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Review

Ion hydration: Implications for cellular function, polyelectrolytes, and protein crystallization

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Abstract

Only oppositely charged ions with matching absolute free energies of hydration spontaneously form inner sphere ion pairs in free solution [K.D.Collins, Ions from the Hofmeister series and osmolytes: effects on proteins in solution and in the crystallization process, Methods 34 (2004) 300–311.]. We approximate this with a Law of Matching Water Affinities which is used to examine the issues of (1) how ions are selected to be compatible with the high solubility requirements of cytosolic components; (2) how cytosolic components tend to interact weakly, so that association or dissociation can be driven by environmental signals; (3) how polyelectrolytes (nucleic acids) differ from isolated charges (in proteins); (4) how ions, osmolytes and polymers are used to crystallize proteins; and (5) how the "chelate effect" is used by macromolecules to bind ions at specific sites even when there is a mismatch in water affinity between the ion and the macromolecular ligands.

Keywords: Ion hydration; Hofmeister series; Polyelectrolyte; Calcium; Magnesium; Protein crystallization; Law of Matching Water Affinities

Contents

1. Introduction

There have been many useful summaries which correlate the physical properties of ions with their biological roles in the cell [1–5]. However, a major advance recently occurred when it was realized that a simple law controlled the tendency of oppositely charged ions to associate spontaneously in aqueous solution: specifically, only oppositely charged ions with matching absolute free energies of hydration (which can be approximated with a Law of Matching Water Affinities) spontaneously form inner sphere ion pairs in free solution [6,7]. For the purposes of this article, an ion may be thought of as a sphere of appropriate size with a point charge at the center. Since the absolute free energy of hydration of an ion is a monotonic function of surface charge density, ions of the same charge sort according to size: large anions form inner sphere ion pairs with large cations and small anions form inner sphere ion pairs with small cations. A crystal radius of 1.06 Å separates small monovalent cations from large, and a crystal

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radius of 1.78 Å separates small monovalent anions from large [6]. Large monovalent ions are weakly hydrated and small monovalent ions are strongly hydrated. Because it is only neutral salts which come out of solution, LiF (small cation–small anion), which readily forms an inner sphere ion pair, has a solubility of only 0.1 M while CsF (large cation–small anion), which resists forming inner sphere ion pairs, has a solubility of 24.2 M. For polyatomic ions, water affinity is controlled by the surface charge density of the specific atom with which the water interacts. The Law of Matching Water Affinities arises because the strength (enthalpy) with which an ion interacts with water closely parallels the strength with which it interacts with other ions; when a small ion interacts with a large counterion, the energetic cost of dehydrating the small ion is greater than the energetic gain of forming an inner sphere ion pair with the large counterion — that is, in the simplest possible model, the large counterion in an unfavorable process must replace a bound water molecule which may be considered a medium size zwitterion [6,7]. Thus the (strongly hydrated) small ion and (weakly hydrated) large counterion remain separated by water and are highly soluble. The Law of Matching Water Affinities imposes a severe constraint upon cellular organization — where can "freedom" arise in a system so strongly determined? One thinks of the rigid compositional constraints of an Arnold Schoenberg or Alban Berg serialist music composition. In this article we shall use the Law of Matching Water Affinities to examine the issues of (1) which ions are compatible with the high solubility of cytosolic components; (2) how cytosolic components tend to interact weakly, so that association or dissociation can be driven by environmental signals; (3) how polyelectrolytes (nucleic acids) differ from isolated charges (in proteins); (4) how ions, osmolytes and polymers are used to crystallize proteins; and (5) how the "chelate effect" is used by macromolecules to bind ions at specific sites even when there is a mismatch in water affinity between the ion and the macromolecular ligands. It may be useful for the reader first to review in more detail the background material, which may be found in Collins [7].

While the Law of Matching Water Affinities is used in this article mostly to characterize the association of ions, it also predicts the association of nonpolar moieties, treating nonpolar solutes as ions in the limit of very low charge density.

2. Ions of the cell

Phosphate and carboxylate anions (and to a lesser extent, the carbonate anion) will, for the purposes of this article, be considered the fundamental ions of the cell. RNA and DNA and phospholipids are phosphate di-esters, built upon the phosphate mono-anion. Phosphate is also important as the monoester in intermediary metabolism, where many small molecules are phosphorylated to keep them in the cell and to provide a "handle" for binding to enzymes. The nucleotide triphosphates, composed of phosphate mono- and di-esters, also play critical roles in cellular metabolism, and phosphate functions as a reversible tag in signal transduction. The carboxylate mono-anion provides all the negative charges on proteins and is found on many small metabolites such as those in the citric acid cycle; it also holds the protein chain together in the form of neutral amides. The carboxylate and phosphate mono-anions each bind about two water molecules tightly [Table 1], indicating that they have very similar hydration properties (oxyanion surface charge densities). Having accepted the requirement of using phosphates and carboxylates to build a cell, it is now useful to examine cations as a function of their surface charge densities and intracellular concentrations, as is done in Fig. 1; the cation surface charge density is plotted on the x-axis and the concentration of total (K^+, Na^+, Mg^{2+}) and free (Ca^{2+}) cation is plotted on the y-axis and given in Table 2.

Ca^{2+} is well matched to carboxylates and phosphates as shown by their similar hydration properties (Table 1): all three ions each bind about two water molecules tightly. Ca^{2+} readily forms inner sphere ion pairs with carboxylates and phosphates, forming insoluble complexes with important biological functions. Calcium carbonate (e.g., eggshells, 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} [4]. The eukaryotic intracellular concentration of free inorganic phosphate is about 5–8 mM [19]. The eukaryotic intracellular free calcium is about 10^{-7} M (Table 2); to maintain this low concentration, calcium must be continually pumped out of the cell at substantial energetic cost [20].

