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Molecular level insight into intra-solvent interaction effects on protein stability and aggregation ☆

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ABSTRACT

Protein based therapeutics hold great promise in the treatment of human diseases and disorders and subsequently, they have become the fastest growing sector of new drugs being developed. Proteins are, however, inherently unstable and the degraded form can be quite harmful if administered to a patient. Of the various degradation pathways, aggregation is one of the most common and a cause for great concern. Aggregation suppressing additives have long been used to stabilize proteins, and they still remain the most viable option for combating this problem. Much work has been devoted toward investigating the behavior of commonly used additives and the resulting models give valuable insight toward explaining aggregation suppression. In a few cases, an explanation for unique behavior is lacking or new insight provides an alternate explanation. Additive selection and the development of better performing additives may benefit from a more refined understanding of how commonly used additives inhibit or enhance aggregation. In this review, we focus on recent molecular-level studies into how a select group of commonly used additives interact with proteins and subsequently influence aggregation. The intent of the review is not meant to be comprehensive for each additive but rather to provide new insights into additive–additive interactions, which may be contributing to protein–additive interactions. This is something that is often overlooked but yet essential to understanding the effect of additives on aggregation. The importance of understanding such interactions is clear when one considers that most formulations contain a mixture of cosolutes and that ideal stability might be better achieved through tuning intra-solvent interactions. We give an example of this when we describe how novel aggregation suppressing additives were developed from the knowledge gained from the reviewed studies.

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Abbreviations: aCgn, α-Chymotrypsinogen; Gdm, guanidinium; SAA, solvent accessible area; TMAO, trimethylamine oxide; VPO, vapor pressure osmometry.
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1. Introduction

Protein aggregation is probably the most common, least understood and most problematic form of protein degradation [1–3]. It occurs in almost every phase of development [4] and essentially all proteins and polypeptides are prone, to some degree, to the formation of non-native aggregates [5]. The presence of aggregates in an injected solution, even in small doses, poses a threat of an immune response that not only can reduce the efficacy of the product over time, but more importantly, has the potential to elicit adverse reactions [1–4,6]. Due to this, it is generally accepted that biopharmaceutical formulations must be substantially free of aggregates and their formation inhibited during storage [7]. This can be a difficult challenge to achieve, especially considering that high protein concentration formulations are often desired [8].With the past 25 years seeing an explosive growth in the number of protein based therapeutics developed, great interest has been sparked in developing improved formulation approaches for preventing aggregation [1,9].

It has long been known that the presence of small molecular weight species (e.g. sugars, polyols, salts, amino acids, etc.) can greatly influence the stability of a protein in solution [10–14]. Therefore, aggregation suppressing additives (often called cosolutes, cosolvents, and excipients) have long been utilized during production, purification, and formulation and they still remain the most viable option for dealing with this problem [3,12,15]. Most often, some type of sugar or polyol (e.g. sucrose, trehalose, glycerol, sorbitol, etc.), in combination with other components that influence protein stability (buffers, salts, surfactants, etc.), is used to inhibit aggregation [16]. However, no one formulation recipe works well for all proteins and other excipients have been used and/or are gaining more attention (e.g. amino acids, polymers, proteins, etc.) [17]. Furthermore, there is a desire to discover or create better performing excipients because for many proteins, a stable liquid formulation cannot be created. In such cases, the product must be lyophilized for storage and reconstituted prior to injection. Lyophilization, for the most part, is undesirable due to higher cost and the difficulty of application, not to mention that in some cases, producing a lyophilized product is a difficult challenge to achieve [1].

To discover or create better performing excipients, a better understanding of how commonly used additives inhibit aggregation needs to be established. Such information will also help to improve the methodology by which additives are selected because selection is almost always made via an ad hoc trial-and-error process using empirically derived heuristics. This can be a lengthy process that can delay the release of a product, resulting in a loss of potential sales. By incorporating detailed mechanistic understandings of various excipients with the known structure of a protein, ideal formulation recipes will more likely be developed in a timely manner and maybe predicted prior to the formulation phase. However, mechanistic inquiries tend to be narrow in focus in terms of the contributing factors that influence stability and often serve to promote previously proposed models. Here, we review recent molecular-level inquiries on a few select additives to highlight overlooked contributions to the suppression of protein aggregation. Most notably, but not exclusively, we take an interest in intra-solvent interactions (i.e. interactions between additive molecules), which are often ignored or not considered when studying protein–additive interactions. However, such interactions have been gaining more attention lately due to the availability of molecular dynamics simulations, which can give a detailed perspective on molecular interactions not obtainable from experimental procedures. The need to study such interactions is evident because proteins are seldom formulated with just a single cosolute and stability might be improved if intra-solvent interactions are tuned appropriately to enhance stabilizing effects.

2. General effects of solution additives

Though the topic is well established and has been reviewed many times before (see the collective work of Serge N. Timasheff, of which, references [13,18–31] are discussed in this review), it will be helpful to briefly address the concept of preferential interactions and the resulting general effects on proteins. This is because the most dominant influence an additive may have on the physical properties of a protein stem from whether the additive is attracted or repelled from the surface of the protein [13,18–20]. Moreover, this concept is fundamental in addressing protein–additive interactions on a molecular level [32]. In addition, as new insight is shed on the mechanisms of various aggregation suppressing additives, it is important to compare new results to what has generally been accepted as contributing factors in the stabilization. This is critical in developing a clear mechanistic picture because many current mechanistic explanations have been built on indirect evidence.

2.1. Preferential binding and exclusion

It is well established that if the concentration of an additive in the local domain around a protein differs from the concentration in the bulk solution, significant changes in the thermodynamic properties of the protein will arise that influence solubility [30] and conformational stability [13]. The most often used method to quantify such behavior is via measuring [33–37] or calculating [32,38] the preferential interaction coefficient, Γ_{23}, which is a measure of the preference a cosolute has for the protein surface [39–41] and is defined by the following expression,

$$\Gamma_{23} \equiv \left(\frac{\partial m_3}{\partial m_2}\right)_{T,P,\mu_3} = -\left(\frac{\partial \mu_2}{\partial \mu_3}\right)_{T,P,m_2}, \qquad (1)$$

where m, T, P and μ represent molal concentration, temperature, pressure, and chemical potential, respectively. The subscripts used indicate solution components in Scatchard notation: water (subscript 1), the protein (subscript 2), and the cosolute (subscript 3) [42]. Additives with a positive Γ_{23} are typically described as being preferentially bound to the protein surface due to an increase in the concentration of the cosolute in the local domain and this favorable interaction, as indicated by Eq. (1), lowers the chemical potential of the protein. The opposite is true for additives with a negative Γ_{23}, which are typically described as being preferentially excluded from the surface of the protein.

Preferential binding or exclusion can arise from either nonspecific interactions with the protein surface or interactions specific to individual amino acid residues, patches on the protein surface, or the peptide backbone [13]. Most notable of the nonspecific interactions are volume exclusion and perturbation of surface free

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energy. To elucidate, additives larger than water will tend to induce preferential hydration, which is equivalent to preferential exclusion, due to there being a solvation layer around the protein which is accessible to water but not the additive [21]. Furthermore, if the additive changes the surface tension of water, it will tend to be either depleted (higher surface tension) or accumulated (lower surface tension) at the protein–solvent interface in accordance to the Gibbs absorption isotherm given that the protein surface constitutes a liquid interface [22]. However, one is cautioned in drawing conclusions directly from surface tension measurements at an air–water interface given that air is a poor model for a protein surface [14].

