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Protein-Protein Interactions in Salt Solutions

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1. Introduction

Protein-protein interactions drive many biophysical processes of proteins in solutions, such as aggregation, solubility, and phase transitions including crystallization, gelation, and amorphous precipitation. Many of these processes are of significant research interest because of their practical importance. In the biopharmaceutical industry, it is crucial to prevent therapeutic proteins from aggregation during the manufacturing process and storage in order to maintain safety and efficacy (1). In addition, protein crystallization and precipitation are used for industrialized recombinant protein purification process (2). In the field of structure biology, it is still a daunting task to produce diffractive quality protein crystals for determining protein 3-D structures because there is lack of clear understanding of the mechanisms for protein crystallization (3). Furthermore, studying protein-protein interactions could shed light on the mechanism of protein condensation (or phase transition) diseases, such as cataract and sickle cell disease (4). Finally, protein-protein interactions may play essential roles in many human neurodegenerative diseases attributed to protein aggregation, such as Parkinson and Alzheimer diseases (5).

In solutions, salts are ubiquitously used to control pH, ionic strength and osomlality in scientific research and industry applications. It is important to understand how salts modulate protein-protein interactions so that solution behavior, such as protein crystallization, precipitation, and solution stability, can be controlled and manipulated. However, the exact interaction mechanisms between salt ions and proteins are poorly understood (6, 7). As a consequence, modulations on protein-protein interactions by salt ions and their implications for protein solution behavior cannot be completely rationalized. The challenges rise because of (i) the sheer complexity of physical and chemical properties for both salt ions and proteins and (ii) the wide range of salt concentrations, which can be varied up to 1000 fold from millimolar to molar. It cannot be emphasized better than how Kunz and Neueder mentioned in their book with regards to salt solutions: “In total, it is still a fact that over the last decades, it was still easier to fly to the moon than to describe the free energy of even the simplest salt solutions beyond a concentration of 0.1 M or so”(6). Proteins probably belong to the most complex colloidal system in terms of variations in surface charge, surface chemistry, and size. Specifically, a protein could be net positive-charged, neutral, or negative-charged at pH conditions below, near, and above its pI (Isoelectric point), respectively. Additionally, protein surfaces are heterogeneously composed of

positive and negative charged, polar and nonpolar amino acid residues. Finally, the size of proteins in the range of 1-5 nm (estimated by the minimal radius of a sphere containing a given mass) would significantly impact the surface charge density(8).

Intermolecular interactions between protein molecules can have different origins, such as electrostatic, hydrophobic, van der waals, and hydrogen bonding (9). It is difficult to pinpoint the exact relative contributions from each type of interaction to the (overall) protein-protein interactions. In this review, I focus on explaining the modulations of electrostatic protein-protein interactions by the simple salt ions (shown in Figure 1) through their specific interactions (or binding) from both cation and anion with protein surface at salt concentrations below 0.5- 1 M. In addition, the complete picture of salt ion's effects on the intermolecular interactions may be better understood by considering the following biophysical properties of proteins and salt ions: (i) the net charge, surface charge density and hydrophobicity of a protein; (ii) hydration, size, polarizability and valency of salt ions. The discussion is based on the recent experimental results reported in literature and findings from Amgen using the following experimental techniques, such as protein solubility measurement, phase transition temperature of $T_{critical}$ (critical temperature) or T_{cloud} (cloud temperature) for liquid-liquid phase separation and small angle X-ray scattering (SAXS) (10-13). It has been demonstrated that there is a strong correlation between protein solubility and protein-protein interactions: protein solubility decreases when the protein-protein interactions become less repulsive or more attractive (for a protein for which its solubility increases with temperature)(12, 13). Also it is generally accepted that for a protein solution with an upper consolute point, an increase in phase transition temperature, as a result of change in the solution condition, indicates that protein-protein interactions become less repulsive or more attractive.

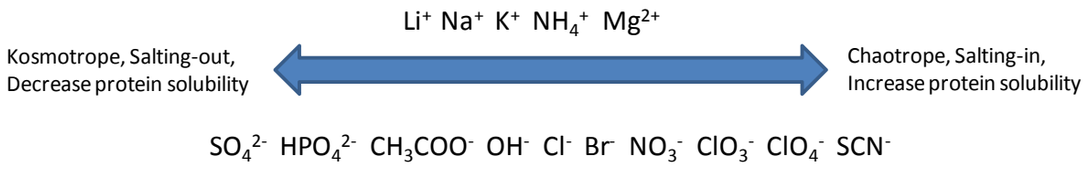


Fig. 1. Hofmeister series adapted from (14).

2. Historical background

2.1 Direct and reverse Hofmeister series

The most important experimental work on protein-protein interactions in salt solutions can be traced back more than 100 years ago when Franz Hofmeister and his coworkers studied salt effects at high salt concentrations on protein precipitation of hen egg white proteins whose main component is ovalbumin (pI=4.6). At that time, he hypothesized that the protein precipitating (salting-out) capability for the salts was dependent on their ion hydration properties (6). Later on, an empirical ranking for both cations and anions in their effectiveness, as shown in Figure 1, for precipitating proteins was named as (direct) Hofmeister series (14). Typically, the anions' effects are more dramatic than cation (14). In 1989, a surprising and complete reverse Hofmeister series was discovered by Ries-Kautt and

Ducruix in solubility measurement of lysozyme in salt solutions at pH below its pI where the protein was net positively charged (15).