Na^+ (eukaryotic intracellular concentration = 10 mM, Table 2) binds only 0.22 water molecules tightly, but high concentrations of intracellular Na^+ are also toxic, as demonstrated by the common adaption of halophilic Archaea who substitute, at substantial energetic cost, high internal KCl (over 2 M) for high external NaCl [21]. Na^+ at high concentration is toxic because of its tendency to form moderately soluble inner sphere ion pairs with phosphates and carboxylates; for

Table 1
Ion–water affinity as measured by Jones–Dole viscosity B-coefficients (B) and the number of tightly bound water molecules (apparent dynamic hydration numbers, ADHN)

Cations	B	ADHN	Anions	B	ADHN
Mg^{2+}	0.385	5.8	PO_4^{3-}	0.590	5.1
Ca^{2+}	0.285	2.1	HPO_4^{2-}		4.0
			$H_2PO_4^-$		1.9
			$CH_3CO_2^-$	0.250	
Ba^{2+}	0.22	0.35	SO_4^{2-}	0.208	1.8
H^+		1.93			
Li^+	0.150	0.6	F^-	0.10	5.0
			HO^-		2.8
Na^+	0.086	0.22	HCO_2^-	0.052	2.0
K^+	−0.007	0	Cl^-	−0.007	0
NH_4^+	−0.007	0	Br^-	−0.032	0
Rb^+	−0.030	0	NO_3^-	−0.046	0
Cs^+	−0.045	0	ClO_4^-	−0.061	0
			I^-	−0.068	0
			SCN^-	−0.103	0

Sources. Jones–Dole viscosity B coefficients: phosphate, formate and perchlorate from Krestov [8]; all others from Robinson et al. [9]. Apparent Dynamic Hydration Numbers from Kiriukhin and Collins [10].

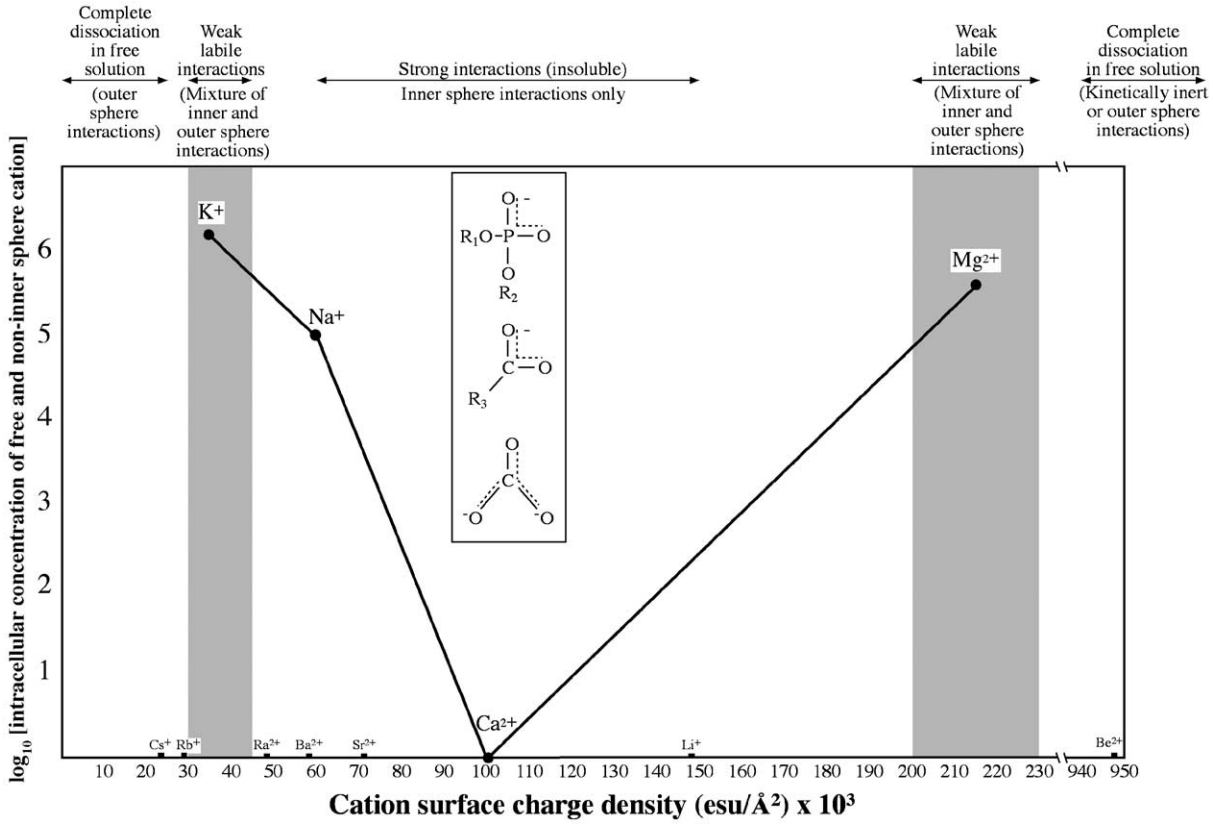

Fig. 1. The major intracellular anions and cations of the cell: correlation of intracellular cation concentration with cation surface charge density. The major intracellular anions — the phosphate monoanion, the carboxylate, and carbonate have similar hydration properties. The law of matching absolute free energies of hydration describes the spontaneous tendency of these anions to form inner sphere ion pairs of low solubility with calcium; therefore membrane embedded ion pumps must keep the intracellular concentration of calcium very low — about 10^{-7} M. Cations of lower and higher surface charge density are less likely to form inner sphere ion pairs with these intracellular anions; therefore the intracellular concentration of these cations can be substantially higher (intracellular cation concentrations are given in Table 2).

