1. INTRODUCTION

The stability of protein solutions is governed not only by the macromolecular net charge, salt concentration and valency, but also on the chemical nature of the dissolved ions (1). Traditionally, the latter falls under the category of Hofmeister or ion-specific effects which in recent years has seen an appreciable renaissance – both from experimental and theoretical perspectives. In Hofmeister's original studies (2) ions were arranged according to their ability to precipitate or “salt-out” egg white proteins. For the anions, which we focus on here, the following order was found: F->CH₃COO->Cl->NO₃->Br->I->SCN-. The effect of cations are generally less pronounced. The situation is, however, more complicated in that the Hofmeister ordering is in fact dependent on the solution pH and the protein iso-electric point, pI. For example, second virial coefficient measurements of lysozyme with pI=10-11, showed that this protein follows the reverse Hofmeister series at low pH and that anions such as iodide and thiocyanate very effectively induce protein association (3). In a systematic study using small angle X-ray scattering it was demonstrated (4) that a Hofmeister reversal for pH<pI is observed not only for lysozyme but for a range of small proteins including α-crystallins, γ-crystallins, ATCase and Brome Mosaic Virus.

Recent studies indicate that the ion specificity of protein association to a large extent is governed by interactions between salt ions and the macromolecular surface (5). Then for pH<pI any absorption of anions will effectively reduce the repulsion between the cationic bio-molecules and thus assist complexation. The reverse is true for negatively charged proteins (pH>pI) where binding of anions will increase the repulsion and thus stabilize the solution. From the above mentioned experiments (3, 4) we thus conclude that anion binding to proteins follows the reverse Hofmeister series – i.e. thiocyanate binds stronger than fluoride irrespective of the net charge.

It has been shown (6) that combining Poisson-Boltzmann continuum electrostatics with ion-specific dispersion forces between salt ions and spherical macro-ions, the Hofmeister reversal can be obtained. In the dispersion framework the larger ions such as iodide and thiocyanate interact stronger with the (averaged) macromolecular surface than does for example chloride. While dispersion is indeed ion specific (see chapter by Boström et al.), other important mechanisms are at play. Protein surfaces are far from uniform and consist rather of an intricate network of polar and non-polar groups to which salt ions have widely different affinities. For example, large anions are attracted to hydrophobic interfaces via surface modified solvation and polarization (7, 8). Direct ion-pairing (9, 10) between salt particles and charged surface groups also give rise to ion specific phenomena (11). In this chapter we will focus mainly on the contributions from these mechanisms using a combination of atomistic and mesoscopic simulation techniques.
2. ION-PAIRING

Ion-pairing and simple electrolytes

Shortly after the introduction of the Debye-Hückel (DH) theory, it was recognized that bulk properties of strongly associating ions are difficult to reproduce. This, in 1926, led Bjerrum to introduce the concept of “ion-pairing” where the association of two ions is described by an equilibrium constant \((9, 12)\). While this partitioning of bound and unbound states is always subject to an arbitrary definition – and invented to mend an incomplete microscopic description – the concept of ion-pairing remains useful for understanding ion specific effects. In the following we outline the basic concepts for bulk electrolyte solutions and, later, show that these apply to proteins as well.

One way of probing the interactions between solvated ions is to study aqueous bulk electrolyte activity coefficients, \(\gamma\). For the most common choice of reference state the mean activity coefficient is a measure of the excess free energy, \(\mu = k_B T \ln \gamma\), of transferring a solvated salt pair from an infinite dilution to a solution with a finite salt concentration. In the present context, we are interested in the ion specificity and therefore – for a fixed salt concentration – define a free energy of exchanging one counter-ion with another. For example,

\[
\Delta \mu_{\text{KCl-KI}} = \mu_{\text{Kl}} - \mu_{\text{KCl}} = k_B T \ln (\gamma_{\text{Kl}}/\gamma_{\text{KCl}})
\]

The range of activity coefficient measurements is vast and we can thus use the change in excess chemical potential as a direct measure of relative ion specificity for a large number of species. In Figure 1 we show the chemical potential change for exchanging chloride with fluoride (left) and potassium with sodium (right). From these experimentally obtained data it is clear that the larger alkali cations like Cs\(^+\) and Rb\(^+\) prefer iodide over fluoride while the opposite is true for the smaller sodium ion. Potassium shows an equal preference for both halides. Likewise, we also observe that acetate – a useful proxy for acidic amino acid side-chains – prefers sodium over potassium. Interestingly, the free energy difference varies almost perfectly linearly with the solute concentration, \(\text{i.e. } \partial \Delta \mu/\partial c = \text{const}\). By plotting the excess chemical potential difference, one can argue that we have subtracted the generic DH-type, salt screened contribution and are left with the specific part, only.

