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Arginine and the Hofmeister Series: The Role of Ion-Ion Interactions in Protein Aggregation Suppression

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Abstract

L-arginine hydrochloride is a very important aggregation suppressor for which there has been much attention given regarding elucidating its mechanism of action. Little consideration, however, has been given toward other salt forms besides chloride, even though the counterion likely imparts a large influence per the Hofmeister Series. Here, we report an in depth analysis of the role the counterion plays in the aggregation suppression behavior of arginine. Consistent with the empirical Hofmeister series, we found that the aggregation suppression ability of other arginine salt forms on a model protein (α -chymotrypsinogen) follows the order: $\text{H}_2\text{PO}_4^- > \text{SO}_4^{2-} > \text{Citrate}^{2-} > \text{Acetate}^- \approx \text{F}^- \approx \text{Cl}^- > \text{Br}^- > \text{I}^- \approx \text{SCN}^-$. Mechanistically, preferential interaction and osmotic virial coefficient measurements, in addition to molecular dynamics simulations, indicate that attractive ion-ion interactions, particularly attractive interactions between arginine molecules, play a dominate role in the observed behavior. Furthermore, it appears that dihydrogen phosphate, sulfate, and citrate have strong attractive interactions with the guanidinium group of arginine, which seems to contribute to the superior aggregation suppression ability of those salt forms by bridging together multiple arginine molecules into clusters. These results not only further our understanding of how arginine influences protein stability, they also help to elucidate the mechanism behind the Hofmeister Series. This should help to improve biopharmaceutical stabilization through the use of other arginine salts and possibly, the development of novel excipients.

Keywords

Arginine; Hofmeister Series; Preferential Interaction Coefficient; Osmotic Virial Coefficients; Vapor Pressure Osmometry; Protein Aggregation

Introduction

Protein aggregation is a major problem that pharmaceutical and biotech companies face when producing protein based therapeutics.^{1,2} Not only does aggregation during production reduce yield, it also places a huge burden on downstream purification processes.³ More importantly, aggregation during formulation and storage can have serious consequences because aggregates can elicit an immune response and other adverse side effects if administered to a patient.^{4,5} Over the past 25 years, the number of protein based therapeutics

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Supporting Information Available

A complete and detailed description of each experimental methodology can be found in the supporting information and this information is available free of charge via the Internet at <http://pubs.acs.org>.

on the market or in development has grown exponentially, thus sparking an interest in developing improved methods for preventing aggregation.^{6,7} For decades, the method and materials most often used for inhibiting the aggregation of proteins in solution has essentially remained unchanged; which is to add aggregation suppressing solution additives (often called cosolutes, cosolvents, and excipients) to the protein solution during production, purification, and formulation.^{2,8,9} Though most formulation recipes include a pH buffer, a nonionic surfactant, and maybe some sort of salt, the key excipient for preventing aggregation is usually some type of sugar or polyol (e.g. sucrose, trehalose, glycerol, sorbitol, etc.), which protects the native structure of the protein from unfolding, thus reducing the number of aggregation prone species in solution.^{10,11} However, other excipient types have been used and/or are gaining much more attention (e.g. amino acids, polymers, proteins, etc.). Furthermore, there is a desire to discover or create better performing excipients because for many protein therapeutics, a stable formulation cannot be created and the product must be lyophilized and reconstituted prior to injection, which for the most part is undesirable and, in some cases, a difficult and costly challenge.^{2,7,11}

One excipient that has gained much attention lately is arginine hydrochloride (ArgHCl).¹² Its ability to greatly improve the refolding yield of proteins was discovered, nearly by accident, two decades ago, and since then it has been the topic of many research articles, mainly due to its effectiveness and unique behavior in regards to suppressing aggregation.^{13–22} Arakawa and coworkers have written many research articles and reviews regarding the abilities and applications of ArgHCl, including its use during protein production and purification and as a solution additive for other macromolecules.^{16,23–30} Their collective work provides useful insight into the mechanism of action and highlights all of the observed behavior gathered thus far for ArgHCl. Those studies have revealed much about the arginine mechanism but a complete mechanistic understanding has yet to be determined.

What is known about ArgHCl is that it seems to inhibit protein-protein interactions with little influence on the conformational stability of the protein, thus slowing association rather than stabilizing the native structure.^{14,15,31} Some researchers have attempted to explain this behavior, but no one theory has been generally accepted because most theories cannot explain all of the observed behavior, not to mention that there is often a lack of experimental evidence confirming the proposed mechanism of action.¹² A theory first proposed in our group, suggests that arginine is neutral in regards to preferential interactions, leading to little influence on the folding equilibrium but eliciting an entropic penalty for when two protein molecules aggregate due to the exclusion of arginine molecules from the gap formed between two associating protein molecules.¹⁴ The preferential interaction coefficient values we measured later on seemed to, for the most part, support this theory.¹⁵ However, at high concentrations, the preferential interaction of arginine goes from being neutral or slightly attracted to being highly excluded, and thus, we have to admit that this model only describes a simple theoretical molecule, referred to as a “neutral crowder”, and cannot explain all of the complex behavior ArgHCl exhibits, though the “neutral crowder” effect may play a large role.^{12,22,32}

Moreover, little attention has been devoted towards other salt forms besides chloride. The chloride form has been studied so exclusively that ArgHCl is often referred to simply as arginine.¹² The Hofmeister Series,

first published in 1888, is an empirical ranking of how various ions influence protein solubility (i.e. ions to the left have a greater ability for precipitating proteins), which in turn is related to the thermodynamic stability of the native state.³³ The series has been studied extensively since the time of Hofmeister and serves as the foundation for selecting salts that will influence protein solubility, crystallization, denaturation, and aggregation, though the mechanism for this behavior is not well understood.^{34–37} It has been clearly demonstrated that the choice of anion has a more pronounced effect on these properties than the choice of cation. Furthermore, chloride is often placed in the middle of the series, thus other arginine salt forms are likely to exhibit a drastically different behavior, both positively and negatively, in regards to protein stability.^{37,38} To the best of the authors' knowledge, the only other form of arginine studied in this context has been $\text{ArgH}(\text{SO}_4)_{1/2}$, though the extent of those studies is limited.^{39,40} Recently though, the combination of arginine hydrochloride with sodium glutamate was studied by two different research groups and has received much attention due to a seemingly synergetic effect when the two solutes are combined in an equimolar ratio.^{7,41,42} Moreover, in a recently published review, Lange and Rudolph¹² (Rudolph being one of the researchers who discovered the arginine effect) postulated, based on existing data, about how other arginine salt forms would behave as excipients and also commented on the refolding behavior of the sulfate and phosphate salt forms, stating that those two forms of arginine perform poorly as refolding excipients even though sulfate and phosphate often provide conformational stabilization. However, no citations or experimental results were provided to support those claims. Even though sulfate and phosphate may reduce the refolding abilities of arginine, we have found that they enhance its aggregation suppression ability when our model protein is aggregated under accelerated conditions. Moreover, switching to a thiocyanate salt induces rapid aggregation under similar conditions rather than enhancing aggregation suppression, opposite of what was hypothesized. However, testing on other proteins is required before one could generalize these observations.