example, the solubility of K_2HPO_4 is about 8.6 M, while that of $Na_2HPO_4{\cdot}7H_2O$ is only 0.93 M [22]. If the phosphate mono-anion and the carboxylate mono-anion had different hydration properties, cations with water affinities matched to either anion would have to be kept in low cytosolic concentration by energy requiring membrane embedded ion pumps; since they have similar hydration properties, fewer kinds of cations must be kept in low cytosolic concentration. The sodium–potassium ATPase pumps sodium out of and potassium into the cell [23].

K^+ is the major intracellular monovalent cation (eukaryotic intracellular concentration = 159 mM); it is a chaotrope, binding water only weakly. As such, it is mismatched in water affinity to the strongly hydrated phosphates and carboxylates, therefore forming predominantly highly soluble solvent separated ion pairs with them. However, its ability to promote tubulin polymerization at a rate predicted by its Jones–Dole viscosity *B*-coefficient (Figs. 2 and 3) indicates that inner sphere ion pair formation with carboxylates does occur; its effects on super-coiling in closed circular DNA [25] and on the positive band of the CD spectrum of calf thymus DNA [26] [Table 3] indicate that inner sphere ion pair formation with phosphate mono-anions also occurs. We thus conclude that K^+ has weak, labile

Table 2
Cation properties

Ion	Radius (Å)[a]	Coordination number	Apparent dynamic hydration number[b]	Water residence time (ps)	Jones–Dole viscosity *B* coefficient[d]	pK_a	Intracellular concentration of free and non-inner sphere cation (mM)[f]
NH_4^+	1.50	4.8 – 8[i]	0		−0.007	9.25	1.8[g]
K^+	1.52	6	0	2[c]	−0.007		159
Na^+	1.16	6	0.22 ± 0.06	5[c]	0.086		10
Li^+	0.73	4–6	0.58 ± 0.05	27[c]	0.150		
Ca^{2+}	1.26	6–10	2.09 ± 0.14	$\sim 70 \times 10^3$	0.285	12.6[e]	0.0001
Mg^{2+}	0.86	6	5.85 ± 0.19	$\sim 1 \times 10^6$	0.385	11.4	40[h]
Be^{2+}	0.41	4	5.31 ± 0.16	$\sim 5 \times 10^9$		5.4	

Sources: [a]Cotton et al. [11]; [b]Kiriukhin and Collins [10]; [c]Enderby [12]; [d]Robinson et al. [9]; [e]Burgess [13]; [f]West [14]; [g]Sainsbury [15]; all others from Richens [16].
[h]The free Mg^{2+} concentration is about 0.5 mM [17]; [i]Perrin and Gipe [18].

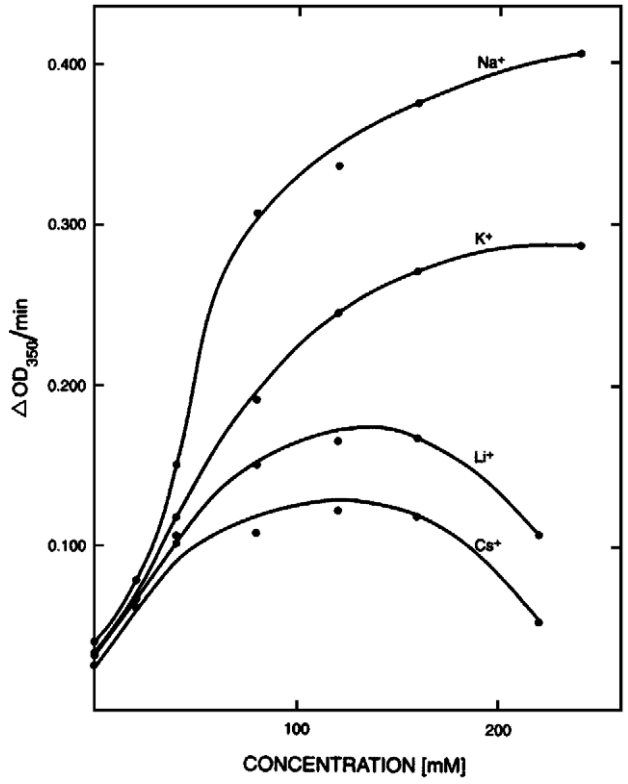

Fig. 2. Alkali metal chloride induced enhancement of rat brain tubulin polymerization. 0.1 M dimethylamino–Mes buffer, pH 6.9, 10 μM taxol, 1% DMSO, 0.8 mM GTP, 25.5 °C, and 2 min temperature equilibration. 18.4 μM tubulin was added to start the reaction. OD_{350} readings were started at 30 s. (Wolff et al., [24]; reprinted with permission). Because both the α and β monomers of nearly all tubulins carry considerable excess anionic charge, particularly at the C termini, added cations allow polymerization by binding to protein carboxylates and eliminating repulsive anionic interactions in the monomer.

interactions with phosphate and carboxylate that is a mixture of inner and outer sphere interactions. Most of the K^+ in *E. coli* [31] (and probably eukaryotes, [32]) is loosely associated with ribosomal RNA.