Interactions specific to the nature of the protein surface may arise due to electrostatic interactions [43], hydrogen bonding [44], cation–π interactions with aromatic residues [45], hydrophobic interactions [44], and solvophobic effects [46]. The last category of interaction is often used to describe additives which enhance the repulsion between the solvent and hydrophobic residues due to how the additive is solvated by water. On a similar note, the term "kosmotrope" and the opposing term "chaotrope" have long been used to describe how additive change protein–water interactions [46,47]. Such interactions are often referred to as indirect (as opposed to direct protein–additive interactions) given that the interaction arises from how the additive changes the bulk structure of water. This mechanism is often used to explain how certain additives can enhance or disrupt the hydrophobic effect that cause proteins to fold and hydrophobic particles to aggregate [46]. Moreover, the "kosmotrope"/"chaotrope" effect can be related directly to the degree an additive is hydrated by water (which is directly related to the charge density of an ion) [48,49]. This facilitates indirect preferential interactions due to how a solute partitions to or from bulk water in the presence of a nonpolar surface [50]. This phenomenon also dictates interactions between ions (e.g. kosmotropic cations tend to interact more strongly with kosmotropic anions), which influences how ions interact with ionic sites on proteins [51].

It should be noted that the long range "structure breaking" and "structure making" mechanism is heavily debated [52] and elucidating the exact mechanism is still an active area of research [53] because there is no clear answer to what extent an additive may change the structure of water [53–57]. Moreover, in the pursuit of answering this important question, it is often overlooked that direct protein–additive interactions [45,58,59] and interactions with water in the first hydration shell of a protein have become central in recent models explaining changes in protein stability [60].

2.2. Intra-solvent interactions

As pointed out above in Section 2.1, preferential interactions can arise from a number of varying interactions between the protein and additive. However, we would like to point out in this section, in prelude to the discussion later in the review, that intra-solvent interactions between additive molecules will influence the interaction with a protein. Additive–additive interactions is a general theme throughout this review, as the implications of such interactions have only recently come to light in determining the mechanism by which a variety of additives influence protein stability [54,61,62]. Of particular interest is the effect a counterion may play in the interaction with a protein [63,64]. Such interactions can interfere with direct interactions with a protein surface or influence how the additive molecules are solvated by water.

The Hofmeister Series [10] is a well-known and often cited empirical ranking of how commonly used ions influence protein solubility and stability. The behavior can be correlated with preferential interactions but it is still unclear what gives rise to the differing behavior between the ions [60]. Recent studies, though, have discovered that the extent of hydrogen bond formation between the cation and anion contribute to this behavior, suggesting that strong attractive interactions will lead to clustering and will inhibit an ion that would otherwise bind to a protein from making such an interaction [54]. This is of particular importance in explaining the behavior of guanidinium [64] (Section 3.5) and arginine salts [63] (Section 3.6). A strikingly similar behavior is also observed for nonionic additive mixtures, such as urea-TMAO [65] (Section 3.3), suggesting that the behavior of a particular additive will be influenced by other cosolutes if the formulation has multiple components.

2.3. Thermodynamic and kinetic effects

2.3.1. Conformational and colloidal stability

It can easily be shown and understood that preferential interactions will influence any reaction the protein may undergo if the extent of the preferential interaction differs between the product (P) and reactant (R) states, as described by the Wyman Linkage Function [66]:

$$-\left(\frac{\partial \Delta G^\circ}{\partial \mu_3}\right) = \left(\frac{\partial \ln K}{\partial \ln a_3}\right) = \Gamma_{23}^{P} - \Gamma_{23}^{R}. \quad (2)$$

The two main reactions of interest, unfolding and precipitation, result in changes in solvent accessible surface area (SAA), which is directly linked to the extent of preferential interactions [36], whether the additive is bound or excluded. As a result, if the nature of the interaction with the protein does not change, bound additives will tend to induce a state with the most surface area exposed (unfolded and dissolved), while excluded additives will tend to induce a state with the least amount of surface area exposed (folded and precipitated). Though the discussed trends are almost always the case for precipitation [30], it is only generally true for unfolding due to that the nature of the protein surface changes upon folding (i.e. hydrophobic groups are exposed and the density of charged residues on the protein surface is reduced, etc.) [13]. Therefore, other thermodynamic techniques which involve thermal unfolding (e.g. DSC, CD Spec., etc.) are required to confirm how a cosolute influences conformational stability.

Such effects play an important role in protein aggregation by two different means: conformational and colloidal stability [11,67]. The most dominant factor that causes protein aggregation is thought to be the reduced exposure of hydrophobic patches [68]. The underlying mechanism for this is entropic because when aggregated, the system entropy increases given that water molecules are no longer structured around the hydrophobic patches [69]. The unfolded or partially unfolded states are more prone to aggregation due the exposure of hydrophobic residues that are typically buried in the native state and the structure of a protein is not static, making it likely that the native state is in equilibrium with a number of different partially unfolded species [70]. Whether or not partial unfolding is the rate limiting step in aggregation, additives which tend to promote a compact, native state (e.g. sugars, polyols, etc.) will often inhibit aggregation by either slowing the unfolding step or reducing the equilibrium concentration of aggregation prone species [5]. On the other hand, if aggregation proceeds through a nucleated polymerization pathway, additives which increase protein solubility may inhibit aggregation by shifting the nucleation equilibrium toward the dissociated state [71]. And lastly, disrupting attractive protein–protein interactions, or making proteins more colloidally stable, is often discussed as a major contributing factor towards the inhibition of aggregation [11,67]. This is something that is not always well captured by the overall preferential interaction coefficient, thus the need to study osmotic second virial coefficients and to perform MD simulations to elucidate molecular level interactions.

2.3.2. Gap effect

While the thermodynamic effects preferential interactions have on the solubility and conformational stability of a protein

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have long been established, up until recently, it was unclear how such interactions directly influence the rate of protein association. Baynes and Trout [72] investigated this through computational methods and developed a model which incorporates additive size and preferential interactions into the relative rate at which two protein molecules associate. This coarse grain model gives insight into protein processes which involve association, such as non-native and native aggregation, reversible association, precipitation, etc. The model is consistent with depletion forces, a well-established aspect of colloid science [73,74]. This because for a given additive size, the model predicts that the more excluded the additive, the more it enhances association. Such a consequence is a fundamental phenomenon in crowded media [75] and something often observed for large additives, such as PEG and dextrin [76,77]. Likewise, bound additives were predicted to inhibit association in relation to the degree of preferential binding, consistent with how denaturants increase solubility and stabilize unfolded proteins against aggregating.

The key observation, though, was that as the additive size was increased while holding the preferential interaction constant, the rate of association decreased by several orders of magnitude for all types of additives via a "Gap Effect" mechanism (see Fig. 1, which shows a drawing based on the original results). That is, as two protein molecules associated, a gap formed in which the additive was too large to penetrate but still large enough for water, thus leading to an increase in the free energy of the encounter complex due to the exclusion of additives from this gap. This effect, of course, was more pronounced for larger additives, due to an increase in the size of the gap, and for bound additives, which have a greater affinity to populate the local domain of the protein. An important consequence of this phenomenon, though, is that additives which are neither preferentially bound nor excluded are predicted to slow association if they are much larger than water, thus exerting a purely kinetic effect on protein association. Such a hypothetical molecule is referred to as a "neutral crowder" and is comparable to the behavior of arginine [78], a unique additive with such behavior.