When attempting to explain the arginine mechanism, many researchers start by addressing the effect of the guanidinium moiety.^{40,43} Indeed, the guanidinium functional group must play an important role, because not only is arginine the only amino acid with a guanidinium moiety, no other amino acid exhibits aggregation suppression characteristics quite like arginine, though other amino acids, particularly proline, have been used as stabilizers.^{11,44,45} However, without a clear understanding at the molecular level, not only of the interaction of arginine with protein molecules, but also of how arginine behaves in solution, the arginine mechanism will remain a mystery.

Recently though, two key breakthroughs, in relation to the solution behavior of guanidinium and arginine, may help to resolve this perplexing question. The first comes from a series of articles published by Mason and coworkers, in which they studied the solution behavior of various guanidinium salts.^{46–51} They initially aimed to explain the denaturing ability of guanidinium but ultimately revealed that guanidinium can form a strong attractive interaction with various anions of similar size that also have hydrogen bond accepting capabilities (e.g. sulfate, carbonate, etc.), which likely explains why some guanidinium salts (e.g. guanidinium sulfate) stabilize proteins rather than denature them because the two ions form clusters in solution rather than bind to the protein surface.⁵⁰ The hydrogen bonding interactions and cluster formations they demonstrated reveal a lot about the behavior of guanidinium. However, some of this behavior was revealed long before the work of Mason and coworkers. Bonner, using osmotic coefficient data and Raman spectra, gave valuable insight into the hydrogen bond interactions between guanidinium and halide ions over three decades ago.^{52,53} Bonner showed that guanidinium and fluoride form a strong hydrogen bond, possibly even a partial H-F bond, in solution and this interaction is weaker for other halides in the order of $\text{Cl}^- > \text{Br}^- > \text{I}^-$, due to ion size and charge density. At the time, these

results were used to speculate on the hydrogen bond formation between guanidinium and proteins in an attempt to explain the denaturing effect of guanidinium. More importantly, Mason and coworkers also revealed that the structure of the poorly hydrated guanidinium molecule allows the positively charged ions to associate, with the planar molecules stacked on top of each other.⁴⁷ These results contradict long held beliefs about how certain cosolutes influence the structure of water (solutes have long been labeled either a chaotrope or a kosmotrope because of this water structure viewpoint) and how the perturbed water structure influences protein stability.³⁷ Their results are compelling and have strong implications for explaining the arginine mechanism, not only with how arginine will interact with various counterions, but how arginine interacts with itself and glutamate (when mixed with that amino acid), given that guanidinium should have a strong attractive interaction with the carboxylate moiety found on both molecules.

This self-association of arginine, the second breakthrough mentioned, has previously been revealed and has been studied extensively in our lab through molecular dynamics (MD) simulations.^{17,22,32,54} These simulations show that the guanidinium functional group does indeed interact with the surface of a protein molecule in a fashion similar to free guanidinium; however, this functional group also forms attractive interactions with other arginine molecules. This self-association of arginine molecules likely prevents arginine from binding too strongly to the surface of protein molecules, preventing it from denaturing the protein but at the same time preventing protein-protein interactions. This self-association also explains the unique trend observed for preferential interaction coefficient measurements (i.e. slightly bound at low concentrations but highly excluded at high concentrations) and explains why the arginine effect diminishes at high concentrations.¹⁵ Therefore, we were motivated to explore other arginine salt forms, not only with the aim of discovering a better performing excipient, but to further our understanding of this mechanism and how guanidinium-anion interactions may contribute to the observed behavior.

In this work, we studied the influence a variety of arginine salt forms have on the heat induced aggregation of α -chymotrypsinogen A. We show that dihydrogen phosphate, sulfate, and citrate enhance aggregation suppression over chloride, while bromide, iodide, and thiocyanate diminish it. This trend is in accordance with the influence these cosolutes have on the conformational stability of the protein, as measured by differential scanning calorimetry (DSC), indicating that changes in the folding equilibrium is an important factor in the aggregations suppression mechanism. However, perturbing the structure of the protein cannot explain everything, since the acetate, fluoride, and chloride salt forms have no influence on the midpoint melting temperature. Trends in the preferential interaction coefficient, as determined by vapor pressure osmometry (VPO) and MD simulations, support the observed behavior and show a key difference between the anions that enhance aggregation suppression and the anions that diminish it. Osmotic second virial coefficient measurements, as determined by VPO and MD simulations, give a clear indication for the observed behavior and support previous reports of ion-ion interactions.^{48,49,55} Dihydrogen phosphate, sulfate, and citrate seem to interact favorably with the guanidinium moiety, with phosphate interacting quite strongly, which reduces the solubility of arginine considerably. These ion-ion interactions seem to limit interactions with protein molecules, while the anions that have a weaker interaction with guanidinium allow arginine molecules to self-associate and the counterion to freely interact with the protein surface, with iodide and thiocyanate forming a very strong interaction, leading to denaturation. These results not only help to elucidate the arginine mechanism, they also have large implications for interpreting the mechanism behind the Hofmeister Series (i.e. ion-ion interactions and other ion specific behaviors are important considerations and the mechanism cannot be completely generalized). In addition, the results will help to improve the production and formulation of

protein therapeutics and aid in the development of novel excipients due to a stabilization method not yet considered.