2.2 Protein-protein interactions for a net charge neutral protein in salt solutions

A protein is net-charge neutral at its pI with the equal numbers of positive and negative charges. This is the most distinctive difference between proteins and the peptides with neutral side chains/small nonpolar molecules, for which extensive and detailed solubility experiments were conducted in salt solutions (16-19). However, there is lack of systematic protein solubility studies in salt solutions near their pIs. It is generally accepted that near the pI an increase in protein solubility (salting-in) is expected when salts are initially added and then a decrease occurs at high salt concentrations (salting-out by kosmotropic salts)(20). Although the mechanism of protein-protein interactions near its pI remains to be determined, it can be inferred from the observation above that the protein-protein interactions may initially become less attractive and then more attractive with increasing salt concentrations.

2.3 Protein-protein interactions for a net positive-charged protein in salt solutions

Lysozyme is a small globular protein with a Molecular Weight (MW) of 14.4 kilo-Dalton (kD) with a high pI value of ~11(12). Despite the fact that the experiments can mostly be conducted at pH conditions below its pI, lysozyme was frequently used as a model protein for studying both protein-protein interactions and protein-salt ion interactions in salt solutions probably due to its availability and easy crystallization propensity. Numerous experiments revealed very complex relationships between intermolecular interactions and salt concentration, salt type and pH; different theories were put into place to interpretate the trends (12, 21, 22).

In monovalent salt solutions under 1.0 M, the intermolecular interactions for lysozyme generally became monotonically more attractive as the salt concentration increased at pH conditions far below its pI(12, 21). These findings are consistent with the no salting-in event, i. e. protein solubility decrease, for lysozyme by NaCl in a pH range from 3 to 9 under the salt concentration up to 1.2 M (23). Acting as counter-ions to the net positively-charged lysozyme and following the reverse Hofmeister series, these monovalent anions imposed profound effects on the intermolecular interactions. But at pH 9.4 closer to pI, a nonmonotonic transition was discovered for SCN^- where the intermolecular interactions initially became more attractive and then less attractive when the phase transition temperature was measured (22). For γD-crystallins, a 20-kD protein, the same reverse Hofmeister series for anions was observed at pH 4.5 below its pI of ~7.0 by using SAXS (13).

Despite the dominant effect of the counter-ions (or anions), the co-ions (or cations) can still significantly perturb the protein-protein interactions. Specifically, comparing the effect by different cation in the salt solutions with the same anion, the intermolecular interactions for positive-charged lysozyme were less attractive and even perturbed nonmonotonically by the strongly hydrated divalent cation (Mg^{2+} and Ca^{2+}) , in comparison to the monotonic effect by the monovalent cations of Na^+ and K^+(12, 21). These findings are consistent with the findings from lysozyme solubility measurement in the multivalent cation salt solutions (12, 24).

2.4 Protein-protein interactions for a net negative-charged protein in salt solutions

Recently, many experiments were conducted to study protein-protein interactions for a net negatively-charged protein in salt solutions where a cation-dominant effect was expected. But the experimental findings were not straight-forward to interpret. Using SAXS and neutron scattering for studying protein-protein interactions of ovalbumin (MW=45 kD) in NaCl and YCl_3 solutions at pH conditions above its pI of 5.2, it was found that NaCl was ineffective in screening the electrostatic repulsive interactions between the proteins while YCl_3 not only suppressed the electrostatic repulsive interactions initially but also raised the repulsive interactions at higher concentrations (25). The ineffectiveness of Na^+ salts to screen the electrostatic repulsion was also confirmed for α-crystallins, a 800-kDa protein, at pH conditions above its pI of 4.5 by using SAXS (13). Similar behaviour was observed for BSA at pH conditions above its pI of 4.6 (26). Interestingly, Petsev et al found that NaAcetate was effective at screening the electrostatic repulsions (protein-protein interactions become more attractive) and then rendered the intermolecular interactions more repulsive for negatively-charged Apoferrtin (MW=450kD) (27).

2.5 Protein-protein interactions for an antibody at different pH conditions

Protein-protein interactions in salt solutions for an antibody with an experimentally determined pI of 7.2 were systematically explored through the measurements of protein solubility and phase transition temperature of $T_{critical}$ in liquid-liquid phase separation (11). The advantage of using this antibody is that the intermolecular interactions can be systemically assessed for the positive-charged and neutral for the same protein, allowing comprehensive experimental investigations of how salts modulate intermolecular interactions. Also, the antibody (MW=147 kD) is a much larger protein than lysozyme, which provides an opportunity for evaluating the surface charge density as a variable in protein-protein interactions(10). These approaches could help us understand how salt ions interact with proteins of different size.

