ELSEVIER

Biophysical Chemistry 127 (2007) 1 – 8

Biophysical Chemistry

http://www.elsevier.com/locate/biophyschem

Review

# Suppression of protein interactions by arginine: A proposed mechanism of the arginine effects

Tsutomu Arakawa [a], Daisuke Ejima [b], Kouhei Tsumoto [c,*], Noriyuki Obeyama [c], Yoshikazu Tanaka [c], Yoshiko Kita [d], Serge N. Timasheff [e]

[a] *Alliance Protein Laboratories, Thousand Oaks, CA 91360, United States*
[b] *Amino Science Laboratories, Ajinomoto Co., Inc., Kawasaki, Kanagawa 210-8681, Japan*
[c] *Department of Medical Genome Sciences, Graduate School of Frontier Sciences, The University of Tokyo, Kashiwa, Chiba 277-8562, Japan*
[d] *Department of Pharmacology, KEIO University School of Medicine, Tokyo 160-0004, Japan*
[e] *Graduate Department of Biochemistry, Brandeis University, Waltham, MA 02254, United States*

Received 19 November 2006; received in revised form 12 December 2006; accepted 12 December 2006
Available online 21 December 2006

**Abstract**

Arginine has been used to suppress protein aggregation and protein–protein or protein–surface interactions during protein refolding and purification. While its biotechnology applications are gradually expanding, the mechanism of these effects of arginine has not been fully elucidated. Arginine is more effective at higher concentrations, an indication of weak interactions with the proteins. The effects of weakly interacting additives, such as arginine, on protein solubility, stability and aggregation have been explained from three different approaches: i.e., (1) the effects of additives on the structure of water, (2) the interactions of additives with the amino acid side chains and peptide bonds and (3) the preferential interactions of additives with the proteins. Here we have examined these properties of arginine and compared with those of other additives, e.g., guanidine hydrochloride (GdnHCl) and certain amino acids and amines. GdnHCl is a strong salting-in agent and denatures proteins, while betaine is a protein stabilizer. Several amino acids and amine compounds, including betaine, which stabilize the proteins, are strongly excluded; i.e., the proteins are preferentially hydrated in these solutions. On the other hand, GdnHCl preferentially binds to the proteins. Arginine is intermediate between these two extreme cases and shows a more complicated pattern of interactions with the proteins. The effects of additives on water structure, e.g., the surface tension of aqueous solution of the additives and the solubility of amino acids in the presence of additives also shed light on the mechanism of the effects of the additives on protein aggregation. While arginine increases the surface tension of water, it favorably interacts with most amino acid side chains and the peptide bonds, a property shared with GdnHCl. Thus, we propose that while arginine is similar to GdnHCl in the amino acid level, arginine interacts with the proteins differently from GdnHCl.


*Keywords:* Arginine; Guanidine; Protein aggregation; Preferential interaction; Solubility; Surface tension

**Contents**



*Abbreviations:* GdnHCl, Guanidine hydrochloride; IB, Inclusion body; BSA, Bovine serum albumin; NaGlu, Sodium glutamate; DKP, Diketopiperazine.

* Corresponding author. Tel.: +81 4 7136 5402; fax: +81 4 7136 3601.
*E-mail address:* tsumoto@k.u-tokyo.ac.jp (K. Tsumoto).

0301-4622/$ - see front matter © 2007 Elsevier B.V. All rights reserved.
doi:10.1016/j.bpc.2006.12.007

## 1. Introduction

During the last two decades, arginine has been used for improving refolding efficiency of recombinant proteins produced in *Escherichia coli* as inclusion bodies [1–10]. The effects of arginine on protein refolding are considered to be its ability to suppress aggregation of folding intermediates. We and other groups have shown that aggregation suppression by arginine occurs on the partially unfolded proteins and structure intermediates observed during thermal unfolding or refolding process [11–15]. Arginine now finds wider applications in protein purification and characterization than previously anticipated; e.g., solubilization of proteins from loose "flocculate-type" inclusion bodies (IBs) [16,17], milder elution of antibodies from Protein-A affinity resins [18,19] and improved separation and recovery of proteins in size exclusion, ion exchange and affinity chromatography [20]. However, we have little understanding on the mechanism of these observed effects of arginine on proteins.