Let us note that the DH theory and the underlying primitive model of electrolytes are appropriate for the long ranged nature of the electrostatic interactions – both between ions and between ions and water molecules. It fails, however, to describe the short range oscillations connected with ion-specific hydration and, to capture ion-specific effects, the original model needs extensions.
Figure 1. Left: Measured change in excess chemical potential of exchanging chloride with fluoride in a range of alkali metal solutions. Right: As left, but for the exchange of potassium with sodium for a number of common monovalent anions.

The activity coefficient is of course a macroscopic average of the excess interactions in the solution and as such does not provide a direct picture of the molecular level mechanisms. On empirical grounds it has been suggested (13) that ions with matching water affinities – which according to the Born solvation energy translate to “matching sizes” – tend to prefer each other. Generally, this notion is in good agreement with the data shown in Figure 1 where pairs of small-small or large-large are preferred over large-small combinations.

To unravel the molecular level mechanisms, calculations have provided important insight (10). Classical Molecular Dynamics (MD) simulations of pairs of ions in a molecular solvent as well as \textit{ab initio} calculations in a continuum solvent show that the principle of matching sizes indeed has a molecular explanation. Examining the radial distribution functions (see Figure 2) between sodium and fluoride – two rather small ions – we note that the large peak corresponds to an attractive configuration of a contact ion pair. At larger separations there is a second peak, corresponding to a solvent-separated pair. The attraction between the larger cesium ion and fluoride is much less pronounced since forming a contact ion pair involves a shared solvation shell and – due to the size differences between Cs\(^+\) and F\(^-\) – this is an unfavorable configuration. As expected, the situation is reversed when fluoride is exchanged with the larger iodide anion. These observations are in qualitative agreement with the monovalent activity coefficient data shown in Figure 1, and provide a molecular interpretation of the empirical idea of matching water affinities.
Ion binding to amino acid residues

We now attend ion-pairing in more biologically relevant systems. Simulation work on proteins, oligopeptides, and amino acid residues have shown that sodium and potassium bind differently to bio-molecules (11, 14). In general, the affinity of sodium to protein surfaces is more than twice that of potassium. The cationic attraction stems mainly from pairing with acidic carboxyl groups on glutamate and aspartate, and with backbone carbonyl groups. In the following we will focus on complementary anion binding which generally exhibits stronger ion specific behavior than the cations.

Let us first ask ourselves how halides bind to isolated positively charged amino acid residues such as arginine, lysine, and protonated histidine. To obtain a molecular level insight of the ion-specific interactions, we investigate the spatial distributions of halides around the positively charged amino acid regions (see Figure 3). This type of analysis provides information about both the strength of interaction as well as the spatial configuration. On one hand, fluoride exhibits a strong affinity for positively charged groups, while that of the larger halides is much weaker. Among the investigated side chains the order of binding to F⁻ is guanidinium > imidazolium > ammonium. Iodide, on the other hand, and to a lesser extent also bromide and chloride, are weakly attracted to non-polar regions as well as to amide hydrogens on the backbone.
Figure 3. Density plots showing the distributions of chloride and iodide ions around aqueous methyl-terminated basic amino acids. From left to right: arginine, lysine, and histidine.

Quantitatively, fluoride interacts most strongly with the guanidinium group of Arg, followed by the imidazolium group of (protonated) His, and the ammonium group of Lys. This affinity ordering is observed when using both non-polarizable and polarizable force fields, being weaker – by about a factor of two – in the latter case. For non-polarizable force fields fluoride is the clear winner among the halides for all positively charged amino acid residues, with the dominant part of the halide-amino acid affinity being due to the charged side chain groups. Upon including the polarization term, the interaction of F$^-$ with the amino acids is reduced. This is opposite to the larger halides where polarization effects tend to enhance the affinity for non-polar regions. This leads to a situation where the overall affinity for the amino acid surface can be higher for iodide than for fluoride. This is caused by a sizeable propensity of the former ion for the interface between water and non-polar groups when using a polarizable force field.