NH_4^+ is also a chaotrope, binding water only weakly, with hydration properties very similar to K^+ (Table 2). NH_4^+ is part of the side chain of lysine, and the arginine and imidazole side chains are also NH_4^+ derivatives. Incorporation of the NH_4^+ into an amino acid hydrocarbon side chain can only lower its charge density, and thus all of the positively charged amino acid side chains are weakly hydrated. In contrast, all of the major intracellular anions are strongly hydrated. In vitro, NH_4^+ is the most effective monovalent cation in activating *Bacillus subtilis* Ribonuclease P RNA [33], perhaps because, like $Mg^{2+}{\cdot}6H_2O$, NH_4^+ has a pK_a that makes it a moderately effective proton donor (Table 2), facilitating its interaction with phosphate anions; since both NH_4^+ and $Mg^{2+}{\cdot}6H_2O$ are multiple hydrogen bond donors, they can also "crosslink" phosphate anions. A negative charge spread over two oxygens produces a strongly hydrated anion, while a negative charge spread over three or four oxygens produces a weakly hydrated anion (Table 1). Sulfate mono-esters, in which the negative charge is spread over three oxygens, are weakly hydrated [34], and appear to be the only common example of a weakly hydrated negative charge found on a biological macromolecule. Therefore heparin sulfate, which profoundly affects mammalian development, interacts strongly with the weakly hydrated positive charges of specific proteins [35]. Cell-penetrating peptides are short peptides of less than 30 amino acids that contain only weakly hydrated positive charges; they readily penetrate membranes, coming into intimate contact with the weakly hydrated lipids of the membrane, and can be used to transport various cargoes across the membrane [36]. Peptides containing positive charges have also been used to inactivate transmembrane transport proteins by blocking the normal interaction between intramembrane intraprotein segments of the transporter; in this case, a (strongly hydrated) negative charge was used to anchor one end of the inhibiting peptide onto the surface of the membrane [37].

Mg^{2+} (eukaryotic intracellular concentration = 40 mM, Table 2) binds almost six water molecules tightly and has a large positive Jones–Dole viscosity *B* coefficient [Table 1] indicating that it has a higher affinity for water than do the phosphate and carboxylate anions. It therefore forms predominantly outer sphere complexes with nucleic acids and proteins [38,39]; that is, Mg^{2+} would rather have a water oxygen as a ligand than a phosphate or carboxylate oxyanion. Magnesium plays a major role in the folding and stabilization of RNA, both as "diffuse" magnesium without a specific binding site, and by binding tightly at discrete sites [33,38,40–42]. Magnesium prefers six oxygen ligands in an octahedral configuration, and in well defined nucleic acid binding sites has 0–3 phosphorus oxyanion inner sphere ligands, with zero or one being the most common number for DNA and slightly more for RNA

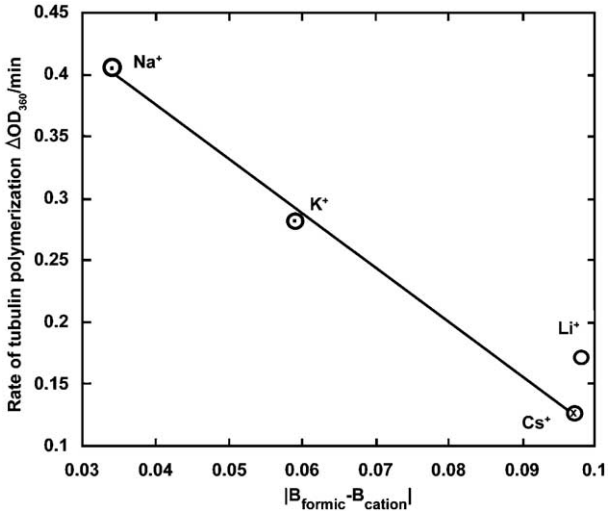

Fig. 3. The maximal rate of tubulin polymerization (from Fig. 2) vs. the difference in Jones–Dole viscosity *B* coefficients between added monovalent cations and the tubulin carboxylates. The Jones–Dole viscosity *B* coefficients for the carboxylate (0.052), Na^+ (0.086), K^+ (−0.007), Cs^+ (−0.045) and Li^+ (0.150) [from Table 1] are measures of the water affinity of these ions. The Law of Matching Water Affinities states that those ions with matching water affinity are those which will most readily form inner sphere ion pairs. The difference in Jones–Dole viscosity *B* coefficients of two ions is a measure of their mismatch in water affinity. Therefore Na^+ has the greatest tendency to bind to carboxylates, followed by K^+, followed by Li^+, followed by Cs^+.

Table 3
Interaction of monovalent cations with anionic charged groups on macromolecules

Type of macromolecule	Strongly hydrated cation exchangers e.g., phosphocellulose, carboxymethylcellulose [a]; inositol hexaphosphate [b]; compact RNA [c].	DNA	Proteins
Charges on macromolecule behave as	Ensemble of charges (polyelectrolyte)	Mixed	Isolated charges
Mechanism of cation neutralization of macromolecular anionic groups	Charge transfer to solvent	Mixed	Inner sphere ion pair formation
Interaction of macromolecular charges with counterions is	Indirect: outer sphere interactions mediated by water molecules	Mixed	Direct: inner sphere ion pair formation
Most effective monovalent cation for charge neutralization is the one	With the highest surface charge density		With an absolute free energy of hydration most closely matched to that of the anion on the macromolecule
Effectiveness of macromolecular negative charge neutralization by free cations (most effective cation is listed first)	$Li^+ > Na^+ > K^+$ [*ref* a,b,c]	$Na^+ > K^+ > Li^+ > Cs^+$ (same as for proteins) [*ref* d,e]	$Na^+ > K^+ > Li^+ > Cs^+$ [*ref* f,g]
Local concentration of negative charges on macromolecule	High, e.g. (−)	Intermediate (−)	Low, e.g. (−)

(a) Bregman, [27] (b) De Stefano et al., [28]. (c) Day-Storms et al., [29] (d) Anderson and Bauer, [25] (e) Chan et al., [26]. (f) Wolff et al., [24]. (g) Inouye et al., [30].