Arginine is more effective at higher concentrations. For example, increasing arginine concentration to 2 M resulted in high recovery of antibodies from Protein-A columns above pH 4.0 [18,19]. A requirement for additives at high concentrations means that the interactions between the additive and proteins are weak. The effects of weakly interacting additives on protein solubility, stability and aggregation have been explained primarily from three different approaches: i.e., the interactions of additives with amino acids (amino acid solubility) and proteins (preferential interaction) and the effects of additives on water structure (e.g., surface tension or ion hydration effect). Preferential interaction measurements for various additives with the proteins have shown that they can be used to explain the effects of the additives on the protein solubility and stability [21–30]. Melander and Horvath [31] extended the Traube's observation [32] for the effects of salts on the gas solubility into the cavity theory as a manifestation of the surface tension changes of water by the salts. Nozaki and Tanford [33] pioneered the solubility measurements of amino acids in aqueous solutions of various additives and the thermodynamic analysis of the solvent effects on protein solubility and denaturation. Although limited data for arginine are available [30,34], we will examine the available data here to attempt an elucidation of the mechanism of the effects of arginine on protein aggregation.

## 2. Surface tension effects

Solvent additives affect the structure of water and often classified as water-structure maker or water-structure breaker, thereby affecting protein stability and solubility. In 1918, Traube [32] proposed a theory that the surface tension of aqueous salt solutions determines the effects of the salts on the solubility of inert gasses; i.e., those salts, which increase the surface tension of water more strongly, are more effective in decreasing the gas solubility. Melander and Horvath [31] later expanded this observation into a cavity theory, which correlated the effects of salts on the surface tension of water with their effects on protein solubility. Thus, the surface tension-raising salts should decrease the protein solubility and in fact, many salting-out salts follow this rule.

Does arginine follow this rule? As an aggregation suppressor, it is expected to decrease, or at least not to increase, the surface tension of water. As given in Table 1, arginine increases the surface tension of water. The molal surface tension increment is positive, a value similar to sodium glutamate (NaGlu), which is a strong protein stabilizer and enhances protein–protein interaction and protein aggregation [33,35]. It is evident that the surface tension effect cannot explain the effects of arginine as a suppressor of protein–protein or –surface interactions and protein aggregation. The same is true for a much stronger salting-in solute, GdnHCl. The surface tension of aqueous arginine (open triangle) and GdnHCl (solid diamond) solutions is plotted against molar concentration of the additive [30,36] (Fig. 1). While both arginine and GdnHCl increase the surface tension of water, arginine does so more strongly. Breslow and Guo [36] arrived at a conclusion that the salting-in and denaturing effects of GdnHCl must be due to factors other than the surface tension effects and proposed its binding (i.e., solvation) as a driving force for salting-in or aggregation suppressive effects of GdnHCl. Thus, solute binding for GdnHCl overcomes the unfavorable surface tension effects. As shown in

Table 1
Various parameters of solvent additives examined here

| Compound | Surface tension increment [a] | Radius in Å | Partial specific volume [b] | Molecular weight |
|---|---|---|---|---|
| Water | – | 1.4 [c] (1.9) [d] | (1) [d] | 18 |
| α-alanine | 0.92 | 2.9 | 0.688 | 89 |
| Betaine | – | 3.4 | 0.836 | 117 |
| ArginineHCl | 2.23 [e], see Fig. 1 | 3.8 | 0.671 | 210 |
| Arginine | – | 3.6 | 0.671 [f] | 174 |
| GuanidineHCl | see Fig. 1 | 3.0 | 0.732 | 95.5 |
| Guanidine | – | 2.6 | 0.732 [f] | 59.0 |
| NaGlutamate | 2.58 [g] | 3.3 | 0.529 | 169 |
| Glutamic acid | – | 3.1 | 0.529 [f] | 147 |

[a] The surface tension increments for other salts are KSCN (0.45), NaCl (1.64), $Na_2SO_4$ (2.73), $MgCl_2$ (3.16), $MgSO_4$ (2.10). Taken from Ref. [31]).
[b] When the partial specific volume showed a significant concentration dependence, the value at dilute solution was used.
[c] Taken from Ref. [35]).
[d] The radius, 1.9 Å, was calculated based on the partial specific volume of water as 1 ml/mg.
[e] The surface tension of aqueous arginine solution is concentration-dependent and the value shown is at dilute arginine solution (slope at 0.2 M). The surface tension increment sharply decreases with arginine concentration.
[f] The partial specific volume of basic or acidic form was assumed to be identical to their salt form.
[g] The surface tension linearly increased with sodium glutamate concentration.