Experimental Methods

Proteins and Reagents

Bovine α -chymotrypsinogen A type II (C4879) and all reagents were obtained from Sigma-Aldrich (St. Louis, MO) in the highest available grade. All arginine salt forms other than arginine hydrochloride (ArgHCl) were prepared by titrating an L-arginine solution or exchanging the chloride ion of ArgHCl. The concentration of aCgn was determined spectrophotometrically with a PerkinElmer Lambda 35 UV/Vis spectrometer using an extinction coefficient of $1.97 \text{ mL} \cdot \text{mg}^{-1} \text{cm}^{-1}$ at 282 nm.⁵⁶

Accelerated Protein Aggregation

The aggregation of α -chymotrypsinogen A (aCgn) was accelerated by incubating samples at an elevated temperature. aCgn was selected as a model protein because it is a well characterized protein that has been used extensively as a model protein in aggregation studies due to it having aggregation characteristics similar to therapeutically relevant proteins.⁵⁷ A temperature of 52.5°C was found to be optimal, in that it was well below the onset of unfolding but accelerated aggregation enough to allow an aggregation experiment to be completed in less than a day. In addition to all aggregation experiments being conducted at that temperature, all samples contained 10 mg/mL aCgn, held at pH 5.0 using a 20 mM sodium citrate buffer. Monomer loss was monitored via size exclusion chromatography using an Agilent 1200 series HPLC, equipped with a Zorbax GF-250 (4.6×250 mm, 4 micron) size exclusion column and a UV-Vis detector. The HPLC mobile phase contained 25 mM citric acid monohydrate, 25 mM sodium acetate, and 200 mM sodium chloride with the pH adjusted to 4.0 using sodium hydroxide. The flowrate was set at 1 mL/min and samples were monitored at a wavelength of 280 nm. For all aCgn concentrations studied, the monomer loss rate exhibited 2nd order kinetics, thus rate constants were determined by fitting each data set to a 2nd order rate law.

Preferential Interaction Coefficient Values from VPO Measurements

Preferential interaction theory is the thermodynamic framework to quantify the effect of cosolutes on protein stability. The preferential interaction coefficient, expressed as either Γ_{μ_3} or Γ_{23} depending on use, is a measure of the preference cosolutes have for the protein surface and is defined by the following expression,

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

where m , T , P and μ represent molal concentration, temperature, pressure, and chemical potential, respectively.⁵⁸ The subscripts used indicate solution components in Scatchard notation: water (subscript 1), the protein (subscript 2), and the cosolute (subscript 3).⁵⁹ We have previously used vapor pressure osmometry (VPO) to determine the preferential interaction coefficient of ArgHCl.¹⁵ In that article, we explain the implications of this parameter and how to obtain values utilizing the VPO methodology, thus complete details of the procedure will not be repeated here. In this report, a Wescor 5520 vapor pressure osmometer was utilized to determine the osmolality of three separate series (16 samples in each series) of carefully prepared solutions (i.e. cosolute only solutions, protein only

solutions, and protein-cosolute solutions at a constant protein concentration), the goal being to determine the slope with respect to concentration.

Differential Scanning Calorimetry

The thermodynamic stability of aCgn in the presence of the arginine salts was determined by DSC (Microcal VP-Differential Scanning Calorimeter). aCgn was analyzed at a concentration of 1 mg/mL in a 20 mM sodium citrate pH 5 buffer containing the cosolute of interest and a scan rate of 90°C/hour. For each arginine salt, three concentrations (including zero concentration) were analyzed and denaturation midpoint temperature values, T_m , with respect to cosolute concentration were fitted to a linear trend.

Molecular Dynamics Simulation Methodology

Molecular dynamic simulations of protein-cosolute solutions and cosolute only solutions were performed to give a molecular perspective on how arginine interacts with aCgn, how arginine interacts with itself, and how the counterion influences these interactions. All simulations were performed using the NAMD package, with the CHARMM22 force field.^{60,61} The TIP3P water model was used.⁶² The force field parameters for arginine were taken from the CHARMM force field with the N terminal and the side chain protonated, and C terminal deprotonated. The parameters for the N and C termini were taken from the CTER and NTER parameters available in CHARMM. Force field parameters for sulfate, citrate and thiocyanate ions were taken from the literature.^{63–65} Force field parameters for acetate, chloride and dihydrogen phosphate ions were available in CHARMM. Fluoride, bromide and iodide were not computationally studied due to the absence of reliable force field parameters in CHARMM. For the purposes of understanding the ordering of anions in the Hofmeister series, the two opposite cases of sulfate and thiocyanate, with few anions in between the two extremes, were studied.

Osmotic Virial Coefficient Values from MD Simulations

To characterize the nature of interaction between specific ion pairs, the osmotic second virial coefficient was calculated from MD simulations by integrating the Mayer function:

$$B_{22} = -2\pi \int_0^{\infty} (g_{ij} - 1) r^2 dr. \quad (2)$$

The McMillan-Mayer B_{22} parameter is obviously not the same as the Pitzer B^ϕ parameter determined from VPO measurements. However, both parameters describe binary intermolecular interactions that lead to nonideal osmotic coefficient behavior, thus it is expected that the trends in both parameters will be identical. The finite size of the simulation box leads to erroneous normalization of a simulated radial distribution function (RDF).⁶⁶ Therefore, the asymptotic behavior of the simulated RDF's are corrected by introducing a normalization factor, $f_{ij}(\rho)$,

$$g_{ij}(r, \rho) = f_{ij}(\rho) g_{ij}^{sim}(r, \rho). \quad (3)$$

The procedure used for the normalization of RDF's is included in the supporting information. The same technique has been used for estimating the osmotic second virial coefficient of monoatomic salts such as NaCl, KF, etc. Once the correct normalization factor is obtained, eq 2 is used to estimate the osmotic second virial coefficient for individual ion pairs using the normalized RDF. The upper limit of the integral was set to a finite cut-off

distance (dependent on the system). The cut-off value was chosen as the distance around which the osmotic second virial coefficient becomes constant or the corresponding RDF reach a value close to 1. The cut-off values lie within the range of 1.2–2 nm, with the sulfate salt having the highest cut-off value.

Preferential Interaction Coefficient Values from MD Simulations

The method for calculating preferential interaction parameters based on a statistical mechanical method applied to an all-atom model with no adjustable parameter was developed by Baynes and Trout.⁶⁷ The number of cosolute and water molecules as a function of distance from the protein surface is used to calculate $\Gamma_{23}(r,t)$, where Γ_{23} is an alternate and common way to represent $\Gamma_{\mu 3}$, until it approaches a constant value. The MD simulation is saved at periodic time intervals and these saved frames are used to find $\Gamma_{23}(r)$. The expression used to calculate the coefficient is^{21,68,69}

$$\Gamma_{23}(r, t) = n_3(r, t) - n_1(r, t) \left(\frac{n_3 - n_3(r, t)}{n_1 - n_1(r, t)} \right), \quad (4)$$

where n_3 is the total number of cosolute molecules, and n_1 is the total number of water molecules. The extent of the local domain around a protein in which $\Gamma_{23}(r,t)$ varies is found to be around 6 Å in our earlier work.^{21,22,65,67,68} The predicted value of the preferential interaction coefficient $\Gamma_{23}(r = 6 \text{ Å})$ is the average of the instantaneous $\Gamma_{23}(r,t)$ values. For arginine salts, which are electrolytic, preferential interaction coefficient calculations would require an estimate of Γ_{23} for both the cation and the anion. The preferential interaction coefficient for a binary electrolyte is given by

$$\Gamma_{23} = 0.5 \left(\Gamma_{2,-3} + \frac{v_+}{v_-} \Gamma_{2,+3} \right), \quad (5)$$

where $\Gamma_{2,-3}$ is the preferential interaction coefficient for the anion, $\Gamma_{2,+3}$ is the preferential interaction coefficient for the cation, and v_+/v_- is the ratio of the number of cations and anions in the chemical formula of the salt. The net charge of the protein divided by the valency of the ion with the same charge is also subtracted from the preferential interaction coefficient of the corresponding ion.