[32,43,44]. This mixture of inner and outer sphere ligands can provide binding sites with substantial stability but also with exchange rates high enough to be kinetically competent in rapid biological processes such as enzymatic catalysis. The higher affinity of water for magnesium than for phosphate and carboxylate is demonstrated in the kinetics of Mg^{2+} combining with oligonucleotides: Mg^{2+} shows a slow dehydration step, while the binding of Ca^{2+} (which is well matched to the phosphate in water affinity) does not [45]. For DNA in solution, Fourier transform infrared spectroscopic methods reveal direct interactions of phosphorus oxyanions with Ca^{2+}, but indirect interactions with Mg^{2+} [46].

Li^+, which binds 0.58 molecules of water tightly, also forms moderately soluble phosphate salts. Li^+ is used to treat manic depression, and is toxic at levels only slightly higher than the therapeutic level of about 1 mM. Li^+, with a size close to that of Mg^{2+}, appears to be neuroprotective, providing protection from a wide variety of insults such as neurotrophin withdrawal, amyloid-β peptide, prions, polyglutamine toxicity, ischemia, and many others [47]. It was suggested [48,49] that this broad-spectrum neuroprotection provided by Li^+ stems predominantly from inhibiting glycogen synthase kinase 3 [47]. Li^+ inhibits GSK-3 at least partly by acting as a competitive inhibitor of Mg^{2+}. "GSK-3 regulates many transcription factors that control gene expression, influences cellular signaling and architecture, and impairs the survival of stressed cells" [47]. Li^+ inhibits other enzymes as well, and is also known to compete with K^+ for protein binding sites [50].

Be^{2+} (Beryllium), one of the most toxic elements in the periodic table, is responsible for the often-fatal chronic inflammatory lung disease Chronic Beryllium Disease or berylliosis, and is listed as a Class A EPA carcinogen [51]. Beryllium inhibits glycogen synthase kinase-3 by competing for Mg^{2+} sites [52], and may be immunogenic by binding to carboxylate rich regions in the peptide binding cleft of the HLA-DP protein [53]. Beryllium fluoride (BeF_3^-) forms persistent complexes with the active site aspartate of enzymes which transfer phosphate via an aspartyl-phosphate intermediate, thus mimicking the phosphoenzyme [54]. At physiological pH, beryllium has only four inner sphere ligands, one of which is hydroxide, and these ligands exchange only slowly (on the millisecond time scale as compared to the microsecond time scale for magnesium) (Table 2); thus beryllium is inert to the rapid biochemical processes that characterize cellular metabolism.

3. Interaction of cytosolic components

The purpose of the membrane embedded ion pumps of the cell is to create a mismatch between the absolute free energy of hydration of the charges on intracellular macromolecules and their counterions. Fig. 1 shows that the major ions of the cytosol are moderately mismatched in terms of water affinity, and are thus characterized by weak, labile interactions involving a mixture of inner and outer sphere contacts. Since all of the positively charged amino acid side chains are weakly hydrated and all of the negatively charged amino acid side chains (carboxylates) and negative charges on nucleic acids (phosphates) are strongly hydrated, then protein–protein and protein–nucleic acid interactions mediated by charge–charge interactions will also be weak. This means that association or dissociation can be tipped in a given direction with a small input of energy, as from an environmental signal. The strength of charge–charge interactions can also be modulated by the polarity of the local environment, helping to drive the reaction in a given direction. That the major intracellular small molecule ions tend not to form inner sphere ion pairs with the major

macromolecular charged groups helps keep the macromolecules in solution (since only neutral species crystallize) and leaves the macromolecular charged groups free to act as binding determinants for interaction with other macromolecules or metabolites. For example, Record and co-workers [55] have shown that binding of the *lac* repressor to DNA in vitro is 40- to 300-fold stronger when the non-physiological, weakly hydrated Cl^- ion (eukaryotic intracellular concentration = 3 mM) is replaced by the strongly hydrated carboxylate-containing glutamate$^-$ (the major intracellular anion in *E. coli*). Only the strongly hydrated F^- has an effect comparable to that of glutamate$^-$ [56,57]. Record and co-workers [55], conclude that glutamate$^-$ is an "inert" anion, whereas Cl^- competes with DNA phosphate groups by binding to the positively charged residues of the *lac* repressor.

The cation surface charge densities of a number of non-physiological cations are also indicated in Fig. 1. The cations with normal physiological roles all have rapid exchange with water, and the water bound to these cations remains unionized at physiological pH (Table 2). Ions can be inert to rapid biochemical processes because of slow ligand exchange rates (e.g., Beryllium, Table 2) or because of an excessive mismatch in absolute free energies of hydration — for example, if Cs^+ were the major intracellular monovalent cation, there would be very little inner sphere ion pair formation with phosphates or carboxylates (Fig. 2).

4. From isolated charges (in proteins) to polyelectrolytes (nucleic acids)

The charges on proteins almost always act as isolated charges. That is, counterions form inner sphere ion pairs with specific protein charged groups according to how well matched they are in water affinity.