It should be pointed out for clarity that the "Gap Effect" model is fundamentally different from how depletion forces arise when large colloidal particles (e.g. proteins) are immersed in a fluid of smaller colloidal particles (i.e. "hard sphere" additives). In such a case, only steric exclusion interactions exist (i.e. $\Gamma_{23} = \Gamma_{23}^{\text{Steric Exclusion}} \cong -C_3V_s$, where C_3 is the molar concentration of the additive and V_s is the volume of the excluded shell) and thus, when the excluded volume shells overlap when two proteins approach each other, a gap of pure water forms at a separation distance less than the diameter of the "hard sphere" additive. This essentially creates an osmotic pressure force on the two protein molecules, forcing them together and thus, enhancing the rate of association (see Fig. 1, which includes a prediction for "Hard Sphere" additives, showing that the Baynes and Trout model incorporates depletion force effects). This phenomenon is entropic in nature since the association of the protein molecules decreases the volume of exclusion, giving the "hard sphere" additives more volume to solvate. However, the case when $\Gamma_{23} > \Gamma_{23}^{\text{Steric Exclusion}}$ (all values below the "Hard Sphere Additive" curve in Fig. 1) implies that attractive interactions exist between the protein and the additive that counteract the steric exclusion. Since large additives cannot penetrate the excluded volume shell, such attractive interactions increase the concentration of the additive above that of the bulk concentration in the solvation layers immediately outside the excluded volume region (observed as peaks in radial distribution functions). Such an increase in concentration in the solvation layers around the protein will obviously counteract the depletion effect because of the loss of this more concentrated volume when two protein molecules associate. In other words, as Γ_{23} increases for a given additive size, there is an ever increasing concentration difference between the solvation layers and the gap of pure water, an entropically unfavorable condition. At some point this exclusion will exceed the depletion force effect and the additive will then inhibit association, giving rise to the "Gap Effect" mechanism described by Baynes and Trout. As shown in Fig. 1, for large additives, this occurs at Γ_{23} values well below zero, making it possible for additives with a $\Gamma_{23} = 0$ capable of inhibiting association.

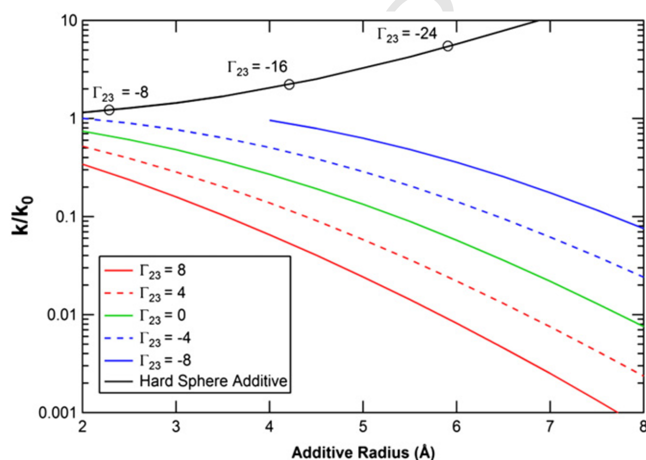

Fig. 1. "Gap Effect" predictions of the change in the rate of protein association, shown as the relative rate constant, as a function of additive size and preferential interaction. The model was applied to the association of two spherical protein molecules with a radius of 20 Å in the presence of a 1 M solution of spherical additives. The plot also includes predictions using a "hard sphere" potential, which serves as a limit for enhanced association via a depletion force resulting from preferential exclusion.

The "Gap Effect" mechanism has been used to describe the influence arginine has on the reversible association and aggregation of proteins [37,63,78–80]. As will be discussed later in Section 3.6, arginine does not change the folding equilibrium for most proteins, thus it likely suppresses aggregation by inhibiting attractive protein–protein interactions. Furthermore, using the current understanding of excipient behavior, it is unclear how arginine can enhance protein solubility but at the same time, not destabilize protein conformation. In an initial experimental study, Baynes and Trout were able to show that the magnitude of the effects of arginine hydrochloride on protein association, dissociation, and aggregation were quantitatively consistent with "Gap Effect" predictions [78]. For example, for the reversible association of insulin to anti-insulin, the "Gap Effect" predicts the association rate constant to be reduced by a factor in the range of 0.10–0.51 and the experimentally observed value was shown to be 0.27. At the time, there was limited data on the preferential interaction behavior of arginine. More experimental measurements have since been conducted [37,63], which show a complex behavior with concentration (neutral at low concentrations but excluded at high concentrations). This has been characterized as the result of arginine–arginine interactions [63,79,80], which have been taken into consideration into the "Gap Effect" mechanism [79].

3. Additives of interest

Now that the general effects of solution additives have been discussed, the remainder of the review will focus on the current understanding of the mechanisms by which a select group of commonly used additives affect protein stability (see Table 1 for an overview of these additives). Of course, these additives have been the subject of numerous reviews and research articles over the past three decades. The intent of this section is to report recent mechanistic insights obtained from a molecular interactions perspective. In particular, we have focused on the effects of additive–additive and direct protein–additive interactions, which up to this point have not been considered heavily in the pursuit of understanding how

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Table 1
Overview of additives reviewed.

Class of additive	Examples reviewed	Action	Source of action
Polyols	Glycerol	Conformational stabilizer, association suppressor	Steric exclusion, repulsive electrostatic interactions, binding to hydrophobic patches
Sugars	Trehalose	Conformational stabilizer	Steric exclusion, cohesive force, enhanced intra-protein interactions
	Sucrose		resulting from clustering
Denaturants	Urea	Conformational destabilizer, solubilizer	Preferential binding from hydrogen bond and hydrophobic interactions
	Guanidinium chloride		
	2:1 Urea–TMAO mixture	No influence on conformation	Intra-solvent interactions inhibiting preferential binding
	Guanidinium sulfate		
Amino acids	Proline	Refolding enhancer, solubilizer	Unclear, supramolecular assemblies disputed
	Arginine hydrochloride	Association suppressor	"Gap Effect", Arg–Arg attractive interactions
Arginine salts	Arginine sulfate	Conformational stabilizer, association suppressor	"Gap Effect", Arg–Arg and Arg–sulfate attractive interactions
	Arginine thiocyanate	Conformational destabilizer	Preferential binding of counterion
	1:1 Arg–Glu mixture	Association suppressor, solubilizer	"Gap Effect", Arg–Glu attractive interactions

additives inhibit aggregation. In a typical protein formulation, a variety of additives might be present because of the high cost of removing an additive added to the protein solution during processing. However, the presence of multiple additives in a formulation leads to a scenario where the interplay between multitudes of interactions determines the overall stability of the drug. Therefore, it is critical to understand the effect of a particular additive on all other molecular interactions in the formulation.