Results and Discussion

Aggregation Suppression

An example of the data collected during an accelerated aggregation experiment is shown in Figure 1A. In the figure, the aCgn monomer loss profiles for four different arginine salt solutions are shown, all at a concentration of 150 mM, in addition to the reference profile (i.e. no cosolute). It can be seen that ArgH(H_2PO_4), ArgH(Citrate)_{1/2}, and ArgHCl reduce the rate of aggregation, while ArgHSCN increases it. The rate of monomer loss in the presence of ArgHCl at 150 mM is approximately twice as slow as the reference monomer loss rate. However, at the same concentration, ArgH(Citrate)_{1/2} and ArgH(H_2PO_4) slow the rate by a factor of approximately 3 and 8, respectively. On the other hand, ArgHSCN at 150 mM increases the rate by a factor of 6. This, however, only represents a fraction of the data collected, which is best represented in Figure 1B. The figure shows the relative rate constant for monomer loss (i.e. the rate constant relative to the rate constant for no cosolute) over a

wide range of cosolute concentrations. It should be noted that the profile for $\text{ArgH}(\text{H}_2\text{PO}_4)$ is limited due to its poor solubility (a limit of approximately 0.45 M at room temperature).

The results shown in Figure 1B demonstrate that dihydrogen phosphate, sulfate, and citrate significantly improve the aggregation suppression ability of arginine when compared to chloride. More importantly, at low concentrations (less than 150 mM), the aggregation suppression ability of $\text{ArgH}(\text{H}_2\text{PO}_4)$ is significant. At a concentration of 150 mM, the rate of aCgn aggregation in the presence of $\text{ArgH}(\text{H}_2\text{PO}_4)$ is 2.5 times slower than when in the presence of either $\text{ArgH}(\text{SO}_4)_{1/2}$ or $\text{ArgH}(\text{Citrate})_{1/2}$. However, the aggregation suppression ability of $\text{ArgH}(\text{H}_2\text{PO}_4)$ levels off significantly as the concentration is increased beyond 200 mM and the aggregation suppression ability of $\text{ArgH}(\text{SO}_4)_{1/2}$ and $\text{ArgH}(\text{Citrate})_{1/2}$ likely exceeds it at concentrations above 300 mM. As will be discussed below, this trend seen for $\text{ArgH}(\text{H}_2\text{PO}_4)$ is likely the result of the strong attractive interaction between the guanidinium moiety of arginine and the phosphate ion. These interactions seem to cause arginine-phosphate clusters to form at higher concentrations, leading to high preferential exclusion and a strong thermodynamic stabilization but also limited cosolute solubility and reduced association suppression.

As for the remaining arginine salts to the left of chloride in the Hofmeister Series, the relative rate constant for fluoride and acetate are essentially the same as that for chloride. At concentrations below 100 mM, the aggregation suppression ability of these three salt forms are comparable to sulfate and citrate. However, as the concentration increases, the aggregation suppression ability of sulfate and citrate continue to improve, having a relative rate constant value between 0.02–0.04 at the highest concentration tested (0.5 M), while the relative rate constant for fluoride, acetate, and chloride levels off, having a near constant value of about 0.2 for concentrations above 0.4 M. ArgHF seems to level off the least and has a slightly better relative rate constant at a concentration of 0.5 M, which will likely improve slightly as the concentration is increased further, however, such concentration are considered quite high and would rarely ever be used.

As for the arginine salts to the right of chloride in the Hofmeister Series, the relative rate constant for bromide remains fairly close to unity for all concentrations tested, while iodide and thiocyanate have nearly identical profiles, which show a large increase in the rate of aggregation, up to 10 times faster at a concentration as low as 200 mM. The relative rate constant for ArgHBr dips down to around 0.7 at 150 mM before it reverses, exceeding a value of 1 at higher concentrations. This indicates that the slight denaturing effect of bromide balances out the aggregation suppression effect of arginine, while the strong denaturing effect of iodide and thiocyanate completely overpower the effect of arginine. The large size and low charge density of these ions lead to them being poorly hydrated and therefore, in thermodynamic terms, they tend to partition to the surface of a protein rather than being solvated in the bulk solution, even more so if hydrophobic residues are exposed.⁴⁶ This favorable preferential interaction leads to protein denaturation, with iodide and thiocyanate having a stronger preferential interaction than bromide due to their smaller charge density. In fact, iodide and thiocyanate are so destabilizing that ArgHI and ArgHSCN are stronger denaturants than guanidinium chloride (GdmCl), as represented by relative rate constant values (see Figure 1) and changes in the denaturation midpoint temperature (see discussion below). The relative rate constant for GdmCl initially decreases when it is added to the solution (similar to ArgHBr), reaching a minimum of about 0.6 at 200 mM and then increases thereafter, reaching a value of 9 at 0.7 M, the highest concentration tested. This shows that at low concentrations, GdmCl has an ability to reduce aggregation by inhibiting protein-protein interactions, similar to arginine, but at high concentration, the denaturing effect dominates and causes aggregation to proceed more rapidly. However, ArgHI and ArgHSCN show no stabilizing effect from the presence of arginine. They instead increase

the rate of aggregation at all concentrations tested, doubling the rate at a concentration as low as 50 mM. All of this exemplifies the pronounced effect the choice of anion has on protein stability and shows that ArgH(H₂PO₄), ArgH(SO₄)_{1/2} and ArgH(Citrate)_{1/2} might be more useful than ArgHCl for preventing protein aggregation.

Conformational Stability

The thermodynamic stability of aCgn in the presence of the arginine salts was assessed by determining the change in the denaturation midpoint temperature, T_m , with respect to cosolute concentration. Shifts in the melting temperature of the protein are indicative of shifts in the folding equilibrium of the protein, with increases in the melting temperature corresponding to a shift toward the native state, which in return reduces the number of aggregation prone species. Table I shows the denaturation midpoint temperature increment, $dT_m/d[3]$, defined as the slope of T_m with respect to the molar concentration of the cosolute (component 3), over the concentration range tested, as determined by DSC. Over this short initial concentration range, T_m exhibited a linear trend, giving constant values for $dT_m/d[3]$. The values obtained exhibit the pronounced effect the anions have on the thermodynamic stability of the protein and they correspond directly to the ordering of the relative rate constant values obtained during the aggregation study.