The positive charges on proteins are all derived from the weakly hydrated NH_4^+ ion embedded in weakly hydrated hydrocarbon chains. Therefore anions bind to the positive charges on proteins [58] with the same specificity that anions show when adsorbing to nonpolar surfaces [59]. That is, the anions with the lowest surface charge density adsorb most strongly: thiocyanate binds more strongly than bromide, which binds more strongly than chloride, which binds more strongly than fluoride. This is known as a reverse Hofmeister series.

The negative charges on proteins are all carboxylates. Monovalent cations bind to protein carboxylates depending upon how well matched to the carboxylate they are in water affinity. One way to measure the water affinity of ions is through the use of Jones–Dole viscosity *B*-coefficients. 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 strong binary electrolytes:

$$\eta/\eta_0 = 1 + Ac^{1/2} + Bc$$

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. Nearly all tubulins carry considerable excess negative charge, particularly in their C termini, and the ability of various monovalent cations to promote tubulin polymerization is a reflection of their ability to neutralize the negative charge on the protein carboxylate groups. Na^+ is the most effective monovalent cation in promoting tubulin polymerization [24] (Fig. 2) and its Jones–Dole viscosity B coefficient (a measure of water affinity) is closest to that of the carboxylate (i.e., formate; see Table 1). In Fig. 3 we use the differences in Jones–Dole viscosity B coefficients (on the x-axis) to measure the difference in water affinity between the carboxylate and the various monovalent cations. As the difference in Jones–Dole viscosity B coefficients increases (on the x-axis), their tendency to form inner sphere ion pairs decreases and the maximal observed rate of tubulin polymerization (on the y-axis) decreases proportionally, in the order $Na^+ > K^+ > Li^+ > Cs^+$.

Clusters of negative charges are common on proteins and often indicate a metal binding site; clusters of positive charges on proteins are rare [60].

Nucleic acids show at least a partial polyelectrolyte effect [61,62]. In polyelectrolytes, the monovalent cation remains hydrated (it interacts via an outer sphere interaction) and is not localized to a specific charged group (the polyelectrolyte charges act as an ensemble of charges). The monovalent cations associated with nucleic acids thus retain substantial freedom of motion. It has been hypothesized that "the assembly of RNA helices during collapse and folding is likely to be driven by the delocalization of condensed counterions." [63]. Table 3 shows that DNA manifests the same specificity for cations as do proteins, indicating at least a small amount of inner sphere ion pair formation of monovalent cations with DNA phosphate anions. When the negative charges on the macromolecule become even closer together (as in a cation exchanger or compact RNA, Table 3), the specificity for cations changes. In this case, the cations of highest charge density are associated most strongly with the cation exchanger.

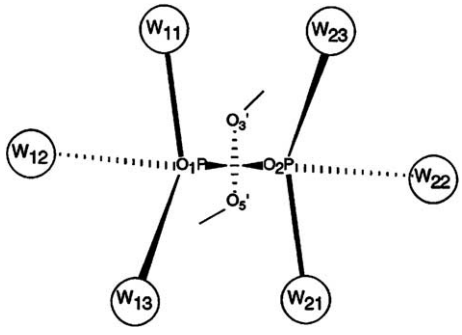

Fig. 4. A scheme of the first hydration shell around a phosphate group in double-helical DNA. This water distribution was obtained from analysis of 59 B-, A- and Z-DNA crystal structures. Schneider et al., [64]; reprinted with permission.

This occurs because there is substantial charge transfer to solvent for strongly hydrated ions. The strongly hydrated anions on the cation exchanger transfer negative charge to the attached water molecules, which interact most strongly with nearby cations of high charge density. Fig. 4 shows the six water molecules attached to the strongly hydrated phosphate di-ester of DNA [64]; approximately two of these six water molecules are strongly attached [Table 1].

5. Protein crystallization

Ions, osmolytes ("compatible solutes"), and polymers are used to crystallize proteins [65–68]. The effects of these additives on proteins can be divided into direct and indirect effects. We shall first examine the direct effects.

5.1. Direct effects

The most important direct effect of additives on protein crystallization is the adsorption of ions onto protein charged groups to produce a net neutral species; only net neutral protein molecules crystallize, and the minimum solubility of a protein occurs at its isoelectric point. A protein at a pH above its isoelectric point has a net negative charge and must adsorb cations to produce a net neutral species; a protein at a pH below its isoelectric point has a net positive charge and must adsorb anions to produce a net neutral species.

5.1.1. Cations

Two general points must first be made about the adsorption of cations onto a negatively charged protein. (a). Cation effects are almost always smaller than anion effects, probably due mostly to the fact that the cations are smaller than the anions. This can be seen in simple model systems such as gel sieving on Sephadex G-10 [59] as well as in complex biological systems such as protein binding to DNA [69]. (b). Protein surfaces contain many adventitious binding sites for cations [70–72]; metal ions such as Li^+, Ca^{2+}, and Mg^{2+} form complexes with amides [73–81]. Because of these facts, cation effects on protein crystallization are usually small and typically do not follow the preference order of cations for the free carboxylate; nevertheless, large effects of *multivalent* cations on protein crystallization have been observed [82]. And, since binding sites for specific monovalent cations such as Na^+ have been observed in X-ray structures of proteins [83,84], crystallization may in some cases require the presence of a specific cation.

