F^- are strongly hydrated, and thus tend to form contact ion pairs (the first step in the process of coming out of solution), whereas Cs^+ is weakly hydrated [109], and will tend to stay away from F^-. The solubility of LiF in water is only 0.1 M at 18° C. In contrast, the solubility of CsF at 18° C is 24.2 M, or 48.4 M in ions, and since pure water is about 55.5 M, a saturated solution of CsF contains only about one water molecule per ion." [101] It is worth noting that The Law of Matching Water Affinity provides a reasonable explanation for the solubility of the alkali halides [6] whereas continuum electrostatics (lattice enthalpy) models do not [108].

6.3.3. The solubility of the biologically important Na^+, K^+, Mg^{2+}, and Ca^{2+} salts is determined mostly by the water affinity of their constituent ions

"The strongly hydrated Ca^{2+} and Na^+ ions are pumped out of living cells, whereas the weakly hydrated K^+ is pumped in. This is because Ca^{2+} and Na^+ are well matched to the strongly hydrated major intracellular anions (phosphate, the carboxylate, and carbonate), and tend to form contact ion pairs with them (and then come out of solution). [Ca^{2+}, $H_2PO_4^-$, and HCO_2^- each bind about 2 water molecules tightly [15].] In fact, insoluble Ca^{2+} complexes play important biological roles. Calcium carbonate (*e.g.*, egg shells, oyster shells) has a solubility product of $10^{-8.5}$; calcium oxalate (*e.g.*, kidney stones) has a solubility product of $10^{-10.5}$; and calcium hydroxyphosphate (hydroxyapatite) (*e.g.*, bones and teeth) has a solubility product of 10^{-58} [110]. K^+, in contrast, stays away from the major intracellular anions [as shown by molecular dynamics simulations of Na^+ and K^+ interactions with the carboxylate [111–113] increasing the net charge and thus the solubility of molecules containing these anionic groups, and also leaving these anionic groups available for acting as binding determinants." [101] The solubility of K_2HPO_4 which contains "mismatched" ions is about 8.6 M [114], whereas that of $Na_2HPO_4 \cdot 7H_2O$ which contains more closely "matched" ions is about 0.93 M [114]. Mammalian body fluid concentrations for K^+ are about 159 mM intracellular and about 4 mM extracellular; for Na^+ they are about 10 mM intracellular and 150 mM extracellular [16]. Dog erythrocytes, which do not have nuclei, do not maintain Na^+ or K^+ gradients across their membrane [115], supporting the argument that the original purpose of the Na^+, K^+ ATPase was to replace inner sphere Na^+-nucleic acid phosphate oxyanion ion pairs with solvent separated K^+-nucleic acid phosphate oxyanion ion pairs. Mg^{2+}, whose transport into the cell is facilitated, is very strongly hydrated (*i.e.*, more strongly hydrated than phosphates and carboxylates), and forms salts with carboxylates and phosphates that are much more soluble than those of calcium. For example, the solubility of magnesium oxalate (containing "mismatched" ions) is 155 times that of calcium oxalate (containing "matched" ions). The intracellular Ca^{2+} concentration is about 10^{-7} M [116], and extracellular Ca^{2+} is about 2.5 mM, whereas intracellular Mg^{2+} is 40 mM (0.5 mM of which free) and extracellular Mg^{2+} is 1 mM [16]. As summarized above, ions interact strongly with water over only short distances through electro-chemical mechanisms which determine water affinity, and thus ion-pairing preferences are determined largely by hydration–dehydration.

Table 3
Solubilities of Group I halides

Solubility (molar value first, g/100 g H_2O given in brackets).

	MF	MCl	MBr	MI
Li	0.1 (0.27)	19.6 (830)	20.4 [177]	8.8 [165]
Na	1.0 (4.22)	6.2 (36)	8.8 [91]	11.9 [179]
K	15.9 (92.3)	4.8 (34.7)	7.6 [67]	8.7 [144]
Rb	12.5 (130.6)	7.5 [91]	6.7 [110]	7.2 [152]
Cs	24.2 (367)	11.0 [186]	5.1 [108]	3.0 [79]

Source: Ref. [106].