As shown in Table I, ArgH(H₂PO₄), ArgH(SO₄)_{1/2} and ArgH(Citrate)_{1/2} increase the melting temperature of the protein with an initial rate of 6.3, 5.4, and 3.3 °C*M⁻¹, respectively. Clearly, these salts are able to stabilize the native structure of the protein and inhibit the formation of partially unfolded species. This thermodynamic stabilization is on top of the association suppression effect of arginine, leading to the significant aggregation suppression shown in Figure 1. ArgH(Acetate), ArgHF, and ArgHCl exhibit no thermodynamic stabilization, which is consistent with previous reports for ArgHCl, thus these salts inhibit aggregation solely through an association suppression mechanism. ArgHBr has a denaturing effect on aCgn, lowering the melting temperature at a rate of 7.7°C*M⁻¹. This reduction in conformational stability, which increases the number of reactive species in solutions, seems to nearly balance out the association suppression effect of arginine, as exhibited by the relative rate constant having a value near unity over a wide concentration range. This reduction in thermodynamic stability is slightly greater than that for GdmCl, which might explain why they have near identical profiles at low concentrations. However, the association suppression effect of arginine is greater than that for guanidinium, thus, at high concentrations, the rate of aCgn aggregation is higher in the presence of GdmCl than in the presence of ArgHBr. As for the other arginine salts, ArgHI and ArgHSCN lower the melting temperature significantly, both with a rate greater than 20 °C*M⁻¹. This denaturing effect overpowers the association suppression effect of arginine and as a result, these salts increase the rate of aggregation at all concentrations. These results, along with aggregation suppression data, support the claim that ArgH⁺ acts as an association suppressor and shows that the choice of anion will either enhance or counteract this aggregation suppression by influencing the stability of the native structure.

Ion-Ion Interactions

As mentioned in the introduction, ion-ion interactions may be contributing to the observed behavior of the arginine salts. To quantify this behavior, osmotic 2nd virial coefficient values were determined for aqueous solutions of the arginine salts via VPO and MD simulations. Experimental osmotic data were analyzed by using the Pitzer model (see supporting information), while MD simulations were analyzed using the McMillan-Mayer model. Pitzer ion interaction parameters for each of the salts are shown in Table S2 in the supporting information, which shows that the second virial coefficient parameters obtained for ArgHCl closely matches values reported in the literature. Since the magnitude of like and unlike ion

interactions is ionic strength dependent, it is generally held that only cation-anion binary interactions make contributions to the value of $\beta^{(1)}$. Both like and unlike ion interactions make contributions to $\beta^{(0)}$, making it difficult to interpret this parameter unless radial distribution functions are available for each ion pair. The trends observed for $\beta^{(1)}$ are in accordance with the discussion above, which are highlighted in Table I. The values for the dihydrogen phosphate, sulfate, and citrate salts are large and negative, indicating a strong attractive interaction between arginine and those anions. The value for ArgHCl is negative but smaller in magnitude, indicating a weak attractive interaction. The values for the other halide salts show repulsive interactions for those salt forms with the strongest repulsion for thiocyanate and iodide and the weakest for bromide. It should be noted that these trends are similar to the virial coefficient values for guanidinium halide salts, which show a weak repulsive interaction with fluoride, a moderate repulsive interaction with bromide, and a strong repulsive interaction with iodide.⁷⁰

During the simulations, significant ion pairing was observed in the aqueous arginine salt solutions, which supports the above experimental results. The ion pairing was also observed to be dependent on the choice of anion. Ion pairs in thiocyanate, chloride and acetate solutions are observed to be randomly distributed throughout the solution while citrate, phosphate and sulfate show a marked tendency to form hydrogen bonded clusters. Snapshots of the MD simulation boxes of arginine salts are shown in Figure 2. It can be seen that the structures formed by sulfate, citrate and phosphate salts are worm-like chains of sizes comparable to the box-size. Clustering observed in chloride, thiocyanate and acetate solutions is mainly due to Arg-Arg interaction as reported in our recent work on aqueous arginine hydrochloride solutions.^{22,71} Similar ionic clusters were also observed in aqueous guanidinium salt solutions.^{47–49} Brady and coworkers have shown that clustering in Gdm salts is not dependent on the choice of the water model and is not a simulation artifact. Due to the presence of Gdm group as a side-chain in arginine, the clustering behavior of arginine salts is expected to be similar to Gdm salts. The only difference between Gdm and arginine salts is the presence of additional charged groups and a carbon chain in arginine. These additional charged groups (N-terminal amino group and C-terminal carboxylate group) further enhance the clustering in solution.

Radial distribution functions (see Figure 3) between ion pairs help to further characterize the solution structure. Arg-Arg pairing, as illustrated by the RDF between the Gdm carbon atoms in the arginine molecule (see Figure 3a), shows a tendency of the Gdm side chain to stack with each other or form ion-pairs separated by the anion acting as a bridge between two Gdm side chains. The presence of an anion does not significantly affect the Arg-Arg pairing except in the case of citrate, where the large size of the anion crowds out the formation of Arg pairs. The pairing between cation and anion is strongest for the sulfate ion due to the exceptionally strong interaction between the Gdm group and sulfate (see Figure 3b). The acetate ion also has a strong interaction with arginine, however, the strong binding of sulfate and acetate is in stark contrast to the weak binding of thiocyanate and chloride. The pairing between anions (see Figure 3c) is significantly weak, as compared to other ion pairs in solution. Phosphate ion pairing exhibits a peak around 0.5 nm due to the presence of hydrogen bonding acceptors and donors in the same molecular anion. Sulfate also shows a peak around 0.6–0.7 nm but this is not due to direct interaction between sulfate ions; rather, the peak is due to the presence of multiple sulfate anions associating with adjacent or the same arginine cation. The peak heights for sulfate and chloride salts are comparable to the peak heights observed by Brady and coworkers for guanidinium salts. They also observe rugged RDF's due to the extensive clustering in guanidinium carbonate and sulfate salt solutions.^{47–49} The RDF's between ions give an idea about their clustering behavior but these plots cannot be used as a direct measure of observed clustering in different salt solutions. Peak heights and positions are dependent on the charge of the ion, the number and

type of hydrogen-bonding groups, the choice of reference atom for calculation of RDF and bulk densities. Therefore, second osmotic virial coefficient values provide a better measure to compare different salts.