At pH 7.1 close to its pI of 7.2, antibody solubility measurement revealed a general salting-in effect by all the anions as shown in Figure 2. More importantly, the specific anion

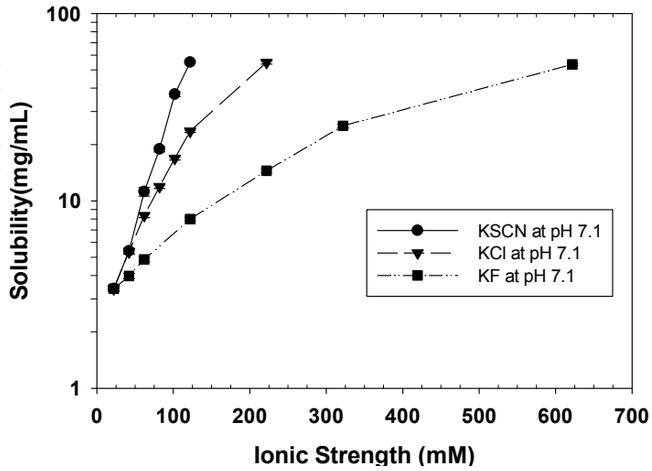


Fig. 2. Antibody solubility at pH 7.1 in KSCN, KCl and KF solutions [reprint with permission from ref (11)].

effect was observed in which SCN^- was the most effective at raising the antibody solubility, following the direct Hofmeister series. These observations are consistent with the ranking of these anions for disrupting the attractive intermolecular interactions as revealed by the results of $T_{critical}$ measurement (10).

At pH 5.3 below its pI, nonmonotonic behavior where protein solubility decreased and then increased with salt concentrations (in Figure 3) was observed for all the salts studied, suggesting that intermolecular interactions became less repulsive and then more. In addition, the effectiveness of the anions for reducing the protein solubility followed the reverse Hofmeister series, in which SCN^- was the most effective at reducing the antibody solubility. Then strikingly, the effectiveness for the anion to increase the protein solubility reverted back to the direct Hofmeister series as the salt concentration further increased. The above nonmonotonic transitions are in agreement with the protein-protein interactions pattern revealed by the measurement of $T_{critical}$ for liquid-liquid phase separation in the same salt solutions (10).

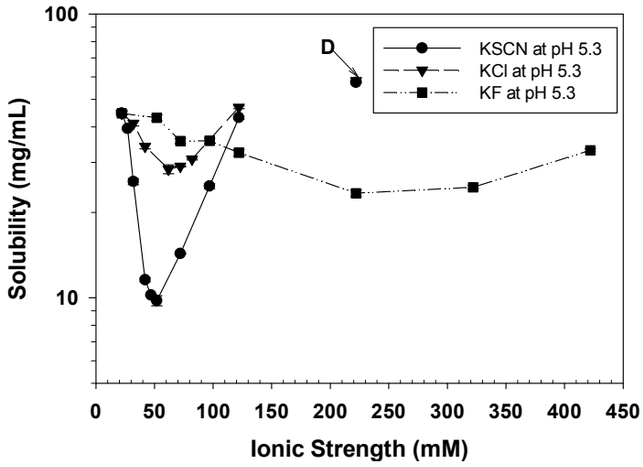


Fig. 3. Antibody solubility at pH 5.3 in in KSCN, KCl and KF solutions [reprint with permission from ref (11)].

It should be interesting to further study how salts affect the antibody solubility at pH values above its pI. Currently, experiments are on-going to do that.

3. Some theoretical explanations for protein-protein interactions in salt solutions

Recently Curtis and Lue wrote a comprehensive review of different theoretical treatments for understanding protein-protein interactions in salt solutions, pointing out that there is no single unified theoretical framework to rationalize the specificity of salt ion effects on protein intermolecular interactions (14). One of the important theories is the DLVO theory, in which proteins are treated as colloidal particles because their sizes are in the nanometer

range (9). The DLVO theory was named after the scientists: Derjaguin and Landau, and Verwey and Overbeek (9). This theory lays the foundation for explaining the interparticle electrostatic interactions in low salt concentrations below 0.1 M in the most simplified way when the protein is net-charged. Specifically, the intermolecular interactions between two protein molecules in low salt concentrations can be described by the following equation (28):

$$w_2(r) = w_{ex}(r) + w_{disp}(r) + w_{elec}(r) \quad (1)$$

Where r is the center-to-center distance from two molecules; w_{ex}(r) is the repulsive protein hard-sphere (excluded-volume) potential; w_{disp} (r) is the attractive dispersion potential; w_{elec} (r) is the electric double-layer repulsion potential, which can be further described by Debye-Huckel theory as the following:

$$w_{elec}(r) = \frac{(ze)^2 \exp[-\kappa (r-d)]}{4\pi r \varepsilon_0 \varepsilon_r (1+\frac{\kappa d}{2})^2} \quad \text{for } r > \sigma \quad (2)$$

Where ze is the net charge of a protein, e is the elementary charge, ε_0 is the dielectric permittivity of vacuum, ε_r is the dielectric constant of water, and κ is the inverse Debye length calculated by

$$\kappa^2 = \frac{2e^2 N_A I}{kT\varepsilon_0 \varepsilon_r} \quad (3)$$

Where I is the ionic strength of the solution, k is the Boltzmann's constant, T is the absolute temperature, and N_A is the Avogadro's number.