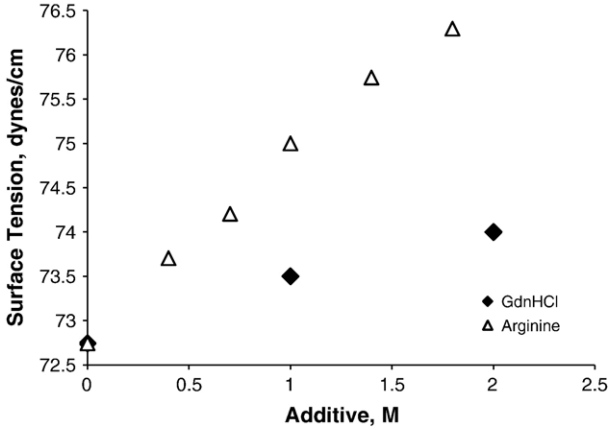


Fig. 1. The surface tension of aqueous arginine and GdnHCl solution. Arginine data taken from Ref. [30]. GdnHCl data from Ref. [37].

Fig. 1, arginine increases the surface tension more effectively than does GdnHCl, suggesting that it would require more binding energy for arginine than for GdnHCl to exert the salting-in effects of similar magnitude. Alternatively, if arginine and GdHCl are similar in affinity for proteins, the overall salting-in effects on proteins should be stronger for GdnHCl; GdnHCl increases the surface tension less than does arginine. Although arginine increases the surface tension of water, positively charged amino acid side chain, such as arginine side chain, has been shown to be chaotropic (a property characteristic of water structure breaker) due to its weak hydration potential [37].

A similar argument was developed to explain the salting-in effects of $MgCl_2$, which increases the surface tension of water as does arginine [24,26]. In fact, $MgCl_2$ is much more effective in increasing the surface tension of water than GdnHCl (Table 1): in fact, the surface tension increment of $MgCl_2$ is more positive than the strong salting-out salts, i.e., $Na_2SO_4$ and $MgSO_4$. By the same argument, $MgCl_2$ must bind to the proteins to overcome the increased surface tension. The preferential interaction measurements confirmed the binding of $MgCl_2$ to the proteins and in fact were successfully used to interpret the salting-in effects of this particular salt [24,26]. Therefore, the following two sections describe the binding of arginine to the proteins.

## 3. Amino acid solubility

Having established that arginine binding should overcome the unfavorable surface tension effects, we experimentally determined the solubility of amino acids in aqueous arginine solution. The solubility change by the addition of additives reflects the interaction of the additives with the amino acids. If the interaction is favorable between the additive and the amino acids (i.e., the additive binding occurs), then the additive should increase the solubility. On the other hand, the interaction is unfavorable (the additive is excluded or water binding is more favorable), the additive should decrease the solubility. The results of amino acid solubility in 1 M arginine are summarized in Fig. 2 (the experimental detail will be published elsewhere). Briefly, the solubility was determined using densimetry to measure the concentration of amino acids in aqueous arginine solution as a function of the amount of each amino acid added [26]. The inflection point, at which the density becomes constant, was used as a point of saturation. The data show the transfer free energy of amino acids from water to 1 M solution of arginine; i.e., it is the free energy required to transfer an amino acid from water to 1 M arginine solution. For comparison, the transfer free energies of amino acids in GdnHCl, and of a few amino acids in $MgCl_2$ (salting-in salt) and $MgSO_4$ (salting-out salt) at 1 M are also shown. The downward bar in the graph means that the interaction is favorable (decrease in free energy), while the upward bar means the unfavorable interaction (increase in free energy). A glance of the data reveals two striking features; i.e., 1) a majority of amino acids interact favorably with arginine (white bar) and GdnHCl (black bar) and 2) the interaction pattern is very similar between arginine and GdnHCl. Only amino acid, which showed a significant positive transfer free energy (unfavorable interaction with arginine and GdnHCl), was Val. Thus, when the effect of arginine on the solubility of an amino acid is stronger, it is true for GdnHCl. In other words, these two co-solutes are similar in interacting with the amino acids and hence their side chains. Both arginine and GdnHCl interact with aromatic amino acids more favorably than with other amino acids. Arginine appears to interact more favorably with Tyr and Trp than does GdnHCl. The interactions of arginine and GdnHCl with hydrophobic amino acids, Leu, Ile and Val, are less favorable or even unfavorable, while those with hydrophilic amino acids are intermediate (moderately favorable). These results suggest that arginine and GdnHCl are similar in binding the amino acid side chains; i.e., favorable interactions with the most amino acid side chains, in particular aromatic side chains. Such favorable interaction should be reflected on their bindings to protein surfaces.