6.4. The water affinity of protein charges determines their biological role

6.4.1. The positive charges on proteins are weakly hydrated; the negative charges on proteins are strongly hydrated

The ammonium-based positively charged amino acid side chains are weakly hydrated whereas the negatively charged carboxylate amino acid side chain is strongly hydrated as measured by Jones–Dole viscosity B-coefficients and dynamic hydration numbers [6,15]. Cell penetrating peptides contain almost exclusively (weakly hydrated) positive charges [117] which can penetrate model membranes when driven by pH gradients (inside acidic) [118]; a negative charge can act as a "stop" sign for peptide insertion into membranes [119]. Similarly, intermediary metabolites are phosphorylated, creating a strongly hydrated "handle", to keep them inside the cell. The ability of positively charged peptides to cross cell membranes is affected by the counterion chosen [120]. Cell penetrating peptides can enter cells by a variety of mechanisms, including transient focal membrane deformation [121] and endocytosis after complexation with heparin sulfate [122], and can be harnessed to deliver cargo across cell membranes [117]. The closely related antimicrobial peptides exploit the facts that "bacterial membranes possess a comparatively large fraction (up to 20 mol%) of negatively charged lipids and maintain high electrical potential gradients (a transmembrane potential ($\Delta\Psi$) of approximately −120 mV) that attract positively charged substances like antimicrobial peptides, whereas the membranes of plant cells and animal cells are enriched in cholesterol and lipids, have no net charge and maintain weak $\Delta\Psi$ "[123]. Almost the total surface of a protein may be covered with (strongly hydrated) negative charges [24], but clusters of (weakly hydrated) positive charges are rare [124]. The introduction of a patch of positive charges on a mammalian protein results in energy- and heparin sulfate-dependent endocytosis of the protein across the plasma membrane [125–127]. Sulfate esters are the only weakly hydrated negative charges found on biological macromolecules [128] (such as on heparin sulfate), which thus bind positively charged peptides and proteins strongly because of the Law of Matching Water Affinity. The high salt concentration in halophilic bacteria increases the surface tension near the protein surface, encouraging the proteins to minimize their solvent exposed surface area by aggregating. This aggregation can be resisted by covering the surface of the protein with strongly hydrated carboxylate groups, which is found to be the case for proteins from halophilic bacteria [129].

7. The role of polarizability

7.1. The water affinity of ions appears to be much more important than polarizability for ions of biological significance

The polarizability of ions (and of the solvent) can be turned on and off in molecular dynamics simulations, providing a means of evaluating its importance; however, in molecular dynamics calculations, polarizability is sometimes used to compensate for the neglect of quantum effects such as charge transfer to solvent. The importance of the polarizability of the halide anions *vs.* the importance of their water affinity in their nucleophilic reactivity has been evaluated by measurements of their nucleophilicity in the gas phase, $F^- \gg Cl^- > Br^-$ [130], which is the same as that in the non-protic solvent acetone $Cl^- > Br^- > I^-$ [131], but the reverse of that in water $I^- > Br^- > Cl^-$

[131]. Since the intrinsic polarizability of the ions does not change with solvent, the observed reversal in the order of nucleophilicity must result from the stronger solvation of the smaller anions in protic solvents such as water, as revealed by Jones–Dole viscosity B coefficients [6] and by Apparent Dynamic Hydration Numbers (ADHN) from gel sieving chromatography [15]; thus water affinity is the controlling factor for their behavior in water in this case. Molecular dynamics simulations suggest that iodide and water polarizability combined play a large role in driving iodide to the air/water interface [132,133], but the effect is substantially reduced after correcting for electrostatic dampening [38]. An electrospray mass spectroscopy study of the accumulation of anions of low charge density at the air/water interface found a dependence on ion radius (size) but not polarizability, establishing the water affinity of the ion as the driving force for migration to the interface [134]. The polarizability of H_3O^+ appears to provide about half of the driving force for its slight preference for the air/water interface [135]. Therefore the polarizability of the more polarizable ions may play a role in their preference for the air/water interface, but the matter is not completely resolved. Ion pairing, in contrast, appears to be largely controlled by water affinity [6,136], and also by the affinity of the ions for acidic protons or unshared electron pairs on the counterion.

8. Current summary of ion–protein interactions

8.1. Forces controlling ion–protein interactions

The interaction of ions with proteins is a subject of intermediate complexity. Three simple generalizations (1–3, below) can explain a bewildering array of observations.