3.1. Glycerol

One of the most commonly used polyols for protein stabilization is glycerol [13,23], one of the smallest members of the polyol family. The mechanism of protein stabilization by this one type of polyol could potentially shed light on other members of the family. The solvophobic effect has long been the primary explanation for the mechanism of polyol induced protein stabilization because of the preferential hydration induced by glycerol despite the fact that it lowers the surface tension of water [13,23]. The solvophobic effect, as mentioned in Section 2.1, is a type of exclusion from the protein surface that is dependent on the nature of the protein surface. When the solvophobic effect occurs, the favorable interactions between water and polyols creates a scenario in which the interaction between hydrophobic patches and the solvent are more unfavorable than the interaction with pure water. As a result, the protein becomes preferentially hydrated. For polyols, this phenomenon is facilitated by the favorable interaction between water and the multiple hydroxyl groups found on such molecules. However, this mechanistic explanation was inferred from indirect evidence, particularly the lowering of the surface tension of water, which is not true for all polyols [81]. Also, this mechanism relies on the reduction of SAA of the protein to explain the enhanced protein stability. However, as mentioned in Section 2.3.1, the same mechanism would promote reversible protein association because of the smaller total SAA for the associated complex as compared to unassociated proteins. The enhancement by glycerol of both reversible protein association and protein assembly is true for some proteins [31,82,83] but not true for all [83–85] and therefore, the mechanism is more complex than once thought. Even though glycerol has been shown to increase the solubility of several proteins [86], and despite the need for a higher concentration of a salting-out agent, it is often used in protein crystallization [87]. This is because its ability to promote a compact native state facilitates the formation of crystals rather than amorphous precipitates, which often form if the protein structure is too flexible.

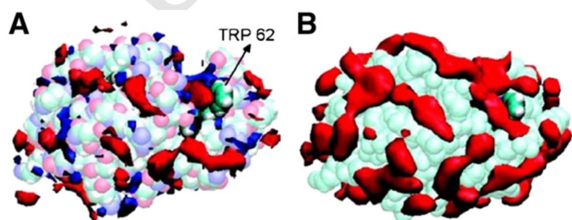

Fig. 2. Local concentration map showing preferential hydration (dark blue) and preferential binding of glycerol (dark red) for hen egg lysozyme (HEL) (A) and for a hydrophobic surface with the same topology as HEL (B). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
Image taken from ref [43] with permission.

Recently, Vagenende et al. [43,88] performed a detailed molecular dynamics study to obtain mechanistic insight into the effect of glycerol on protein stability. This study highlights the importance of electrostatic interactions with polar and charged groups on the protein surface in the exclusion of glycerol and the interaction of glycerol with hydrophobic patches on the protein surface. They showed that the overall preferential interaction coefficient, Γ_{23}, of the protein in a glycerol solution is mainly determined by the contribution from electrostatic interactions. These electrostatic interactions come from the interaction of glycerol with the polar groups (N and O atoms) on the protein surface. Furthermore, during the simulation, glycerol molecules tended to adopt a particular conformation around the polar sites such that only one of the outer oxygen atoms is hydrogen bonded to the protein and oriented perpendicularly to the surface. The excluded volume effect for this particular conformation is considerably larger as compared to randomly oriented glycerol molecules. Therefore, the protein compaction resulting from preferential exclusion is significantly enhanced due to this additional excluded volume contribution.

More importantly, the MD simulation predicted that glycerol would form an amphiphillic surface between contiguous hydrophobic patches found in depressions on the protein surface and water (see Fig. 2), which is in contrast to previous mechanistic explanations. Glycerol and other polyols have a carbon backbone with hydroxyl groups attached to each carbon. For a large hydrophobic patch found in a depression (Fig. 2b), glycerol was found to be in a conformation where the carbon backbone interacted with the hydrophobic patch and the hydroxyl groups faced and interacted with water molecules in the solvent. This phenomenon was not observed for all hydrophobic surfaces and was more pronounced for extended hydrophobic depressions with a limited number of nearby polar groups. Thus it was hypothesized that if the protein did partially unfold, glycerol could stabilize such a conformation against aggregating by favorably interacting with exposed hydrophobic depressions. These two effects could explain how glycerol inhibits protein aggregation, that is by enhancing the native state conformational stability and inhibiting the association of partially unfolded proteins.

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3.2. Sugars

Carbohydrates have been extensively studied both experimentally and computationally from a mechanistic viewpoint. Seminal work by Timasheff and coworkers on sugars has established enhanced hydration of protein due to the preferential exclusion of sugars as their primary stabilization mechanism [22,24]. Some recent studies have focused on sugar–water and sugar–sugar interactions to understand the molecular origins of the observed preferential exclusion and to investigate the role of specific protein–carbohydrate interactions in protein stability. Sugars can form hydrogen bonds with water and with other sugar molecules in solutions due to the presence of multiple hydroxyl groups. The interaction between sugars and water has been shown to affect the structural and dynamical properties of sugar solutions. In particular, trehalose and sucrose have been shown to significantly affect the water tetrahedral coordination and form extensive hydrogen bonded networks in solution as compared to other sugars [61,89,90]. Lerbret et al. [61] show that trehalose clusters in solution increase in size as a function of concentration due to the formation of cages that trap water molecules. All of these studies on intra-solvent interactions in sugar solutions show that sugars form clusters in solution but there are limited studies which link the observed clustering behavior to the molecular mechanism responsible for protein stabilization [90]. Simulations of a protein (lysozyme) in a trehalose solution show that trehalose–trehalose and trehalose–water interactions affect protein–water interactions [91]. There is a loss of trehalose–water and protein–water hydrogen bonds with an increase in trehalsoe–trehalsoe hydrogen bonds. The water-layer around a protein has different solvation properties due to the presence of surrounding trehalsoe molecules. The overall effect of trehalose self-association and trehalose–water interaction is the enhancement of intra-protein interactions at the expense of protein–solvent interactions.

The proposed stabilization mechanisms for sugars are mainly indirect and nonspecific in nature, though there are two direct protein–carbohydrate interactions which could play a role in protein stabilization. Carbohydrate–aliphatic interactions are similar to the previously mentioned interaction between glycerol and hydrophobic patches on protein surfaces. Therefore, we will not discuss them in detail here. The other direct interaction is between a carbohydrate and aromatic amino acids. This has been mainly studied from a viewpoint of carbohydrate recognition by proteins. The strength of carbohydrate–π interactions is found to be greater than the cation–π interaction between lysine and tryptophan amino acids [92]. It has been shown that phenyl rings preferentially interact with sugars as compared to the cyclohexyl group [93]. The interaction between carbohydrates and aromatic groups has been explained using the hydrophobic effect, CH–π interactions, etc. The α face of the carbohydrate interacts with the face of aromatic ring due to the presence of a partial positive charge on the hydrogen atoms pointing out of the α face. It is surprising that this interaction has never been investigated from a protein stabilization viewpoint. The possible reasons could be that carbohydrate–π interactions are strongly dependent on the nature of the sugar and the aromatic group. Therefore, it may not be a favorable interaction for commonly used sugars. However, the strength of carbohydrate–π interaction can be tuned by attaching an electron withdrawing group to a hydroxyl oxygen, which could be helpful in tuning the overall protein–carbohydrate interaction [93].