As presented in Equation 2, it is obvious that the more net charges a protein carries, the stronger the electrostatic double-layer repulsive force becomes. Also, Equation 2 indicates the addition of the salts monotonically decreases (or screens) the double-layer repulsion, and then reaches a plateau (the exponential term approach zero). The general screening effect is consistent with the initial drop in protein solubility and rise in liquid-liquid phase transition temperature as described above for the charged proteins. The DLVO theory was used to explain the protein solubility decrease of lysozyme (23). It should be pointed out that it is difficult to differentiate between the direct binding of salt ions to their opposite-charged partners on the protein surface and the screening by the salt-ion layer near the protein surface. The reason is that the first type of interaction decreases the double layer repulsion through balancing out the "ze" term in Equation 2 while the second type of interaction work through κ, the inverse Debye length. One of the major limitations of the DLVO theory is lack of ion-specificity as presented in Equation 2 and both cation and anion contribute equally as far as they have the same valency. Therefore, the DLVO theory cannot explain the anion-specific modulations on protein-protein interactions, i.e. the direct or reverse Hofmeister series at pH 5.3 for the antibody (3). In addition, the DLVO theory suggests that the double-layer repulsion decreases and levels off with salt addition, in contrary to the numerous nonmonotonic behavior mentioned above in Historical Background.

For a charge-neutral species (i.e. proteins at their pI), many other theoretical considerations were developed to explain the initial salting-in and later salting-out behavior (19, 29, 30). They

can be used to explain the general pattern of protein-protein interactions. In essence, the electrostatic interactions and hydrophobic interactions are the two major types of intermolecular forces (20, 31). The effects from the electrostatic interactions on the free energy of a protein in a low salt concentration solution may be described by Debye-Huckel theory in combination with Kirkwood's expression of the protein dipole moment as follows (20, 31):

$$\Delta G_{e.s.} = A - \frac{B(I^{1/2})}{1 + C(I^{1/2})} - DdI \tag{4}$$

Where A, B, C and D are constants, I is the ionic strength of the solution, d is the dipole moment for the protein. This theory predicts the salting-in effect: as the ionic strength increases, protein solubility rises. This idea is consistent with the observations of salting-in of proteins near pI. The main limitation of this theory is that it does not consider ion-specificity.

The free energy change for a protein involving the hydrophobic interactions may be illustrated by the cavity theory as follows(20):

$$\Delta G_{cav} = \left[N * Area + 4.8N^{1/3}(\kappa^e - 1)V^{2/3}\right]\left(\frac{\partial \sigma}{\partial m_3}\right)m_3 \tag{5}$$

where N is Avogadro's number, $Area$ is the surface area of a protein molecule, κ^e corrects the macroscopic surface tension of the solvent to molecular dimensions, V is the protein's molar volume, $\left(\frac{\partial \sigma}{\partial m3}\right)$ is the molal surface tension increment of the salt, and m_3 is the molality of the salt. This cavity theory describes how much free energy is needed to form a cavity in the solution to accommodate a hydrophobic protein molecule. Therefore, the surface tension of the solution is an important parameter and its modulation by salts impacts protein solubility and therefore protein-protein interactions. It predicts that the addition of kosmotropic salts, which increase the solution surface tension, will result in the salting-out effect and effectively strengthening of attractive protein-protein interactions. Therefore, these salting-in and salting-out effects in combination modulate protein solubility and protein-protein interactions in salt solutions (20, 31). Specifically, near the pI the salting-in effect dominates initially (protein solubility increases) and the addition of salts disrupts attractive protein-protein interactions. Then, further increase in (kosmotropic) salt concentration results in strengthening attractive protein-protein interactions as the salting-out effect begins to dominate (protein solubility decreases).

4. Molecular mechanism for protein-ion interactions

The simple ions shown in Figure 1 have different sizes, diverse hydration properties and polarizabilities (32). The interaction strength between an ion and water molecule in comparison to that between water-water determine the ion hydration property: an ion is strongly hydrated when it interacts with water molecules more strongly than the water-water interaction while the opposite makes an ion less hydrated (33-36). Shown in Figure 4 is the ranking of hydration property for the selected salt ions. Specifically, the large and more polarizable anion, i.e. SCN^-, is less hydrated while the small and less polarizable anion, i.e. F^-, is strongly hydrated.

The law of matching water affinities is the hallmark theory for defining the interaction strength between salt ions and proteins thermodynamically, in which the hydration and size properties of the ions and their counterparts on the protein surface are the key for explaining the protein-protein interaction behavior (33-36). Specifically according to the law of matching water affinities, oppositely charged ions in solutions form inner sphere ion pairs spontaneously when they have similar water affinities (36).

The chemistry of protein surface is heterogeneous, composed of both positive and negative-charged residues, and polar and nonpolar groups. As shown in Figure 4, monovalent anions of SCN^- and halides, except F^-, were weakly hydrated because of their large size, in comparison to the small-size monovalent cations being reasonably hydrated. On the protein surface, the positive-charged side chains on Arg, Lys and His are all derivatives of ammonium and therefore they are all weakly hydrated, matching well with the weakly hydrated SCN^-. According to the law of matching water affinity, the weakly hydrated anions, such as SCN^-, have the strongest interactions with the positive-charged side chains from the protein and neutralize them, followed by CI^- and F^-. On the other hand, the negative-charged side chains from Asp and Glu are strongly hydrated carboxylate, mismatching with Na^+ and K^+ whose interaction strengths are similar to that between water molecules (33-36). To the contrary, the divalent cation, i.e. Mg^{2+}, interacts with water molecules more strongly than Na^+ and K^+ and is strongly hydrated. It is then expected that the divalent cation interacts with the carboxylate more strongly than both Na^+ and K^+.