8.1.1. The Law of Matching Water Affinity is a useful generalization

In general, the binding of ions to proteins is controlled by the Law of Matching Water Affinities: that is, ions of opposite charge tend to form contact ion pairs when they have the same water affinity. This phenomenon is particularly clear in the aqueous behavior of the Group I halides, but there are some additional complexities when this law is applied to proteins. Since the positive charges of proteins are composed of various forms of weakly hydrated ammonium embedded in a neutral hydrocarbon skeleton, the more weakly hydrated an anion, the more strongly it binds (because these weakly hydrated anions also bind to neutral hydrocarbon surfaces) [5]. A comparative study of seven anions [69] shows that thiocyanate binds most strongly to and crystallizes hen egg white lysozyme (which has a net charge of about +10 at pH 4.5), Cl^- binds with intermediate strength to and crystallizes lysozyme (a typical Cl^- dissociation constant for proteins is 150 mM [61,62]), while the strongly hydrated di-anion sulfate binds weakly to lysozyme (see below) but is a poor crystallizing agent for lysozyme [137–140] probably because it overcharges the protein (puts a net negative charge on it, increasing its solubility) as has been shown to occur by adding excess positive charge with the trivalent cation Yttrium [141], as well as with Ni^{2+}, Mn^{2+}, Co^{2+} and Yb^{3+} [45,46]. Some chloride anions bound to hen egg white lysozyme do not contact any positive protein charges at all, but instead bind to the asparagine sidechain amide hydrogen atom [40], reflective of the fact that the amide ($pK_a \sim 15$) is slightly more acidic than water ($pK_a \sim 15.74$). Similarly, the retroviral class-1 membrane fusion proteins form a "trimer of hairpins" structure in which three asparagine sidechain amide hydrogen atoms bind a common chloride anion [41]. Presumably these Cl^- amide interactions result in significant transfer of negative charge from Cl^- to amide oxygen.

At 1 mM concentration weakly hydrated anions (such as nitrate) allow a 1 mM (weakly hydrated) cationic peptide [Ac-A_9K-NH_2] to form compact structures, whereas 1 mM strongly hydrated anions (such as citrate) cause the same peptide to form elongated structures, suggesting that contact ion pair formation effectively neutralizes the peptide positive charge whereas solvent separated ion pairs do not, allowing continued electrostatic repulsion between 'un-neutralized" cationic peptide molecules [142]. Similarly, weakly hydrated anions ($I^- > Br^- > Cl^- > F^-$) cause reversible oligomerization of a fusion protein, apparently by contact ion pair formation with protein positive charges [64]. In the case of monovalent cations, the excess negative charge in the C-terminal region of tubulin is neutralized most effectively, allowing polymerization, by $Na^+ > K^+ > Li^+ \sim Cs^+$ [55,143]; the effective neutralization of the carboxylate charge implies contact ion pair formation with the monovalent cations. Supportive of this interpretation is the observation that the relative rates of α-synuclein aggregation reveal a strong monotonic relationship between the C-terminal charge of α-synuclein and the lag time prior to the observation of fibril formation, with truncated (charge obliterated) species exhibiting the fastest aggregation rates. [Furthermore, the number of duplex turns in closed circular DNA increases as a function of counterion type in the same order as for the carboxylate group, $Na^+ < K^+ < Li^+ < Rb^+ < Cs^+ < NH_4^+$, suggesting some contact ion pairing between Na^+ and DNA phosphates [144,145].] The carboxylate preference for Na^+ over K^+ is also supported by solution oxygen 1s X-ray absorption spectroscopy [146,147], cyclodextrin complex formation [113], and both *ab initio* and molecular dynamics calculations [111–113,148–150] as well as by activity coefficients [105]. There is some variation of results except for the relative preference of the carboxylate for Na^+ over K^+, upon which everyone appears to agree. Preformed Na^+ and K^+ binding sites in proteins have been well characterized [20,151,152].

However, it is known that proteins show selective and preferential binding of anions to protein cationic groups, but not cations to protein carboxylates, at their surface in dilute salt solutions [139], and that the association constant of Na^+ for the carboxylate is small ($K_A(NaOAc) \approx 0.07\ M^{-1}$) [153]. This is consistent with recent molecular dynamics calculations which detected no interaction of Na^+ with the carboxylate group [154,155] and with UV resonance Raman measurements of Na^+ and K^+ in the presence of acetate and poly-L-glutamate [156] which detected no interaction of these cations with the carboxylate. [It should be noted that the behavior of poly-L-glutamate is surprisingly complex: "it is not stable in the α-helical form in aqueous solution under any conditions when it is fully charged." [34].] There are at least two obvious reasons why monovalent cations should interact weakly with the strongly hydrated carboxylate whereas weakly hydrated monovalent anions should interact strongly with weakly hydrated positively charged groups on proteins. First, if the water released by contact ion pair formation makes a large contribution to the energetics of the process, the smaller size of the IA cations (Li^+, Na^+, K^+, Rb^+, Cs^+) as compared to the positively charged amino acid side chains means that less water is released when the IA cations are involved. And second, each carboxylate has two tightly bound water molecules [15] onto which leaks 0.22 of the carboxylate negative charge [8], providing an obvious mechanism for water-mediated interactions with a counterion; additionally, if quantum effects are really this large, molecular dynamics simulations may not be capable of accurately describing the behavior of this system. Because the cell expends large amounts of energy pumping Na^+ out of the cell *via* the ($Na^+ + K^+$)-ATPase, we may conclude that high intracellular Na^+ is toxic to the cell, but the toxic interaction may be with nucleic acids.