3.3. Urea

Even though urea is a widely used denaturant, understanding how the molecule forms attractive interactions with proteins will be useful in developing a complete understanding of protein–additive interactions. The mechanism of urea induced protein denaturation has been studied extensively from both theoretical and experimental viewpoints; however, no clear consensus has been established given that the mechanism is still actively debated [94]. The two basic lines of reasoning suggest that urea denatures proteins via either a direct (interaction of urea with the protein) or an indirect (effect of urea on water structure) mechanism, however, as new insights emerge, the two mechanisms are likely not mutually exclusive, though they are often treated as such [65,95]. The direct effect is gaining more attention as the main driving force for denaturation; however, the means by which this interaction arises is still disputed, with recent evidence suggesting stronger interactions with nonpolar groups rather than the often claimed hydrogen bonding to polar residues [44,96].

Given the circumstances of this ongoing debate, we cannot effectively give an exhaustive review of the urea mechanism. However, we would like to highlight an overlooked contribution along the lines of the other interactions highlighted in this review. It has been speculated for more than a half a century [97] and now widely accepted, that urea has a tendency to reversibly self-associate in solution [62,98]. Today, however, this phenomenon is not often taken into consideration in explaining how urea denatures proteins and the implications of urea self-association are far from clear. Recently, Stumpe and Grubmuller [99] showed that the association of urea molecules contributes to the indirect effect but more importantly, their results indicate how urea might be able to preferentially interact with apolar groups, contributing to the direct effect. Urea substitutes well for water, geometrically, in the hydrogen bond network but energetically, the strength of the hydrogen bonds are quite different. Water–water hydrogen bonds are stronger than water–urea or urea–urea hydrogen bonds. This difference in hydrogen–bond strength leads to urea self-interaction and strengthens the water structure, giving insight into how urea may interface between less polar residues and water. Other researchers have since incorporated urea association in mechanistic models that take into account both "indirect" and "direct" interactions, giving rise to the idea that urea association will induce concentration dependent behavior [100,101].

Along the lines of additive–additive interactions, the counteracting effect of trimethylamine N-oxide (TMAO) on urea induced protein denaturation provides a classic and naturally occurring example of the effect intra-solvent interactions have on protein stability [25,102]. Early studies suggested that the two osmolytes acted independently of each other [25]. Later on, MD simulations suggested that TMAO strengthens the urea–water interaction, thereby limiting urea–protein interactions by inhibiting urea–protein hydrogen bonds [103]. Recent studies indicate strong TMAO–urea interactions, with the TMAO–urea hydrogen bond stronger than the TMAO–water hydrogen bond, leading to the hypothesis that urea and water prefer to solvate TMAO, rather than the protein [65]. These studies reiterate the need to understand all possible interactions in aqueous protein–additive solutions.

3.4. Proline

The amino acid proline is another naturally occurring osmolyte that has been found to accumulate in certain organisms when stressed by low water and low temperature conditions [104,105], giving rise to research investigating its protective properties, specifically its role in protein stability. Proline has been shown to inhibit aggregation during refolding [106] and to inhibit the aggregation of an aggregation prone protein, not only in vitro but as it is expressed in vivo [107]. In addition to this, proline solutions exhibit other interesting properties, such as high solubility (the highest among amino acids), unusually high freezing point depression, anomalously high viscosity above a concentration of 3.5 M, and enhanced solubility of proteins and hydrophobic compounds at high concentrations [108].

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Contrary to the mechanistic inquiries of other additives, in which the role of additive–additive interactions gradually developed over time, it was immediately proposed that the unusual behavior of proline was due to its propensity to form semi-ordered supramolecular assemblies in solution [109,110]. The ring structure of proline gave rise to this idea, in which the pyrrolidine rings stacked on top of each other, creating an amphiphilic structure with a hydrophobic backbone and solvent exposed hydrophilic groups on the surface. Some indirect evidence from calorimetric [111], hydrophobic dye binding [106], and light scattering [106] data supported this idea, but no clear mechanistic explanation has been given for how such structures, if they exist, would provide the protective properties exhibited. Furthermore, recent reports (reviewed in ref [108]) have mounted evidence against the long-range ordering of proline and direct evidence of aggregate formation is lacking.

Recently, MD simulations [105,108,112,113], neutron diffraction [104,108], Raman spectroscopy [105], and Rayleigh–Brillouin light scattering [105] techniques were employed to verify the structure of proline in solution. Consistent among the studies is the absence of any structured clustering or ring–ring interactions, though some short-range structures were found via hydrogen bond formation. Diffuse or transient aggregates may exist, which would explain the light scattering results, but they are not observed by small angle neutron scattering. Therefore, the often proposed formation of stacked rings likely does not occur. Proline exhibits stabilizing behavior that could be utilized in formulations and a proline derivative, hydroxyproline, has been shown to be a more potent conformational stabilizer [114]. Molecular level inquires investigating protein–proline interactions are lacking and as is a complete analysis of the applications of proline. Such insight will be useful in understanding, utilizing, and improving upon the compounds nature has used to protect against aggregation.

3.5. Guanidinium

From a preferential interaction perspective, salts are typically treated as single components despite the presence of two or more ions in solution. The behavior of the cation and anion could differ widely, not only in terms of their interactions with the protein surface, but also in terms of their self-interaction. The role of intra-solvent interactions in protein–protein interactions is most obvious for the case of guanidinium (Gdm) salts [50,115]. GdmSCN and GdmCl are protein denaturants, whereas, $(Gdm)_2SO_4$ is effectively neutral in its effect on protein stability [26,54,64,116].

The effect of the ions on proteins have been explained in terms of the changes induced by these salts on the water structure, with sulfate salts labeled as kosmotropes (structure makers) and SCN salts labeled as chaotropes (structure breakers) [54]. However, changes in the water structure seems to be only limited to the first solvation layer around the ion and a relatively new picture of direct interactions of these ions with each other and with proteins has been gaining ground [57,60]. $(Gdm)_2SO_4$ has been shown to form mesoscopic clusters in solution and these clusters are formed due to the ability of Gdm and sulfate ions to form multiple hydrogen bonds with each other, which are stronger than the hydrogen bonds formed between ions and water [54]. GdmSCN shows a marked contrast in terms of ion pairing as compared to the sulfate salts, with limited or negligible interactions between Gdm and SCN ions [50,54]. The difference in the ion pairing behavior of these salts is likely a contributing factor in the reversal or enhancement of the denaturing ability of Gdm. For the sulfate salt, the binding of Gdm to the protein surface is limited due to the strong interaction between Gdm and sulfate ions, which make Gdm molecules unavailable for binding to the protein surface [64,117]. These conclusions were drawn based on not only molecular dynamics simulations but neutron scattering data of the Gdm salts as well. The presence of clusters could affect solution properties such as viscosity, protein diffusivity, etc., that could influence the rate of aggregation, however, the contribution from such changes have yet to be investigated.