To elaborate, the individual contribution of specific ion-pairs to the overall clustering in the solution can be estimated by calculating the osmotic second virial coefficient between ion-pairs in solution. B_{22} values for ion-pairs in each salt solution are reported in Table II. It can be seen that the contribution of cation-cation interaction is similar in magnitude to cation-anion interaction. Σ_{ij} denotes the sum of interactions between all ion pairs. Sulfate, dihydrogen phosphate and citrate form a group with strong overall interactions whereas chloride, thiocyanate and acetate form a group with weak overall interactions. The difference between the collective structures of these two groups of anions apparently results from the differences in the hydrogen bonding of the anions to the arginine cation. The numbers of hydrogen bonds between different ion-pairs in solution are reported in Table III. It can be seen that the highest number of hydrogen bonds between arginine molecules are formed in chloride and thiocyanate solutions due to the minimal interaction of these ions with the cation. The loss of hydrogen bonds between arginine molecules in acetate, sulfate, phosphate and citrate solutions is compensated by the formation of large number of cation-anion hydrogen bonds. Acetate also interacts strongly with the arginine, forming hydrogen bonds with the Gdm group as shown in Figure 4a, but due to the limited number of hydrogen bond acceptors, the acetate anion cannot act as a bridge between multiple cations. On the other hand, citrate, sulfate and dihydrogen phosphate can form bridged structures, as shown in Figure 4b, 4c, and 4d, respectively. The number of hydrogen bonds between arginine ions (with an anion acting as a bridge) is high for sulfate, dihydrogen phosphate and citrate as compared to acetate, chloride and thiocyanate, which have limited to negligible capacity to form such bridged structures. These observations also explain why sulfate, dihydrogen phosphate and citrate form large hydrogen bonded clusters in solution as compared to acetate, chloride and thiocyanate. These results are in accordance to our previous reports, in which we showed that ArgHCl likely forms Arg-Arg clusters in solution at high concentration, which leads to a higher preferential exclusion.

Preferential Interactions

To gain insight into how the arginine salts inhibit protein-protein interactions, the preferential interaction coefficient, $\Gamma_{\mu 3}$ or Γ_{23} , at various concentrations was determined, both experimentally via VPO measurements and computationally via MD simulations. The experimental results are depicted in Figure 5, which shows $\Gamma_{\mu 3}$ values at various cosolute concentrations, and Table S2, which summarizes the polynomial fit and uncertainty of the experimental data. It should be noted that the preferential interaction coefficient could not be obtained via VPO for the acetate and fluoride salts due to these anions being in equilibrium with their respective conjugate acid forms at pH 5. This is because both of these acid forms are volatile and this volatility (though slight due to the low concentration of the conjugate acid) interfered with the VPO measurements. Regardless, the experimental results for the other salt show a clear difference between the anions to the left and to the right of chloride in the Hofmeister Series. The chloride, bromide, iodide, and thiocyanate salts all have preferential interaction coefficient values that start out positive at low concentrations, with an initial slope almost identical to GdmCl, whereas the initial values for the citrate, sulfate, and dihydrogen phosphate salts are negative. The preferential interaction data for GdmCl is depicted in Figure 5 and Table I for comparison, which exhibits a linear trend with respect to concentration.

The initial slope for the chloride, bromide, iodide, and thiocyanate salts show that at low concentrations, when there is likely little ion-ion interactions, the salts interact favorably with the protein surface, with an attractive interaction similar in strength as that for GdmCl.

However, as the concentration increases, the preferential interaction coefficient values for all of these arginine salts begin to decrease and ultimately become negative, whereas the trend for GdmCl continues to increase linearly, indicating that at high concentrations, these arginine salts exhibit a net repulsive interaction with the native protein structure, opposite of what is observed for GdmCl. Furthermore, the preferential interaction coefficient values for thiocyanate and iodide salts exhibited the largest values at all concentrations, followed by bromide and chloride, indicating that the thiocyanate and iodide ions cause those salts to have a stronger attractive interaction with the protein surface, followed by bromide, which causes only a slightly stronger interaction.

This nonlinear trend is in accordance with previously reported values for ArgHCl and aCgn, with values differing slightly due to a difference in pH.¹⁵ In our previous report, the surprising behavior exhibited by ArgHCl was initially thought to be the result of the protein surface quickly becoming saturated with the large molecule. MD simulations later revealed that this hypothesis was likely true but only at much higher concentrations (>1.5 M) and thus another mechanism contributed to the nonlinear trend observed at lower concentrations.^{22,32} As mentioned before, studies of aqueous ArgHCl solutions revealed that arginine has a strong propensity to form clusters in solution via hydrogen bonding between the guanidinium and carboxylate moieties, which was found to be stronger than the hydrogen bond formed with water, and as a result, it reduces the interaction with the protein surface. This reduced interaction is due, in part, to the larger net size of the clusters (steric exclusion) but in large part, is due to a favorable interaction with other arginine molecules in the bulk solution, which is similar or greater in strength as the interaction with the protein surface. This self-interaction is concentration dependent, with more clustering obviously occurring at higher concentrations, leading to the nonlinear trend shown in Figure 5.

It should be noted that preferential interaction coefficient measurements and the interactions studied via MD simulations are for interactions with the native protein structure only and are not necessarily indicative of the propensity to denature protein molecules. However, most cosolutes that are attracted to the native state are typically more attracted to the unfolded state and vice versa for preferentially excluded cosolutes, due to most interactions being nonspecific in nature. It is this difference in the preferential interaction between the two states that leads to a change in the thermodynamic stability of the protein, which can be represented by the Wyman linkage relationship. Therefore, even though for most cosolutes there is a direct relationship between the preferential interaction coefficient and the folding stability of the protein, there are, however, some exceptions.⁷² The mechanism for each exception varies but all are the result of preferential interactions being repulsive in one state and attractive in the other. For compounds containing guanidinium and halide anions, exceptions can arise due to the exposure of hydrophobic residues upon unfolding.

Guanidinium, the larger halides, and thiocyanate are poorly hydrated ions that have a strong preference for hydrophobic residues which causes them to interact more strongly with the unfolded state and as a result, they denature proteins.⁴⁶ That is, if they are not inhibited from interacting with the protein surface, as in the case for arginine. The preferential interaction coefficient values obtained experimentally and MD simulations seem to indicate that arginine is inhibited from interacting strongly with the protein (either with the native state or the unfolded state) even though it has a guanidinium moiety, but the halide anions and thiocyanate are free to interact with the protein. This favorable interaction is in order of $\text{SCN}^- \approx \text{I}^- > \text{Br}^- > \text{Cl}^-$, as indicated by T_m and $\Gamma_{\mu 3}$ results. The interaction between F^- and the protein is likely similar in strength as that for Cl^- , which is weak at best, but as explained above, $\Gamma_{\mu 3}$ could not be determined for ArgHF.