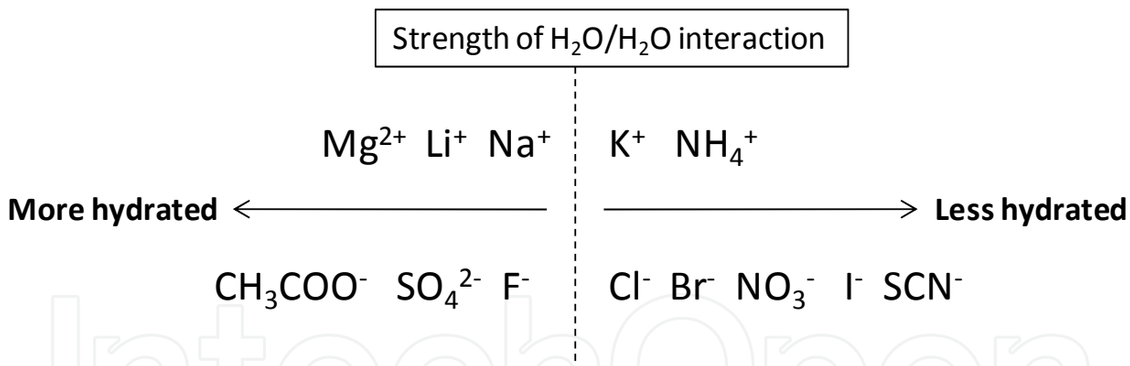


Fig. 4. Hydration properties of selected salt ions (34, 36).

Protein surface is composed of not only polar functional groups from the amide bonds of the exposed peptide backbone and the side chains of Asn and Gln, but also non-polar functional groups from the side chains of Phe, Ile and other amino acids. Both the polar and non-polar groups can be considered as weakly hydrated (37). Collins proposed that the weakly hydrated anions could also interact with both of the groups, besides the charged side chains (33-36). Recently, it was demonstrated, through a molecular dynamics (MD) study of lysozyme in a mixed aqueous solution of potassium chloride and iodide (0.4 M), that weakly hydrated anions, i.e. I^-, preferred to interact with the nonpolar groups besides the positive-charged residues on lysozyme (38). Furthermore, the interaction between

weakly hydrated anions and the amide bonds was also proposed based on the solubility study on poly(N-isopropylacrylamide) in salt solutions (39). For cations, it has been shown that both Ca^{2+} and Mg^{2+} can interact strongly with proteins through the diopolar amide bond (40) (18, 41).

The electroselectivity theory deserves attention when considering salt ion-protein interactions. Developed based on the anions' affinity for the anion exchanger, the electroselectivity theory proposed, purely based on the electrostatic interaction, that the ions with higher valency, such as SO_4^{2-} , interact with the positive-charge residues on the protein surface more strongly than those with a single valence, such as SCN^-(42, 43). The strong electrostatic interactions imparted by SO_4^{2-} were recently demonstrated by exploring specific ion effects on interfacial water structure adjacent to a bovine serum albumin at pH conditions below its pI using vibrational sum frequency spectroscopy (VSFS) (44).

5. From protein-ion interactions to protein-protein interactions

The complexity of protein-protein interactions as modulated by salt ions at low concentrations might be explained from the framework of dominance of specific electrostatic interactions from both cation and anions for the protein surface, concomitantly considering the following biophysical properties including net charge, surface charge density and hydration of a protein, and hydration, size, polarizability and valency of salt ions.

The first key property is the macroscopic net charge (considering the protein as a particle) as modulated by pH. First, a protein is net charge neutral, positively-charged and negatively-charged at pH near, below, or above its pI, respectively. Furthermore, patches of protein surface could be macroscopically weakly-hydrated because of the abundantly exposed nonpolar and polar groups, regardless of whether a protein surface is overall hydrophobic or hydrophilic. It was pointed out that in general 1/3 of the protein surface is hydrophobic, resulting in a partially weakly-hydrated surface(45). Although the net charge of the protein is dictated by the solution pH, its nonpolar or polar surface might maintain its property of weak hydration when the native folding structure is not drastically affected by pH and low salt concentrations. As pH decreases below its pI, the increasingly net positive-charges, from the weakly hydrated side chains of Arg, His and Lys, might render the protein surface even more weakly hydrated. At pH above its pI, the strongly hydrated carboxylates, from the strongly hydrated side chains of Asp and Glu, bring more water onto the protein surface, which results in the surface becoming more hydrated.

5.1 pH near pI

A protein is net charge neutral at pI with the equal number of positive and negative-charged residues. Therefore the protein molecules may approach each other and fully explore complementary interaction configurations (46). It is well-known that a protein has the lowest solubility near its pI and easily precipitates, suggesting the presence of strong intermolecular attractive interactions. The interactions can be highly anisotropic due to ionic-pair interactions, cation-π interaction, hydrophobic interaction and others types of interactions. It is difficult to dissect which type of interaction contributes most to the intermolecular interactions, which might be sequence dependent and protein-specific.