The preferential interaction coefficient values at concentration of 1 M for BSA in the presence of Gdm salts show that the sulfate salt ($Gdm(SO_4)_{1/2}$) is excluded ($\Gamma_{23} = -8$) and the chloride salt is highly bound ($\Gamma_{23} = 18$) [26]. The contrasting clustering behavior for these salts provides a more realistic explanation of these observed preferential interaction values. Before, it was believed that the effects from each ion were additive and the net contribution was responsible for the behavior. To elaborate, for $(Gdm)_2SO_4$ and other Hofmeister salts, the cation and anion were thought to act independently of each other and the resulting preferential interaction coefficient and influence on stability was simply an average of the effect the two solutes imposed. Such an explanation is satisfactory for a mixture of uncharged solutes, however, for electrolytic solutions, such a scenario would result in an unfavorable charge separation for $(Gdm)_2SO_4$, with the Gdm molecules bound and the sulfate ions excluded [64]. From a molecular interaction perspective, cation–anion clustering would not result in such a charge separation and the interaction with sulfate would limit the hydrogen-bonding and cation–π interactions of Gdm group with the protein, thus eliminating its denaturing effect. It is likely that the intra-solvent interactions exhibited in the series of studies featuring Gdm extends to the whole Hofmeister Series, but to a lesser degree given that Gdm and sulfate are on the two ends of the spectrum of hydrogen bond donating and accepting ions, respectively [54].

Strong support for the clustering model over the previous model comes from a case in which a protein was found to have the unique behavior of being sensitive to GdmCl denaturation but insensitive to stabilization from alkali metal sulfates [64,117]. For this particular protein, $(Gdm)_2SO_4$ had no destabilizing effect, which leads to only one possible conclusion, that sulfate inhibited the binding of Gdm due to clustering because sulfate had no stabilizing effect on the protein. These results confirmed that ions could affect the self-interaction of each other and their interaction with the protein. For a binary salt solution, there are ten possible binary interactions in a protein–additive mixture with the possibility of each interaction affecting the other. However, as seen in the case of Gdm salts, only a few of these possible interactions play a dominant role in protein stability.

3.6. Arginine

The amino acid arginine is a fascinating case study in terms of the multitude of interactions it may form that influence how it interacts with proteins. To summarize, it (1) is large relative to water (volume exclusion), (2) increases the surface tension of the solution [28,29], (3) is a salt (chloride form most common), thus electrostatic and counterion interactions come into play (4) is zwitterionic, thus it has two other ionic charge locations, (5) has the hydrogen bond donating and protein denaturing functional group guanidinium, which allows it to interact favorably with the protein surface or hydrogen bond accepting groups, (6) has a hydrogen bond accepting carboxylate moiety, (7) has an amine group, another location for donating hydrogen bonds and (8) has a hydrophobic alkyl chain three carbons long. These interactions culminate in a number of ways but the most striking result has to be that Arg–Arg clusters (see Fig. 3) tend to form in solution, mainly due to the interaction between the guanidinium and carboxylate moieties.

3.6.1. Arginine HCl

L-Arginine HCl is an additive that is not only effective at inhibiting protein aggregation but has important applications in protein

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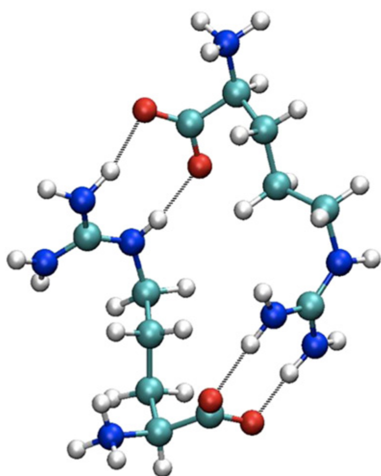

Fig. 3. MD simulation snapshot of two arginine molecules oriented in a head-to-tail fashion. Such an orientation allows for four hydrogen bonds to form between the two molecules and is a leading cause of arginine cluster formation.
Image taken from ref [79] with permission.

purification, refolding, etc. [29,118–123]. The most notable feature about L-Arginine HCl is that it does not alter the folding equilibrium of most proteins [29]. This behavior is in sharp contrast to the mechanism of sugars and polyols, which places it outside the typical class of additives used to inhibit aggregation. Several mechanisms have been proposed to explain the unique behavior of arginine (reviewed in refs [124] and [79]). However, no one theory has been generally accepted because most theories cannot explain all of the observed experimental behavior arginine exhibits.

"Gap Effect" theory [72], described in Section 2.3.2, provides a possible explanation of arginine's unique behavior by proposing that arginine exerts a purely kinetic effect on protein association. The magnitude of the observed aggregation suppression due to arginine was found to be consistent with the "Gap Effect" theory [78]. It was assumed, based on preferential interaction coefficient data available at the time of the study, that arginine is neutral in regards to preferential interaction, thus explaining the negligible effect on the free energy isolated protein molecules. However, recent experimental and theoretical preferential interaction coefficient data shows that arginine exhibits a more complex behavior, in that coefficient values go from being neutral at low concentrations to being highly excluded at high concentration [37,80]. Therefore, "Gap Effect" theory cannot explain all of the complex behavior exhibited by arginine, though the gap effect may contribute to the overall mechanistic picture of arginine.

The "Gap Effect" theory was an attempt to explain the behavior of arginine from a nonspecific interaction perspective. Several attempts have been made to explain the arginine mechanism from the viewpoint of specific interactions of chemical groups in arginine with protein residues [120,125]. It is ironic that the stabilizing behavior of arginine is generally attributed to the presence of a strongly destabilizing guanidinium group present on its side chain [126,127]. It is argued that the Gdm group interacts strongly with aromatic side chains based on fluorescence experiments and simulations [127]. Therefore, any additive, which could mask the exposed aromatic residues, would inhibit protein–protein interaction. This observation does not answer the question of why arginine does not denature proteins despite the presence of the Gdm group. Furthermore, the Gdm group does not interact strongly with aliphatic groups [45], which provide the main driving force for aggregation due to their higher frequency of occurrence as compared to aromatic residues.

Das et al. [128] proposed that the presence of the aliphatic $-(CH_2)_3-$ group in the middle of the arginine molecule could play a role in the arginine mechanism. In an L-Arginine hydrochloride crystal structure, the arginine molecules are hydrogen bonded to each other in a head-to-tail fashion with aliphatic carbon atoms aligned along a crystallographic axis [128]. It was proposed that the arginine molecules could form a similarly aligned hydrophobic surface in solution due to the self-interaction of multiple arginine molecules that could in return mask the exposed hydrophobic residues. It was an ingenious idea based on the self-interaction of arginine in crystals and other indirect evidence, such as hydrophobic dye binding and light scattering. However, similar to proline, molecular dynamics simulations of aqueous arginine solutions and proteins in arginine solution do not show the formation of such large hydrophobic surfaces [79,80]. The idea of interactions between a chain of methylene groups in a molecule and the hydrophobic groups on protein surface is similar to the interaction between the carbon backbone found on glycerol and the protein surface [43]. However, in the case of arginine, the presence of charged carboxyl and guanidinium groups located at opposite ends of the molecules make it difficult for the alkyl chain to interact with the protein while keeping the charged groups solvated. In the case of glycerol, the hydrophilic groups are located on one side of the molecule, which make the energetic cost of such alignment favorable.