The magnitude of the interaction with the protein for these salts not only arises from a difference in the protein-anion interaction but also from a difference in the guanidinium-anion interaction, as discussed above, with thiocyanate and iodide exhibiting a weaker interaction with guanidinium. Thus these ions are less likely to interact with arginine in the bulk solution. Fluoride and chloride not only exhibit a weaker interaction with the protein surface, but they also interact more favorably with the guanidinium functional group on arginine. Therefore, as a result of Arg-Arg interactions and weak Arg-anion interactions, Γ_{μ_3} values exhibit a nonlinear trend for all of the salts discussed and due to the variation in Arg-anion and protein-anion interactions, the magnitude of Γ_{μ_3} and the shift in T_m varies amongst the salts. ArgHCl and ArgHF are neither naturants nor denaturants because there is little difference between the interaction with the native and unfolded states. When a protein unfolds, free ArgH⁺ will likely interact more strongly with the exposed hydrophobic residues but the clusters should become more excluded, counteracting this interaction. The interaction of the fluoride and chloride ions with the unfolded state likely does not change much because of the weak interaction these ions have for hydrophobic residues. As for ArgHBr, ArgHI, and ArgHSCN, the interaction with the unfolded state is stronger due to the stronger attractive interaction the anions exhibit for hydrophobic residues, leading to those salts denaturing proteins.

The salts to the left of chloride in the Hofmeister series exhibit similar interactions but of differing magnitude, resulting in Γ_{μ_3} trends distinct from the other salts. The dihydrogen phosphate, sulfate, and citrate salts have Γ_{μ_3} values that start out negative and remain negative, with values similar to other highly excluded solutes (e.g. glycerol), indicating that the salts have a strong repulsive interaction with the protein at all concentrations.¹⁵ ArgH(SO₄)_{1/2} is the least excluded of these salts, causing the ordering of Γ_{μ_3} data to differ slightly from the ordering of T_m data. Also, the profile for citrate is almost linear for most of the concentration range, likely from the large citrate ion being highly excluded and being a hindrance to Arg-Arg interactions. It is well documented that salts with these anions typically stabilize the folded state, as is the case for the data shown here. Both this and the preferential exclusion can be contributed, in part, to the nonspecific repulsive interaction of the anions with protein molecules that result from the ions being well hydrated and preferring the bulk solution. But as discussed above, dihydrogen phosphate, sulfate and citrate have been shown to exhibit a strong attractive interaction with guanidinium, which further reduces the interaction with the protein.

Theoretical preferential interaction coefficient values computed from the MD simulation are reported in Table IV. The estimated preferential interaction values match well with the trend in the experimental values. The experimental and computational estimates for sulfate, dihydrogen phosphate (if linearly extrapolated to 0.5m) and citrate indicate large, negative preferential interaction coefficient values, while thiocyanate exhibits a large, positive preferential interaction coefficient value. The experimental and computational estimates do not match exactly, which is likely due to the inability of the force field to capture the interactions of these ions with the protein surface. Force fields are optimized for interaction of ions with water molecules. However, as shown above, for arginine salts, interactions with water is only one of the key interactions. In order to obtain an accurate estimate of preferential interaction coefficient values, force fields for electrolytes should also be benchmarked to reproduce bulk solution properties and interactions between individual ions.^{73,74} For the purposes of understanding the interesting experimental trends observed in this study, the current force fields provide reasonable estimates of the trends in the data. On the basis of the preferential interaction coefficient of individual ions, it can be concluded that the number of cations on the protein surface is related to the number of anions. Thiocyanate ions have high positive preferential interaction coefficient values but it does not interact strongly with arginine cations. Sulfate ions have a negative preferential interaction

coefficient but it interacts strongly with arginine. Therefore, there are two driving forces that push and pull arginine towards and away from the protein surface. In order to conserve the local charge near the protein surface, more arginine molecules are drawn towards the protein surface if there are a high number of anions near the protein surface. If the interactions between ions in the bulk solution are not as strong as compared to the interaction between the ion and the protein surface, the ions accumulate near the protein surface. Therefore, sulfate, phosphate and citrate salts have a large negative Γ_{μ_3} because of their exclusion from the protein surface and the strong interaction with arginine molecules and vice-versa for thiocyanate, acetate and chloride salts.

This interaction between arginine and the anion further reduces the preferential interaction of the arginine salt with the surface of the protein and further limits interactions with the unfolded state, thus enhancing thermodynamic stabilization. This cation-anion interaction does not necessarily reduce Arg-Arg interactions though, because the anion bridges together two arginine molecules, similar to guanidinium-sulfate clusters discovered by Mason and coworkers, causing the nonlinear trend observed.⁴⁸ Therefore, the ordering of the Γ_{μ_3} and T_m data is not only ranked by how the individual anions interact with protein molecules but also, with how those anions interact with the guanidinium functional group on the arginine molecule.

Mechanistic Insight

The trends discussed here are related to the impact these salts have on protein aggregation. As discussed in the previous section, both changes in the folding equilibrium of the protein and changes in protein-protein interactions must be taken into consideration. The halide and thiocyanate salts exhibit an attractive interaction with the protein at low concentrations. This, according to the theory developed by Baynes and Trout, should reduce protein-protein interactions, much more so for arginine than for guanidinium due to the larger size of arginine.^{14,75} However, as discussed before, bromide, iodide, and thiocyanate also denature the protein, which counteracts this effect. This is not the case for chloride and fluoride, thus ArgHCl and ArgHF reduce aggregation solely by reducing protein-protein interactions. However, at high concentrations, the ArgHCl shows a net exclusion (which is likely the case for ArgHF as well but to a lesser extent) and the aggregation suppression ability of ArgHCl plateaus because adding additional ArgHCl molecules at that point does not contribute any to protein-protein interaction suppression due to the additional arginine molecules being partitioned into the bulk solution. Even though ArgHCl shows a net exclusion at high concentrations, there are enough arginine molecules in the local domain of the protein that it significantly reduces protein-protein interactions, thus lowering the relative rate constant to about 0.2. This indicates that the effect from the arginine molecule should be significant for the highly excluded salt forms as well, though the effect of arginine might be reduced some. Furthermore, the aggregation suppression is further enhanced by the stabilizing effect the citrate, sulfate, and dihydrogen phosphate salt forms have on the folding equilibrium, leading to the relative rate constant being further reduced beyond that for ArgHCl. Moreover, another contributing factor may be an increased diffusional barrier. The cluster formation induced by the phosphate, sulfate, and citrate ions likely increases the viscosity of the solution in addition to forming a network of bridged ions around the protein, thus inhibiting the movement of protein molecules.