Our previous experiment of antibody liquid-liquid phase separation near its pI suggests that the intermolecular interactions were attractive and sensitive to salts, indicating that there were electrostatic interactions between the antibodies. Our observations of the general salting-in trends in the solubility measurement and disruption of intermolecular electrostatic attractive interactions in the LLPS are in agreement of the solubility data at low salt concentrations for other proteins near their respective pI, i.e. carboxyhemoglobin (47). The idea of attractive electrostatic interactions is especially supported by the salting-in behavior near its pI by KF. Typically, KF only salts out neutral peptides without charged side chains and nonpolar small molecules (16, 17). The general salting-in trend is also consistent with the electrostatic interaction theory as described by Equation 4. However, this theory cannot explain the ranking of the anion's effectiveness for raising the antibody solubility.

In the monovalent K^+ salt solutions, K^+ does not match well with the strongly hydrated carboxylate as discussed above. In contrast, the water affinity of the weakly hydrated positive-charge side chains, polar and nonpolar groups match well with those weakly hydrated anions from SCN^- to Cl^-. It is then expected that K^+ interacts with protein surface fairly weakly and anion could specifically binds to the protein surface in which their specificities are determined by their binding constants for the protein. This idea is consistent with the specific anion's effect, as described by a direct Hofmeister series, of raising the antibody solubility and disruption of the intermolecular attractive interactions at pH 7.1. In addition, this idea is in agreement with the recent findings where a chaotropic monovalent anion bound more strongly to a net-charge neutral macromolecule, like BSA near its pI and polar Poly-(N-isopropylacrylamide), than a kosmotropic monovalent anion(44) (48).

On the other hand, strongly hydrated multivalent cation, such as Mg^{2+} and Ca^{2+}, could bind to the strongly-hydrated carboxylate. In addition, there are strong interactions between the amide bond and multivalent cation (17). The above two modes of binding could make multivalent cations strong salting-in reagents (just like the anions) at low salt concentrations, overshadowing the possible salt-outing of the nonpolar residues on a protein by the multivalent cations.

In short, the electrostatic attractive interactions may dominate at protein-protein interactions in low salt solutions at pH near its pI, where the binding strengths between the protein surface for both cation and anions, working in synergy, determines the salting-in effectiveness of the salts as they are initially added.

5.2 pH below pI

When a protein is net charged at pH above and below its pI, the aforementioned observations of protein-protein interactions initially becoming more attractive or drop in protein solubility suggest that (i) the electrostatic repulsion dominates the protein-protein interactions and (ii) the initial addition of the salts to a charged protein effectively neutralizes the net charge of the protein and reduces the electrostatic repulsion.

Below pI, the positive-charges on proteins are from the weakly hydrated side chains of Arg, Lys or His. In addition, polar and nonpolar sites on the protein surface are also

weakly hydrated. As results, the more weakly hydrated a monovalent anion is, the more strongly it interacts with the positive-charged protein, and the more effectively it neutralizes the protein's net charge. The monovalent anions then follow the reverse Hofmeister series for their effectiveness of weakening the electrostatic repulsive intermolecular interactions and decreasing the protein solubility. This idea is consistent with the solubility measurement and phase transition data for both lysozyme and the antibody. The ranking for the binding strength between the anions and this antibody is also in agreement with what has been observed in monovalent salt solutions for other positive-charged proteins including other antibodies, BSA and lysozyme(49) (44) (22, 50). The binding of SO_4^{2-} to the positive-charged lysozyme and BSA, consistent with the electroselectivity theory, provides convincing experimental evidence that there is strong electrostatic interaction between a positive-charged protein and divalent anions, despite the mismatching water affinity.

The competitive interactions of co-ions against the counter-ions for a positive-charged protein become apparent for the strongly hydrated multivalent cation, i.e. Mg^{2+}. For example, Mg^{2+} may interact strongly at the strongly hydrated carboxylate or peptide groups in comparisons to Na^+ and K^+, effectively raising the positive-charges of the protein and hindering the anion's charge neutralization effect. Then, it appears that $MgCl_2$ will be less effective at weakening the electrostatic repulsive interactions and decreasing the protein solubility than NaCl (with the same molar concentration of Cl^-). Therefore, the protein-protein interactions are expected to be more repulsive in the $MgCl_2$ solutions than in the NaCl solutions, following the direct Hofmeister series. This notion is in agreement with the measurement of the phase transition temperature for lysozyme(21). Similarly, solubility of lysozyme in multivalent cation salt solutions was higher than that in the monovalent cation salt solutions with the same anion(24).

When anions complete their charge neutralization process as suggested by the minimum of protein solubility in Figure 3, the protein can be considered as pseudo charge-neutral. The salt's effect on protein-protein interactions then is expected to follow the direct Hofmeister series, as described above for a protein near its pI. This is the reason for why we observed the nonmonotonic behavior in the aforementioned proteins at pH below their pI.