The computational results show that the halide interactions with positively charged amino acid residues are local and generally not overwhelmingly strong. This is well documented by the fact, that even fluoride anions, which exhibit the strongest interaction, frequently exchange positions in the vicinity of the amino acid and in the bulk. Therefore, additivity can be invoked and analogous ion-specific behavior of halides can be expected at surfaces of aqueous proteins. The overall ion specific effect will then be a net result of non-polar attractions and direct ion-pairing with positively charged side chains. In the former case, large, soft anions such as iodide wins, while for the latter case, small anions like fluoride dominate. In addition, interactions with the backbone amide hydrogens should be considered as these have a considerable preference for larger anions.
3. NON-POLAR ATTRACTION

In the previous sections we discussed how ion pairing influences bulk electrolyte properties as well as controls where and how strongly ions bind to complex biomolecules. We also mentioned that large, “soft” anions can bind to aliphatic regions and we now focus on this mechanism that – perhaps counter intuitively – causes certain ions to be attracted to non-polar or hydrophobic molecular regions.

Experimental as well as theoretical studies of anions close to the vapor-water and molecular interfaces reveal an appreciable ion specific segregation. In particular large, “soft” and poorly hydrated ions such as iodide and thiocyanate are attracted to the interface (7) while small, “hard” and well solvated species are repelled from it. The former observation contradicts the traditional dielectric continuum picture within which a generic, solvated ion close to a low dielectric interface will experience a repulsive force due to partial dehydration (15). Let us revise the mechanisms with which an ion may interact with a non-polar interface:

I. Desolvation or loss of ion-dipole energy as described in classical electrostatics by a reaction field.
II. Association of poorly solvated species is induced due to a reduction of the ordered water network surrounding these (16).
III. Aligned water molecules near the non-polar interface set up an electric field that leads to induced dipole interactions with polarizable ions (17).
IV. Solvent-solute, solute-solute and solvent-solvent dispersion interactions can lead to both an attraction and also a repulsion of ions (see the chapter by Boström et al.)

How much each of the interactions I-IV contributes to the potential of mean force depends to a large extent on the ion type. Let us first consider a small ion, characterized by a high surface charge density and a small polarizability (both static and dynamic). In other words, the ion is strongly hydrated and (I) will be the dominant interaction type upon moving the ion towards a non-polar surface. A large ion has opposite characteristics and, consequently, interactions II-IV become increasingly important.

3.1 An idealized bio-colloid

While the attraction of large anions to water-vapor interfaces is well established, the situation for complex molecular surfaces is less scrutinized. Solvated, globular proteins are mainly hydrophilic in nature but an appreciable number of non-polar residues can be present even at the solvent exposed molecular surface – see Figure 4. With the situation at the water-vapor in mind it indeed is imaginable that large anions exhibit a similar attraction to such non-polar surface patches.
To decipher the interaction mechanisms of small and large anions with bio-molecular interfaces it is, at least initially, advantageous to look at a simplified system that captures the essential physics. With the risk of dismaying readers with a biochemical background, we will now construct an artificial “bio colloid” – a simple nano-sphere with distributed cationic surface charges (see Figure 4). While we do not claim that this toy-model (8) is an adequate proxy for a real protein, it has some appealing advantages in that one can control the hydrophobicity/hydrophilicity and, due the simple surface topology, it can be readily scrutinized. Figure 5, which shows where fluoride and iodide bind to the surface of such a bio-colloid, reveals some interesting features:

- Fluoride (small and well hydrated) binds exclusively to the discrete surface charges.
- Iodide (large and poorly hydrated) is found on the remaining non-polar patches.

Hence, from this simplified model one can extract that specific ion binding is governed by a delicate balance between interactions with charged and non-polar surface patches on the macromolecule. The former groups attract ions via direct pairing while the latter do so via a range of solvent mediated interaction mechanisms (discussed above).
3.2 Binding to a “real” protein

To confirm that different ions segregate also on complex molecular surfaces according to the distribution of charged and non-polar patches, we now substitute the above “bio-colloid” with a real protein in full atomistic detail (see Figure 4, right) and redo the analysis. Since the protein surface is immensely more complex (as well as fluctuating) the binding cannot be represented using spatial density plots as in Figure 5. Instead, one can average the number of ions encountered in non-spherical shells around polar and non-polar patches. The result is shown in Figure 6. As in the simplified model iodide (large and poorly hydrated) indeed prefers non-polar patches while chloride (small and well hydrated) prefers cationic groups.

Figure 6. The ratio between the cumulative sums of chloride and iodide around polar/non-polar surface groups on lysozyme. Results of MD simulations in an explicit solvent (POL3) and a mixture of sodium chloride/iodide (19).