The clustering due to the presence of hydrogen-bonding anions leads to the formation of large clusters, which are either present within the local domain surrounding the protein or attached to the protein surface via hydrogen-bonds. These clusters of hydrogen-bonded molecules would be difficult to remove from the surface as compared to single molecules within the local domain. In earlier studies, we shown that arginine self-interaction limits the binding of guanidinium group to the protein surface, which explained why arginine is not a

protein destabilizer despite the presence of a denaturing guanidinium group.³² In this study, we have shown the effect of enhanced self-interaction in aqueous arginine salt solutions (due to the use of hydrogen-bonding anions) on protein-protein association.

Conclusions

For arginine salt solutions, we have shown that the interaction between ions and the interaction of the anion with the protein surface influences the preferential interaction coefficient value, which is directly related to the conformational and colloidal stability of the protein. Attractive ion-ion interactions in solution lead to the formation of clusters that are larger in size as compared to the individual ions. These large clusters should be more effective at decreasing protein-protein association than small molecules. Furthermore, clustering should lead to an increase in the viscosity of the solution, which would lower the diffusion of proteins in solution. Therefore, ion-ion interactions affect the rate of aggregation via three mechanisms: 1) enhancing the native state conformational stability, 2) increasing the barrier for protein-protein association, and 3) decreasing protein-protein encounters by increasing the viscosity of the solution medium. These results not only help to elucidate the arginine mechanism, they also have large implications for interpreting the mechanism behind the Hofmeister Series, in that changes in the water structure did not seem to come into play. Rather, ion-ion interactions and other ion specific behavior seem to dominate the behavior of each arginine salt. On a last note, for a more definitive picture of how arginine influences aggregation, the research conducted here should be extended to other proteins and possibly a wider range of pH to better understand what other biophysical effects contribute to the arginine mechanism.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 2.

Snapshots of the MD simulation box containing arginine salts at a molal concentration of 0.5 mol/kg. To improve the clarity of the image, water molecules are not shown and only heavy atoms (all atoms excluding hydrogen) in the arginine molecules and counterions are shown. The following color code is used to represent atoms: C (cyan), O (red), N (blue), S (yellow), Cl (light blue), and P (brown). Arginine molecules are shown in silver.

Figure 3.

Radial distribution functions (RDF) between ion pairs in aqueous arginine salt solutions. The Cation-Cation RDF is the RDF between the guanidinium carbon atoms of arginine. For the counterions, the atoms used as centers for estimating the RDF's are: Sulfate – Sulfur atom, Phosphate – Phosphorus atom, Citrate – Central carbon atom, Thiocyanate – Nitrogen atom and Acetate – Carboxylate carbon atom.

Figure 4. Hydrogen bonding interaction between arginine and (a) acetate, (b) citrate, (c) sulfate and (d) phosphate anions. It can be seen that sulfate, phosphate and citrate can interact with multiple arginine molecules forming large hydrogen-bonded structures. The following color code is used to represent atoms: C (cyan), O (red), N (blue), S (yellow), and P (brown).

Figure 5.

Preferential Interaction Coefficient, Γ_{μ_3} , values for the interaction between arginine salts (and guanidinium chloride for comparison) and aCgn, as determined from VPO measurements. Error bars left off for clarity and curves drawn through the plots to aid the eye (see Table S2 for more detail).

Table I

Summary of aCgn denaturation midpoint temperature increment and Pitzer ion interaction parameter for arginine salts and guanidinium chloride.

Salt	$dT_m/d[3]$ K*L/mol	Max. [3] mol/L	$\beta^{(1)}$ kg/mol
ArgH(H ₂ PO ₄)	6.3	0.2	-1.5303
ArgH(SO ₄) _{1/2}	5.4	0.5	-1.5809
ArgH(Citrate) _{1/2}	3.3	0.5	-1.0850
ArgH(Acetate)	0.0	0.5	N/A
ArgHF	0.0	0.5	N/A
ArgHCl	0.0	0.5	-0.3357
ArgHBr	-7.7	0.5	0.0818
ArgHI	-22.6	0.5	0.2778
ArgHSCN	-24.2	0.5	0.5404
GdmCl	-6.6	0.5	N/A

Table II

McMillan-Mayer Second Virial Coefficient, B_{22} (L/mol), values for ion pairs in aqueous arginine salt solutions.

Arg Salt	C (mol/L)	B_{22}^{++}	B_{22}^{+-}	B_{22}^{--}	Σ_{ij}
(SO ₄) _{1/2}	0.49	-6.58	-4.63	-4.89	-20.73
(Citrate) _{1/2}	0.48	-2.13	-3.24	-0.35	-8.96
(H ₂ PO ₄)	0.48	-3.20	-2.00	-1.60	-8.80
(Acetate)	0.48	-1.80	-1.25	-0.2	-4.50
Cl	0.48	-0.99	-0.90	0.45	-2.35
SCN	0.48	-1.40	-0.90	0.40	-2.80

Table III

Number of hydrogen bonds between different ions in aqueous arginine salt solutions.

Arg Salt	C (mol/L)	Cation-Cation	Cation-Anion	Anion-Anion	Anion-Arg-Anion
(SO ₄) _{1/2}	0.49	42.7	51.3	0	67.3
(Citrate) _{1/2}	0.48	35.8	31.7	0.4	28.2
(H ₂ PO ₄)	0.48	40.6	30.1	11.7	19.5
(Acetate)	0.48	29.3	30.6	0	8.3
Cl	0.48	49.4	6.3	0	0
SCN	0.48	38.2	4.0	0	0

Table IV

Theoretical preferential interaction coefficient values for α -Chymotrypsinogen A in aqueous arginine salt solutions.

Arg Salt	C (mol/L)	Γ^{VPO}	Γ^{MD}	Γ^+	Γ^-	Protein-Arg h-bonds
(SO ₄) _{1/2}	0.5	-5.2±1.7	-4.5	-4	-3	15.3
(Citrate) _{1/2}	0.5	-7.7±1.5	-4.5	-4	-3	16.1
(H ₂ PO ₄)	0.2	-5.7±0.4	-3	-2	-4	10
(Acetate)	0.5	N/A	0	1	-1	19.7
Cl	0.5	-2.6±0.3	-2.5	-1	-4	18.9
SCN	0.48	0.2±1.2	4	4	4	26.5

The error bars on the preferential coefficient values are on the order of ±1