5.3 pH above pI

On the other hand, at pH above its pI, the protein is negatively charged. Although the net negative charges are from the strongly hydrated carboxylate side chains on Asp and Glu, its surface still has significant presence of polar and nonpolar residues, attracting weakly hydrated anions. It is anticipated that the competitive bindings of cation and anion for protein surface determine the final effect on protein-protein interactions and solubility. The counterions with strong electrostatic interactions with the proteins, i.e. multivalent cations, can neutralize the net charge, weaken the repulsive electrostatic intermolecular interactions and decrease the protein solubility more effectively than the monovalent cations of Na^+, following the reverse Hofmeister series. Furthermore, in the Na^+ salt solutions, the anion's binding to the weakly hydrated sites, possibly stronger than that between Na^+ and the

carboxylate, may effectively increase the repulsive interactions. This is consistent with the experimental observation of the experimental findings for protein-protein interactions of ovalbumin in NaCl and YCl_3 solutions at pH conditions above its pI. Specifically, in the NaCl solution Cl^-'s binding to ovalbumin preempted that of Na^+, effectively raising the intermolecular repulsive interactions. On the other hand, the trivalent Y^{3+} could bind to the carboxylate strongly, neutralize the net negative-charges and weaken the repulsive intermolecular interactions. After charge neutralization, the salting-in effect by YCl_3 followed.

However, when either strongly hydrated F^- or acetate was used, they mismatched for both the positive-charged side chains and weakly hydrated polar and nonpolar residues on the net negative-charged protein surface. Possibly, Na^+ now might interact with the protein stronger than F^- or acetate and neutralize the negative charges. This could be a reasonable explanation for the nonmonotonic behavior mentioned for Apoferrtin in NaAcetate solution, but not in the NaCl solution.

5.4 Surface charge density

The surface charge density of a protein could dramatically change the above nonmonotonic behavior. At pH close to the pI or a large-size protein with small number of either positive or negative net charges, where the surface charge density is low, only the monotonic salting-in behavior could be observed because the charge neutralization process is less dramatic. On the other hand, when a protein has high surface charge density due to either a small size or a large number of positive charges, the anions might not completely neutralize the positive charges even at molar concentration and therefore only a decrease in protein solubility can occur. As a matter of fact, this might be for the case of lysozyme solubility at pH 4 and 7, especially when a weak chaotropic anion, i.e. Cl^-, was used(22). The reason is that Cl^- could bind to the protein surface less strongly and effectively at weakening the electrostatic repulsive interactions than a strong chaotropic anion, such as SCN^-. But at pH 9.4 where the surface charge density was smaller than at pH 4 and 7, the weakly hydrated SCN^- could neutralize the net charges completely, and as a result the nonmonotonic behavior appeared.

As proteins transition from a high surface charge density system to low, the interaction between a co-ion and charged surface could be explained through the smeared surface charge model and discrete surface charge model, respectively. In a low surface charge density system (discrete charge surface), such as a large-size antibody, the co-ion binding probably becomes more significant, in comparison to a small globular protein, i.e. lysozyme, of a high surface-charged density system. The reason is that the co-ion can approach the surface without experiencing the repulsive electrostatic force. This idea of co-ion adsorption to a low or medium negative-charged hydrophobic surface is supported by the recent molecular simulation for a self-assembled monolayer (51). The simulation results shows that even at a high surface charge density of -2.0×10^{-2} C/m^2, there was significant co-ion adsorption. Therefore, significant presence of co-ion adsorption is expected for a typical protein surface with a surface charge density in the low range of mC/m^2 (10, 52),.

5.5 Additional attractive interaction by polarizable anions

Another important feature of protein-protein interactions in salt solutions is the presence of possible additional protein-protein attractive force caused by the weakly hydrated anions for a positive-charged protein, although the exact mechanism remains to be defined. A recent Monte Carlo simulation reveals that the presence of chaotropic (or polarizable) ions, like SCN^-, introduced this additional interaction of dispersion force in nature between protein molecules (53). More importantly, liquid-liquid phase separation of the antibody at different pHs in a KSCN solution at a pH below its pI indicates that this attractive protein-protein interaction became stronger as the pH dropped and the protein carried more positive charges.

6. Conclusions

Despite the complexity of salt ion and protein interactions and their effects on protein-protein interactions, the rich salt-specific effect at low salt concentrations may be qualitatively explained based on the specific binding of both anions and cations for protein surface with heterogeneous surface chemistry as illustrated in Figure 5. In the future, it would be beneficial to have a quantitative description for the salt ions' effect on protein-protein interactions.

As shown in Figure 5, protein surface may always have hydrophobic patches, which are weakly hydrated and matches well with the weakly hydrated anions. Additionally, the exposed dipolar amide bond of the peptide backbone is the potential site for the divalent cation and weakly hydrated anions. Furthermore, pH change not only modulates the net charge property of the protein but also modifies the degree of surface hydration. Specifically, as the pH decreases away from their pIs, proteins become net positively-charged and even more weakly hydrated because the positive-charges are from the weakly hydrated side chains of Arg, Lys, and His. At pH values close to their pIs, proteins are net-charge neutral. Then as pH increases away from their pI, proteins become becomes net negatively-charged and less weakly hydrated because the negative charges are from strongly hydrated carboxylate from Asp and Glu.