5.1.2. Anions

The positive charges on proteins are derivatives of the weakly hydrated ammonium ion embedded in a hydrocarbon chain. Therefore, the Law of Matching Water Affinities states that weakly hydrated anions should adsorb to these protein cations more effectively than do strongly hydrated anions. The data of Ries-Kautt and Ducruix [58] in Fig. 5 illustrate the effectiveness of various anions in crystallizing the basic protein lysozyme at pH 4.5 and 18 °C; under these conditions

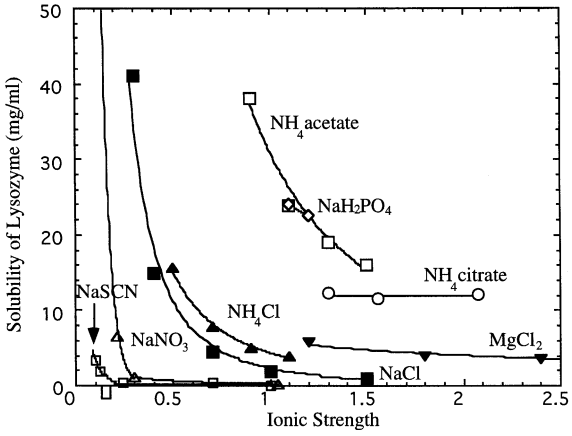

Fig. 5. Solubility of lysozyme as a function of ionic strength in the presence of various salts, at pH 4.5 and 18 °C. Riès-Kautt and Ducruix [58]; reprinted with permission.

at least four anions must bind to each molecule of lysozyme to produce a neutral species. The anion effects are large. The most effective anion, thiocyanate, is the most weakly hydrated, as judged by its Jones–Dole viscosity *B* coefficient (Table 1), its tendency to adsorb to the nonpolar surface of Sephadex G-10 [59], and its diffraction of neutrons in solution [85]. Nitrate, the next most effective anion, is the next most weakly hydrated, as judged by its viscosity *B* coefficient (Table 1). Chloride, the next most effective anion, is the next most weakly hydrated anion, as judged by its viscosity *B* coefficient (Table 1) and chromatography on Sephadex G-10 [59]. The dissociation constant of chloride for binding to protein cations is about 150 mM [86]; that of thiocyanate is about 90–100 mM [87]. Acetate and phosphate are strongly hydrated, as judged by their viscosity *B* coefficient or dynamic hydration number (Table 1). These strongly hydrated anions, which should resist forming inner sphere ion pairs with positively charged groups on proteins, are very inefficient at crystallizing lysozyme. In summary, Fig. 5 shows that thiocyanate is most closely matched to the positively charged amino acid side chains in water affinity because it binds to and crystallizes lysozyme most effectively; and that the greater the mismatch in water affinity between the protein cations and the added anions, the less effective are the anions in crystallizing lysozyme. Thiocyanate binding to proteins has been demonstrated by electrophoresis [88], equilibrium dialysis [70,89], X-ray crystallography [90] and nuclear magnetic resonance [87].

5.1.3. Neutral solutes

Neutral molecules such as 2-methyl-2,4-pentanediol (MPD) and polyethylene glycol (PEG) encourage protein crystallization primarily by several indirect mechanisms: excluded volume effects, lowering of the water activity, and interfacial effects which decrease the solubility of polar surfaces (see below). Consistent with these indirect mechanisms, equilibrium dialysis shows MPD to be excluded from the surface of the protein [91]. MPD and PEG also act as cryoprotectants. But solutes containing a weakly hydrated nonpolar portion would be expected to interact with the weakly hydrated nonpolar

surface of proteins (typically about 57% of the total surface for soluble globular proteins [92]) by the Law of Matching Water Affinities, thus acting as weak detergents and inhibiting nonspecific aggregation. In accordance with this prediction, X-ray crystallography shows nonpolar portions of MPD to be interacting with nonpolar patches on a protein surface [93] while immobilized PEG [94] has been shown to act as a nonpolar chemical chaperone, assisting in the refolding of proteins.

5.2. Indirect effects

Ions and organic solutes affect protein crystallization via intervening water molecules through excluded volume, water activity and interfacial effects.

5.2.1. Excluded volume effects [95,96]

Excluded volume effects are a strong function of the apparent size of the additive [97], and thus appear to be much more important for organic solutes and polymers than for small molecule ions. Also referred to as macromolecular crowding, this phenomenon is a simple consequence of space-filling: since two objects cannot occupy the same space at the same time, solution volume filled with additives is not available to the protein, encouraging it to acquire its most compact configuration and to form protein–protein contacts in the crystal. 2-Methyl-2,4-pentanediol (MPD) is one of the most common organic solutes added to protein crystallization mixtures and polyethylene glycol (PEG) is the most commonly used space-filling polymer [65].

5.2.2. Water activity effects

The water activity is the effective water concentration of a solution, and is determined by measuring the vapor pressure of water over a solution and comparing it to the vapor pressure of pure water at the same temperature. Lowering the water activity of a concentrated protein solution encourages crystallization by two different mechanisms. First "lubricating water" is drawn out of proteins, rigidifying the protein [98]. And second, other processes which release water are favored, such as formation of "closed" or more compact conformations [99] and protein–protein contacts in the crystal.

5.2.3. Interfacial effects

Interfacial effects are indirect effects that occur in the region of the protein–water interface. Interfacial effects near the nonpolar portion of the protein surface are most conveniently considered separately from interfacial effects near the polar portion of the protein surface; the polar surface includes not only the polar portion of the external surface, but also backbone amides and their intramolecular hydrogen bonds exposed upon large "breathing" motions of the protein. Energetically, indirect solute effects on the polar portion of the protein surface are probably more important than those on the nonpolar portion. Osmolytes ("compatible solutes") are known to interfere with the solvation of backbone amide groups [100] and (by simulation) intramolecular hydrogen bonds [101], and a major function of nonpolar amino acid side chains is to "wrap" backbone amide intramolecular hydrogen bonds, protecting them from attack by solvent water [102].