Coming full circle and relating to experiments, the affinity for large ions to non-polar molecular regions are also manifested in bulk electrolyte solutions. Figure 7 shows the growing preference for iodide over chloride when increasing the chain length of the tetra-alkylammonium cations. In contrast, for the bare ammonium ion there is a slight preference for the smaller chloride anion.
4. PROTEIN AGGREGATION

Having dissected the ways in which ions bind to bio-molecular surfaces, we attempt for predictions of ion specific phenomena in protein solutions. In Hofmester’s original work (2), salts were ranked according to their ability to precipitate egg white proteins. Since then, many more experiments have been conducted with a rather general conclusion for protein-protein association (4):

- Cationic proteins (pH<pI) usually follow the reverse Hofmeister series.
- Anionic proteins (pH>pI) usually follow the direct Hofmeister series.

Hence at low pH, lysozyme, pI=10-11, will associate more in the presence of iodide than of chloride. This is manifested in the second virial coefficient, $B_2$, that can be measured using various scattering techniques (3). The thermodynamic virial coefficient is defined as an integral over the angularly averaged potential of mean force between two protein molecules, $w(r)$:

$$B_2 = -2\pi \int_0^\infty (e^{-w(r)/kT} - 1)r^2 \, dr.$$

We now perform Monte Carlo simulations of two lysozyme molecules, coarse-grained to the amino acid level (see Figure 4, middle). Due to the nano-scale length scales involved, we invoke the dielectric continuum model for water and thereby average out all structural features of the solvent. It is, however, precisely water structuring that gives rise to the ion specificity mentioned in Section 3 and we therefore need to include this implicitly (17, 18). Applying effective potentials between hydrophobic surfaces and ions, obtained from explicit solvent simulations, it is possible to account for the solvent-assisted interaction between ions and non-polar amino acid residues (defined as ALA, LEU, VAL, ILE, PRO, PHE, MET, TRP). In this framework, iodide is attracted to these groups while sodium and chloride are repelled.
The specificity of anion binding to the charged residues is included through the distance of closest approach in the water-screened Coulomb potential. A straightforward improvement would be to invoke a more realistic pair-correlation function, obtained from explicit solvent simulations.

As shown in Figure 8, at low pH the inter-protein potential of mean force, \( w(r) \), is more attractive in solutions of sodium iodide than in sodium chloride. This can be attributed to the fact iodide absorbs to the non-polar patches, thus decreasing the effective charge on the cationic proteins (pH < pI). Changing the solution pH from 4.5 to “12.5” the iso-electric point of lysozyme is exceeded and the protein net-charge goes from +9 to -5.5. The quotation marks are used to signal that strong alkaline conditions are hardly practical for protein solutions. Our simulations predict – in accord with measurements (4) – that salting out of anionic proteins indeed follows the direct Hofmeister series while the reverse is true for cationic ones.

![lysozyme-pmf.tif](lysozyme-pmf.tif)

Figure 8. Simulated potential of mean force between two mesoscopic lysozyme molecules in aqueous solutions of NaCl and NaI at low and high pH. Simulated in a continuum solvent with explicit salt- and counterions (18).

Note that the Hofmeister reversal has also been observed in calculations of spherical-macroions via inclusion of specific dispersion interactions between ions and the macromolecules (6). In that and other studies (22, 23) the macro-molecular surface is represented by a uniform, spherical object akin to the renowned DLVO theory (24, 25). This implies that co-solutes experience the macromolecule as a spherical average of the actual, complex topology as found in real proteins. Depending on which properties one is after, this high degree of coarse graining may provide a insight, although it seems less applicable for complex bio-molecules where ion specificity is determined by a subtle balance between ion-pairing and non-polar interactions.

**SUMMARY**

We have seen how ion specific binding to colloids, amino acid residues as well as complex proteins is governed by two main mechanisms that target distinct surface groups on the macromolecule:
• Ion-pairing.
• Non-polar, solvent mediated interactions.

Interestingly, both mechanisms are manifested in bulk solution properties such as the mean activity coefficient. This hints – and is indeed supported by molecular simulations – that ion specificity is controlled by a subtle combination of generic intermolecular interactions, effective for a large range of molecular dimensions. Protein-protein association is governed by salt type and concentration as well as the solution pH. Taking into account the “patchiness” of protein surfaces, the experimental observations can be reproduced using coarse grained molecular simulations. In particular, ion binding to non-polar patches leads to the observed pH dependent reversal of the Hofmeister series.

REFERENCES

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