At a pH close to the pI of a protein, both cations and anions can access the neutral protein and may work in synergy to disrupt the attractive intermolecular protein interactions and result an increase of protein solubility. On the other hand, they work competitive for a sufficiently charged protein (in Figure 5). Specifically, the counter-ion from the salt tends to neutralize the net charge of the protein, weakening the electrostatic repulsive intermolecular interactions while the co-ion is likely to hinder the charge-neutralization effect by the counter-ion, effectively strengthening the repulsive intermolecular interactions. The interaction strength between the ions and protein surface is dependent on both electrostatic and hydration properties for both ions and protein. The final outcome of protein-protein interactions is then determined by a combination of the protein surface charge density and the relative binding strength of both ions for the protein surface. When the counter-ions interact with the charge protein more strongly than the co-ions, the charge neutralization step dominates, resulting in protein-protein interactions becoming less repulsive, after which there could be the salting-in effect as if the protein-counter-ion complex is pseudo

charge-neutral. In the opposite situation, the strong interaction from the co-ions effectively renders the protein-protein interactions more repulsive.

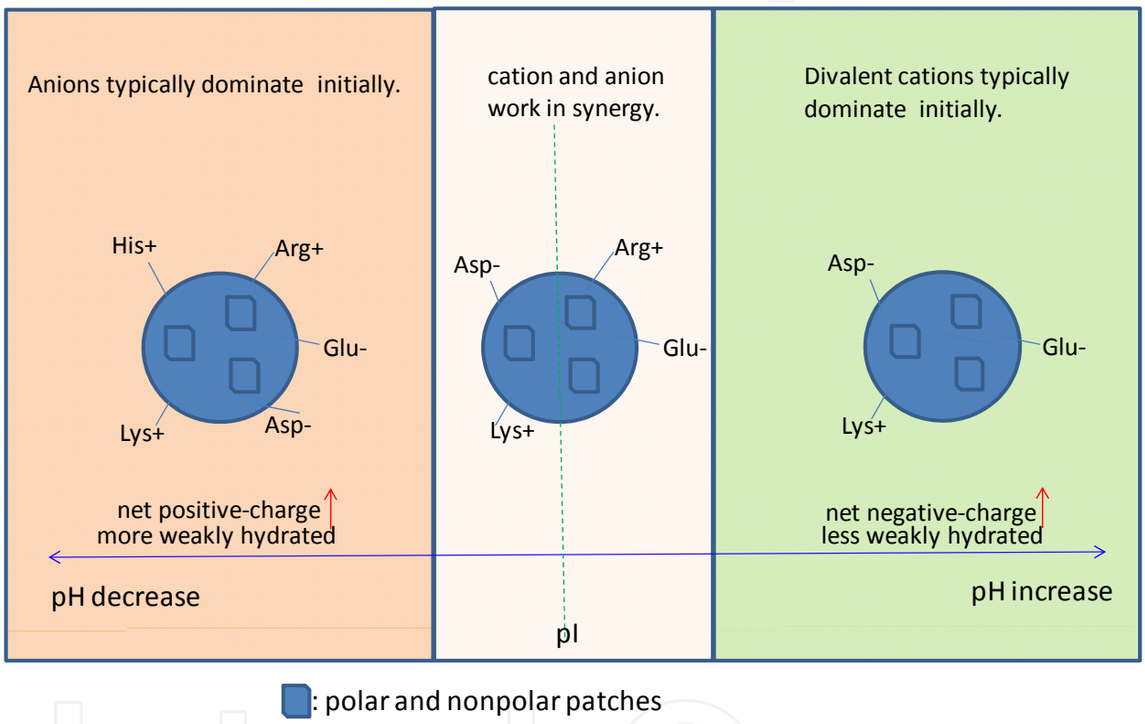


Fig. 5. Schematic illustration of the changes in net charge and hydration properties of a protein as pH varies.

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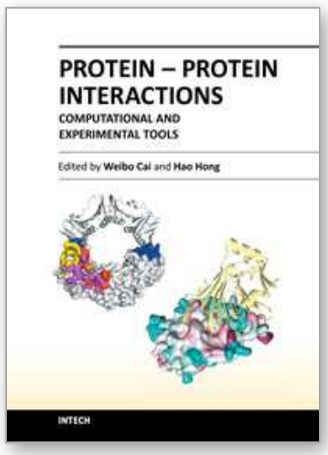
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Protein-Protein Interactions - Computational and Experimental Tools

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Proteins are indispensable players in virtually all biological events. The functions of proteins are coordinated through intricate regulatory networks of transient protein-protein interactions (PPIs). To predict and/or study PPIs, a wide variety of techniques have been developed over the last several decades. Many in vitro and in vivo assays have been implemented to explore the mechanism of these ubiquitous interactions. However, despite significant advances in these experimental approaches, many limitations exist such as false-positives/false-negatives, difficulty in obtaining crystal structures of proteins, challenges in the detection of transient PPI, among others. To overcome these limitations, many computational approaches have been developed which are becoming increasingly widely used to facilitate the investigation of PPIs. This book has gathered an ensemble of experts in the field, in 22 chapters, which have been broadly categorized into Computational Approaches, Experimental Approaches, and Others.

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