8.1.2. Ions of high charge density interact with acidic protons or unshared electron pairs on proteins

Modulating the law of matching water affinities is the tendency of anions of high charge density to interact strongly with the acidic protons of water ($pK_a = 15.74$) [15], the acidic proton of the amide moiety ($pK_a \sim 15$), and the acidic protons of the guanidinium side chain of arginine ($pK_a = 12.5$) [60,157,158]. This interaction has some covalent character and involves some transfer of charge. It leads to the strongly hydrated phosphate oxyanion binding to the neutral amide proton [42], the strongly hydrated carboxylate binding to the weakly hydrated guanidinium group [159,160], and the strongly hydrated sulfate binding

to the weakly hydrated guanidinium group [137–140]. Similarly, cations of high charge density interact with the unshared electron pairs of water [15,49] ($pK_a = -1.74$) and of proteins; for example, the strongly hydrated divalent cation Ca^{2+} destabilizes proteins [34] by forming a strong chemical interaction with the peptide backbone amide oxygen ($pK_a \sim -1$), showing a large charge transfer from Ca 4s states into the peptide backbone as detected by photoemission spectroscopy [161]. Ba^{2+} also destabilizes proteins [34], presumably by the same mechanism (pulling the backbone amide out of the interior of the protein).

8.1.3. Circumstantial evidence suggests that very strong low barrier hydrogen bonds may be used to stabilize transition states at enzyme active sites

The strength of a hydrogen bond in pure water is about 3.7 kcal/mol [162,163]. Low barrier hydrogen bonds in protein structure and function are short, potentially very strong hydrogen bonds in which the proton is suspended midway between two heteroatoms of identical pK_a [164–167]. The strength of the intramolecular hydrogen bond between the carboxylates of hydrogen maleate in the gas phase as determined by photoelectron spectroscopy and electron propagator theory has been determined to be about 30 kcal/mol, which is enormously strong for a hydrogen bond [168]. Proton coupled bi-carboxylates involving amino acid side chains have been observed in 16% of a homology-reduced set of high-quality X-ray crystal structures extracted from the Protein Data Bank [169], and therefore such interactions, probably of modest strength, appear to be common in proteins. They are probably also involved in the binding of phosphate to protein carboxylates in the periplasmic phosphate transport proteins from *E. coli* [22] and *M. tuberculosis* [24], as well as in the in the stabilization of enzymatic transition states [166]. Be^{2+} toxicity may arise from its ability to displace the proton in low barrier hydrogen bonds [170,171]. The Glu-104 side chain carboxylate at the active site of the enzyme cytidine deaminase stabilizes the actual transition state of the reaction by −8.8 kcal/mol, corresponding to a catalytic rate increase of eight orders of magnitude; it also forms a short (2.5 Å) hydrogen bond with the Zn^{+2}-bound hydroxyl group of the tetrahedral transition state analog 3,4-dihydrouridine, which binds to the enzyme active site with a K_i of 1.2×10^{-12} M [172]. The Glu-270 side chain carboxylic acid at the active site of the enzyme carboxypeptidase A forms a very short hydrogen bond (2.2 Å) with the Zn^{+2}-bound phosphonate oxygen of a tetrahedral transition state analog which binds to the enzyme with a K_i of 3×10^{-12} M [173]. Two groups have crystallized the HIV-1 (aspartyl) protease with (different) bound peptide substrates at the active site, in each case distorted into a tetrahedral reaction intermediate stabilized by a short strong hydrogen bond of 2.2 Å [174] or 2.3 Å [175] with one of the catalytic aspartates.