The surface potential difference [103] is a measure of the orientation of the surface water molecules at an air–solution interface; it reflects preferential interactions in the top three layers of water, each layer being one water molecule thick, and appears to be a good model system for understanding the effects of ions and osmolytes on the polar protein surface. The polar protein surface can be modeled by the backbone amide, which has an affinity for water equal to the affinity of water for itself (a viscosity B coefficient of zero) [10]. In the surface potential difference model, the surface layer of water molecules at the air–solution interface corresponds to the layer of water molecules adjacent to the polar protein surface (the solvation layer). The second layer of water into the solution is the transition layer, while the third layer of water into the solution can be thought of as the bulk surface [7,104]. Water is unique in that the second solvent layer (the transition layer) makes a large contribution to the free energy of interaction of the solvent with test solutes such as the protein polar surface [105]. The protein is stabilized (and intramolecular hydrogen bonds are strengthened) when the solution becomes a poorer solvent, causing the protein to minimize its solvent exposed surface area. This occurs when there is a large mismatch in water affinity between the polar protein surface and an ion or osmolyte inserted into the third hydration layer (the bulk surface). Transition layer water molecules maximizing their hydrogen bonding interactions with a strongly hydrated ion (one with a large positive viscosity B coefficient) in the third water layer (bulk surface) are unavailable to help solvate the protein surface, as are transition layer water molecules maximizing their interactions laterally adjacent to the nonpolar portion of an osmolyte in the third water layer [104]. Thus a large mismatch in the water affinity of an ion or osmolyte compared to the polar protein surface leads to a stabilizing indirect interaction encouraging minimization of water exposed protein surface area and formation of crystal contacts. This is illustrated in

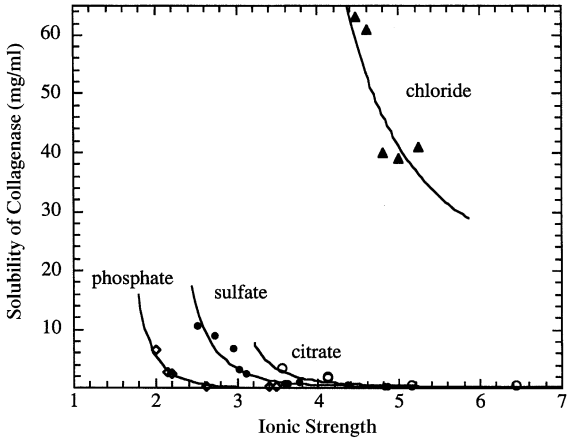

Fig. 6. Solubility of *Hypoderma lineatum* collagenase at pH 7.2, 18 °C, and in the presence of various ammonium salts. Riès-Kautt and Ducruix [58]; reprinted with permission.

Fig. 6, in which Ries-Kautt and Ducruix [58] used a series of ammonium salts to crystallize the acidic protein *Hypoderma lineatum* collogenase at pH 7.2. Since all of the ammonium salts should be comparable in their ability to neutralize protein carboxylates, the observed variation in protein crystallizing efficiency can be attributed to the water binding ability of the anions in the third interfacial water layer. Using the number of tightly bound water molecules associated with each anion from Table 1 (the apparent dynamic hydration numbers), we see that phosphate, the most effective crystallizing anion is the most strongly hydrated, binding between three and four water molecules at pH 7.2, while sulfate, the next most effective crystallizing anion, binds two water molecules. Citrate, the next most effective crystallizing anion, binds less than two water molecules per carboxylate because of intramolecular hydrogen bonded forms (Kiriukhin and Collins, unpublished observations), and chloride, the least effective crystallizing anion, binds no water molecules tightly. Thus the effectiveness of an anion in crystallizing collagenase via indirect interactions is a monotonic function of its mismatch in water affinity with the protein polar surface.

The surface tension at an air–water interface [103] is increased by dissolved particles in the interfacial region, which creates 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 in the solution away from the interface, creating a force which tries to minimize the amount of interface. The surface tension at an air–solution interface serves as a good model system for the effect of ions and osmolytes on a nearby protein nonpolar surface. While multivalent cations in particular have a tendency to bind to the protein surface (lowering the surface tension) (see above), strongly hydrated anions (those with large positive viscosity B coefficients) will be excluded from the nonpolar (weakly hydrated) protein surface, thus raising the surface tension of the interfacial region. This creates a driving force for the minimization of solvent exposed protein nonpolar surface area, stabilizing the protein. The more strongly hydrated an anion, the larger its effective size and the more strongly it is repelled from the nonpolar protein surface. Here again, a large mismatch in water affinity between the protein surface and a nearby strongly hydrated anion leads to a stabilizing indirect interaction.

Diagrams of these anion mediated interfacial effects can be found in Collins and Washabaugh [104] and Collins [7].

6. The chelate effect

In addition to considerations of water affinity which impart specificity to charge–charge interactions in water, macromolecules also use other techniques to create highly specific binding sites for small ions or charged small molecules. In particular, highly specific binding sites on macromolecules arise (a) because the binding site is geometrically complementary to the small ion or charged small molecule, and (b) because there is a large entropic enhancement to binding resulting from multipoint attachment, which has been called the chelate effect, among other names [106–108]. By examining the binding affinities of enzyme substrates and inhibitors in comparison to their fragments, Wolfenden [107] has experimentally demonstrated increased free energies of protein–small molecule interaction of more than 11 kcal/mol which corresponds to effective ligand concentrations of more than 2×10^8 M due to the chelate effect. With these potentially large free energies of interaction available from multi-point attachment, matching the water affinity of individual protein ligands for the bound ion or charged small molecule within the highly specific binding site on the macromolecule becomes a less important consideration.

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