The idea of arginine self-interaction and its implication on protein aggregation has been further explored using MD simulations [63,79,80]. Arginine molecules not only interact via head-to-tail hydrogen bonds (Fig. 3) but also via the unusual stacking of Gdm groups in solution [129]. The Gdm molecule is planar in structure with the faces of each plane poorly hydrated, promoting the molecules to stack on top of each other despite repulsive electrostatic interactions [50]. This stacking has been observed in aqueous GdmCl solutions [115], which such behavior providing a reasonable mechanism for how Gdm favorably interacts with hydrophobic residues, and between arginine residues located on protein–protein interfaces [129]. The extent of clustering can be quantified in terms of the loss of solvent accessible area of arginine molecules in solution as compared to the fully solvated arginine molecule [79]. Fig. 4 shows the loss of surface area, as well as the number of hydrogen bonds formed with α-Chymotrypsinogen (aCgn), as a function of arginine concentration. These results make it clear the importance of determining what the implications of arginine self-interaction are on protein–arginine and protein–protein interactions because arginine clustering seems to limit the number of hydrogen bonds formed with a protein.

Cluster formation is an obvious cause of preferential interaction coefficient values becoming excluded at higher concentrations and is

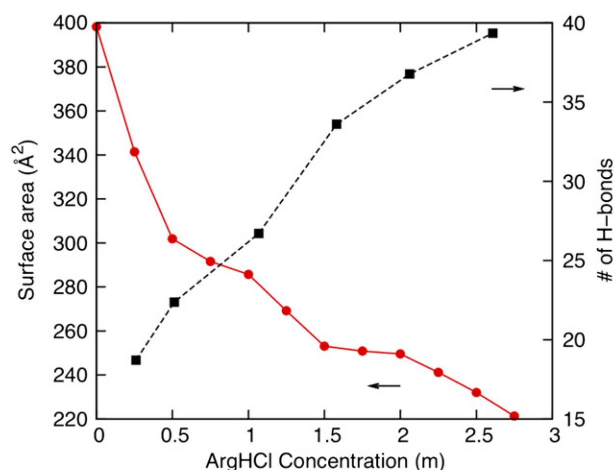

Fig. 4. Change in solvent accessible area (SAA) for arginine and the number of α-chymotrypsinogen–arginine hydrogen bonds versus arginine concentration. The plot shows that clustering reduces arginine solvent exposure and limits the number of hydrogen bonds with the protein.

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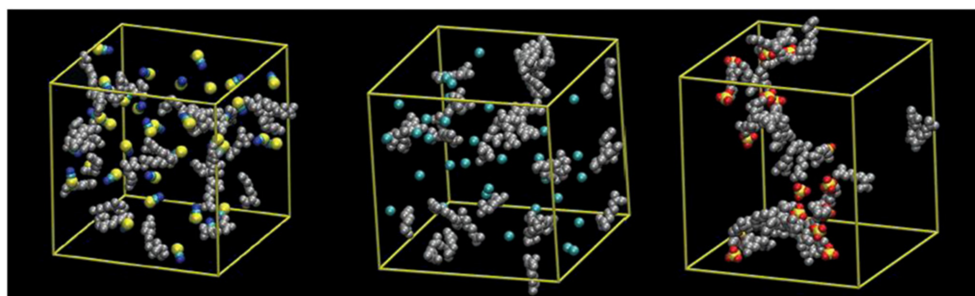

Fig. 5. MD simulation boxes of arginine salts; thiocyanate (left), chloride (center), and sulfate (right). Image taken from ref [63] with permission.

also a leading cause of the observed leveling off of aggregation suppression beyond a concentration of 0.5 M [37,63,79,80]. The formation of clusters in solution indicates that there is a competition between the protein surface and the bulk solution, which affects the partitioning of the arginine molecules. At higher concentrations, cluster formation is more extensive, resulting in more arginine molecules being partitioned to the bulk solution, resulting in increasingly negative Γ_{23} values and diminishing contributions to the "Gap Effect" [79,80]. The end result of this is that the formation of clusters reduces the binding of the Gdm group to the protein surface beyond what the volume exclusion contributes, which is a contributing factor in the aggregation suppression behavior of arginine despite the presence of the destabilizing Gdm group.

3.6.2. Other arginine salts

It is interesting, given what is empirically known about the stabilizing effect of Hofmeister anions, that little interest has been given toward other arginine salts. L-Arginine HCl has been studied so exclusively, that it is commonly referred to simply as arginine without confusion as to the type of salt [124]. Arginine molecules have only one hydrogen bond accepting group (carboxylate) which limits the extent of clustering. Therefore, clustering should be enhanced by introducing hydrogen bond accepting groups which could act as a bridge between arginine molecules. It was recently observed that arginine salts with anions that can accept hydrogen bonds have superior aggregation suppression abilities over arginine HCl [63]. Molecular dynamics simulations of these salts (arginine sulfate, citrate and dihydrogen phosphate salt) show enhanced clustering as compared to the chloride salt (Fig. 5). Other arginine salts, such as thiocyanate, chloride, and acetate, showed limited clustering in solution and were more on the lines of being randomly distributed. In that study, the sulfate and citrate salts, at a concentration of 0.5 M, reduced the rate of α-chymotrypsinogen aggregation at an elevated temperature to 2% of its original value, which is a significant improvement over the chloride salt, which reduces the rate to only 20% of the original value, while the thiocyanate, iodide and bromide salts enhanced the rate of aggregation. The reason for this observed change in aggregation rate is that the salts which formed extensive clusters were more excluded and thus favored a more compact folded state on top of the association suppression induced by arginine, while the ions that did not induce clustering were free to interact with the protein surface. With bromide, iodide, and thiocyanate capable of forming strong interactions with proteins, as compared to chloride, they favored a more unfolded state, which counteracted the aggregation suppression induced by arginine. These observations established a correlation between clustering and aggregation suppression and exhibit a need to understand ion–ion interactions in the pursuit of stabilizing proteins against aggregation.

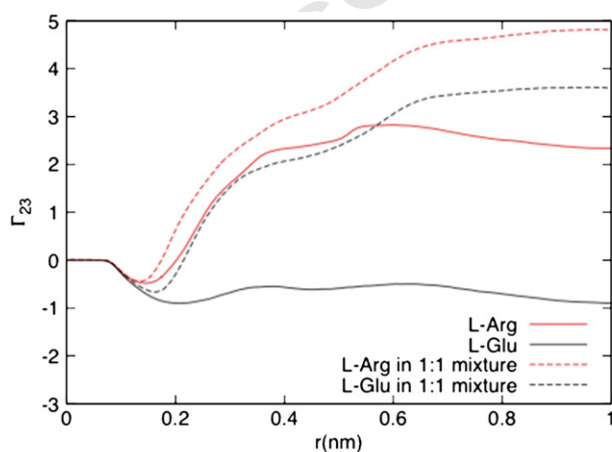

Fig. 6. Cumulative Preferential Interaction Coefficient values, as predicted by MD simulations, for WW34 protein in the presence of arginine, glutamate, and Arg + Glu solutions at a concentration of 100 mM for each cosolute. Image taken from ref [132] with permission.