Enzyme active sites typically have protein loops which fold down over the bound substrate to exclude water [176]. Transition state stabilization by enzymes is known to be largely enthalpic, usually involving polar interactions such as hydrogen bonds [177], and these strong enzyme-transition state interactions produce a more compact and rigid form of the enzyme which "wraps" around the transition state [178]. Therefore, although the existence of strong low barrier hydrogen bonds in aqueous environments remains a contentious issue [179], there should be strong selective pressure to recruit them for stabilizing transition states in the protected, near anhydrous environment of enzyme active sites, and much circumstantial evidence supports their involvement in this context.

8.2. Protein solubility and crystallization

8.2.1. Only net neutral proteins crystallize; below about 0.2 M, ions interact directly with proteins; above about 0.2 M, ions tend to interact indirectly with proteins (mediated by water molecules)

Up to a concentration of about 0.2 M, ion effects on proteins are due mostly to the direct binding of ions to polar and to oppositely charged groups on the protein, as can be seen by X-ray crystallography [40]. Weakly hydrated ions are "sticky", and can adsorb at many sites on the surface of the protein, such as nonpolar patches [136]. Increasing the net charge on proteins increases their solubility; decreasing the net charge on proteins decreases their solubility. Proteins have their lowest solubility when they are net neutral [180,181], because only neutral species can change phases. For example, at pH 7.1, a monoclonal antibody of isoelectric point (pI) 7.2 has approximately equal numbers of positive and negative charges. Up to 100 mM or higher, added monovalent anions ($SCN^- > Cl^- > F^-$, as the K^+ salts) increase the solubility of the antibody as they bind to the positive charges and increase the net charge on the protein. In contrast, at pH 5.3, where the same protein has a net positive charge, the same ions in the same order initially (up to 50 mM) bind to the positive charges on the protein but decrease its solubility as they decrease its net charge to zero; as the salt concentration is further increased, additional binding of the anions increases the net charge on the protein and increase its solubility [11]. The dominance of local effects in protein electrostatic interactions is illustrated by the existence of single "gatekeeper" charges which control the rate of protein aggregation [182]. Certain ions such as Ni^{2+}, Mn^{2+}, Co^{2+}, Yb^{3+} [46] yttrium [141] and probably sulfate [137] appear capable of causing an "overcharging" or "charge reversal" of proteins by direct binding. Above about 0.2 M, water-mediated (indirect) effects begin to dominate, where ions affect the solvation of the protein by competing for interfacial water [14] (probably that associated with the amide backbone) [183]. Weakly hydrated ions (such as guanidinium and thiocyanate) make interfacial water more available to solvate the protein; strongly hydrated ions (such as sulfate and fluoride) make interfacial water less available to solvate the protein [183–185]. These indirect Hofmeister effects can be substantial; for example, 1 M sulfate increases the melting temperature of staphylococcal nuclease by 15 °C (from 54 °C to 69 °C) [186]. At high concentration, neutral or strongly hydrated salts increase the surface tension over nonpolar patches, which can lead to aggregation [187]. Polymers such as polyethylene glycol probably encourage protein crystallization by excluded volume effects and by acting as a weak detergent, preventing nonspecific aggregation.

Strongly hydrated anions such as sulfate and fluoride can also salt in or out *via* direct binding to acidic protons on proteins [11,39,60,67,136,137,188–190] and increasing or decreasing the net charge on the protein, respectively.

The large weakly hydrated thiocyanate ion binds strongly to proteins [69] not only because the positive charges on proteins are weakly hydrated, but also because thiocyanate binds strongly to nonpolar surfaces [5]. Thiocyanate binding is characterized by multipoint attachment [40] and can crosslink protein molecules [40,191,192]. Thiocyanate is an aggressive denaturant and can partially denature proteins, even at 0.2 M [193]. Binding of trace iron [194] and copper [195] to the surface of proteins is associated with oxidation of the protein.

9. Conclusion

9.1. Solvent molecularity and the chemistry of ion–water interactions must be included to accurately model ions in water in complex environments and concentrated solutions

Continuum electrostatics models describing the long range forces on ions in water do not acknowledge the importance of the local water affinity of the ions or of chemical processes involving the ions. However the experimental and computational evidence for the dominant role of both is clear and convincing. It is the ability of water to keep the influence of charges mostly local that provides so much flexibility in designing biological structures. It will require explicit modeling of aqueous solutions with quantum chemistry to accurately represent the local forces that control the behavior of ions in water and make life possible. Additionally, we are now able to

list the principle forces and processes that give rise to the complex behaviors associated with ion-protein interactions, simplifying the planning and interpretation of experiments whose goal is to increase the stability and to increase the solubility of proteins.

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