3.6.3. Arginine–glutamate mixture

The addition of an equimolar mixture of the amino acids L-Arg and L-Glu to a protein solution has been shown to enhance the maximum achievable concentration of the protein by a factor of 8 [130]. A follow-up investigation by Valente et al. [131] showed that the combination of L-Arg and L-Glu is more effective at reducing intermolecular attraction between proteins (as measured by an increase in osmotic second virial coefficient, B_{22}, values) than either component by itself. It has been speculated that this synergistic effect is the result of arginine and glutamic acid interacting with oppositely charged residues on the protein surface and masking the surrounding exposed hydrophobic patches, thereby reducing protein–protein interactions [130]. Due to the presence of oppositely charged side chains in L-Arg and L-Glu, it is expected that these additives will interact with each other via charge-assisted hydrogen bonds in the solution. The hydrogen bond network between these additives is also observed in the Arg–Glu crystals. However, the reported synergistic enhancement of protein solubility was only investigated up to a maximum concentration of 50 mM for each component. At such low concentrations, there will be negligible clustering in solution. Therefore, the synergistic effect of Arg + Glu should be related to direct protein–additive interactions. We have performed molecular dynamics simulations of WW domains 3 and 4 from *Drosophila* Su(dx) protein in the presence of a Arg + Glu mixture and single component solutions (results to be published elsewhere [132]). It has been reported that the solubility of this protein is enhanced up to 4 times in equimolar mixture (50 mM for each component) [130]. It was found that in the equimolar mixture, the concentration of L-Arg and L-Glu on the protein surface is much higher than in either single component solution at the same concentration. Fig. 6 shows the preferential interaction coefficient values for L-Arg and L-Glu in equimolar

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mixtures and single component solutions calculated using the statistical mechanical method developed in our group (complete results to be published elsewhere) [38]. L-Glu is typically an excluded additive and therefore, it is expected that the presence of an excluded solute would reduce the local concentration of L-Arg on the protein surface, but the results are in opposition to this prediction [27,36]. The enhancement of the local concentration around protein in an equimolar mixture is likely due to the following reason. L-Glu has two carboxylate groups, therefore, when one of these groups forms a hydrogen bond with the protein surface, the other group acts as a negatively charged site for interaction with the L-Arg side chain. Similar behavior is also observed for when L-Arg binds to the protein surface. Therefore, the presence of one additive on the protein surface creates a favorable site for the interaction with the other additive. From a mechanistic viewpoint, it has to be established whether the local concentration enhancement is related to the synergistic effect on protein solubility. Arg + Glu mixtures not only have a higher local concentration around the protein but both the Arg and Glu contribute to the "Gap Effect" due to their larger size. For L-Arg, only the arginine cation contributes to the "Gap Effect". The association suppression due to the crowding of L-Arg and L-Glu on the protein surface cannot be directly related to the maximum achievable protein concentration reported by Golovanov et al. [130] but it provides an alternate mechanistic explanation for the observed synergistic effect.

4. Designing novel additives

Developing new additives which are more potent at inhibiting aggregation might be a means for improving biopharmaceutical formulations. However, there have only been a limited number of attempts at designing novel additives based on the current mechanistic understanding of commonly used additives. Several new compounds, which are derivatives of a particular additive, have been shown to enhance aggregation suppression [133–135]. The selection of these compounds is done mainly on the basis of their structural similarity with some commonly used additive. For example, L-Argininamide has been shown to increase the refolding yield of lysozyme more than arginine [135]. In a recent study in our group, we present a rational design approach based on the acquired mechanistic understanding of arginine and the "Gap Effect" theory (results to be published elsewhere [136,137]).

From a molecular interaction perspective, the additive design involved tuning protein–additive and additive–additive interactions in order to reduce protein–protein interactions. According to the "Gap Effect" theory, large molecules that have the same concentration on the protein surface as the bulk solution should be effective at inhibiting protein association. We call such additives "neutral crowders". However, large molecules naturally tend to be excluded from protein surfaces (e.g. polyethylene glycol) due to steric exclusion. We theorized, though, that if functional groups which tend to preferentially bind to proteins (e.g. guanidinium, urea, etc.) were added to the surface of a large, core structure that the resulting molecule could potentially behave as a "neutral crowder". Therefore, creating a "neutral crowder" molecule requires a balance between attraction and repulsion with respect to the surface of a protein. From this rational, our first approach was to modify the surface of PAMAM dendrimers to guanidinium and to produce polyarginine peptides. However, as the study progressed, it became clear that intra-solvent interactions, such as those highlighted in this review, play an important and crucial role in the stabilization mechanism for such compounds, more so than for other additives. This is due to such molecules having multiple guanidinium groups that could either cooperatively bind to the protein surface or form extended networks in solution depending on how intra-solvent interactions are tuned by exchanging the counterion. Choosing a proper balance of interactions allowed us to produce compounds (PAMAM dendrimers with a guanidinium sulfate or phosphate surface [136] and polyarginine sulfate salts [137]) which are shown to be potent aggregation suppressors, slowing aggregation by an order of magnitude more than the additives discussed in this review. Such potent aggregation suppressing additives might be useful during production and formulation, as they could improve yield and extend the shelf-life of protein therapeutics.

5. Conclusions

In summary, we have reviewed a broad spectrum of commonly used additives (see Table 1 for a brief overview) and highlighted some recent mechanistic inquiries. Most of these inquiries focused on intra-solvent interactions in developing a complete mechanistic explanation. For traditional conformational stabilizers, such as polyols and sugars, a behavior more complex than the simple nonspecific exclusion that is often cited has been elucidated. Glycerol exhibited a behavior where electrostatic interactions contributed to preferential exclusion. Furthermore, hydrogen bonding to the protein surface and water induced the molecule to be oriented perpendicular to the surface, enhancing the excluded volume effect. But more importantly, glycerol was able to bind to hydrophobic patches, enabling it to inhibit protein–protein interactions. As for sugars, intermolecular interactions between sugar molecules, which lead to cluster formation, has an indirect effect on protein–water interactions and enhances intra-protein interactions, stabilizing the protein structure. There is also an observed carbohydrate–π interaction between sugar molecules and aromatic residues, the strength of which can be tuned by adding electron withdrawing groups to the sugar molecule.

Denaturants are probably the most affected by intra-solvent interactions. Self-association of urea and guanidinium molecules provides a rational for how they contribute to the "indirect" effect and preferentially bind to hydrophobic residues. Both urea and guanidinium are observed to form strong attractive interactions with hydrogen bond accepting cosolutes. In particular, urea interacts with the stabilizing additive TMAO and guanidinium interacts with stabilizing counterions, such as sulfate, phosphate, citrate, and carbonate. Such interactions interfere the preferential binding to the protein surface, counteracting the denaturing effect such additives exhibit. Such results have been shown to be crucial in explaining the behavior of the aggregation suppressing additive arginine, which contains a guanidinium moiety. The self-association of arginine molecules is a contributing factor for its unique behavior and the linking together of arginine clusters into bigger clusters by hydrogen bond accepting counterions enhances its aggregation suppressing ability. Furthermore, intermolecular interactions with glutamate lead to synergistic behavior, such as enhanced binding and protein solubility.

Lastly, intra-solvent interactions may not always be the case even though an additive might have a structure that suggests intermolecular interacts can occur. Proline has numerous traits that cannot be fully explained and ring stacking was, for a long time, a leading theory for explaining its behavior. However, recent studies found no evidence for such interactions, making proline's mechanism a mystery that still needs to be elucidated. All of the examples reviewed here exhibit the need to conduct molecular-level inquiries and consider all possible interactions in the attempt to explain how an additive influences protein aggregation. Such knowledge has allowed us to rationally design novel additives which are more potent as suppressing aggregation.

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