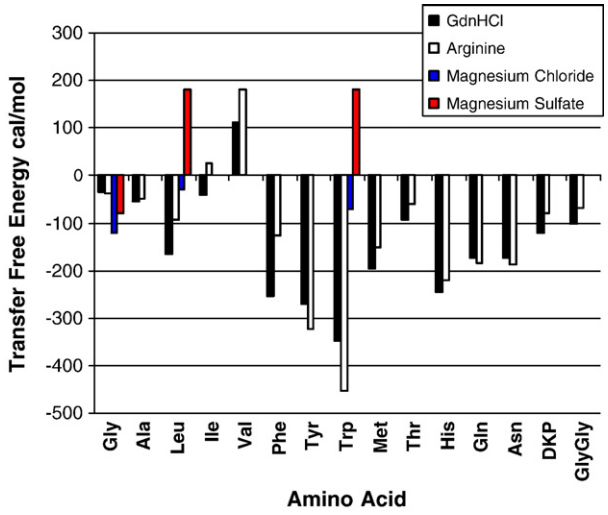


Fig. 2. The solubility of amino acids in 1 M additive solution. The data are expressed as transfer free energy, calculated by $\Delta G_{transfer} = -RT \ln (S/S_{water})$, where $\Delta G_{transfer}$ is the transfer free energy of amino acid from water to additive solution, $R$ is the universal gas constant, $T$ is the absolute temperature and $S$ and $S_{water}$ are the solubility of amino acid in additive solution and water (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.).

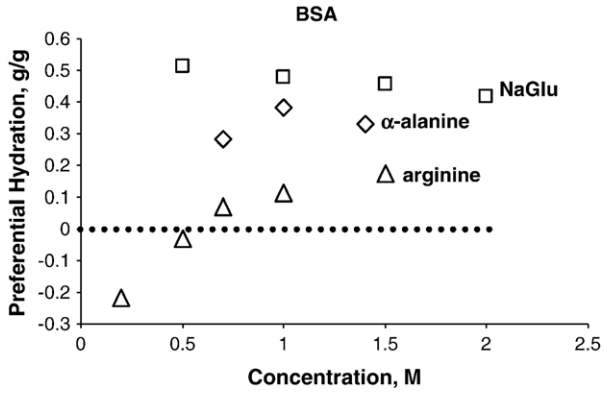


Fig. 3. Preferential hydration of bovine serum albumin (BSA). This figure shows binding of arginine to BSA at the concentration of less than 0.5 M. The preferential hydration, if any, is much lower in arginine than in α-alanine or sodium glutamate. The latter two amino acids show preferential hydration greater than 0.3 g/g, suggesting that they may be excluded from the protein surface. Square, sodium glutamate; diamond, α-alanine; triangle, arginine. Taken from Refs. [23,30,38].

$MgCl_2$ was also suggested to bind to the proteins [26]. Fig. 2 indicates that the mechanism of its binding to the protein surface must be different from arginine and GdnHCl. As shown in Fig. 2 (blue bar), the interaction pattern, although limited to only three amino acids, is different from that of arginine and GdnHCl. First, $MgCl_2$ increases the solubility of glycine more than arginine. Since glycine is a dipolar and the solubility should increase with ionic strength, the results indicate stronger ionic nature of $MgCl_2$, which may be consistent with its divalent property. Thus, $MgCl_2$ may interact favorably with charged and polar side chains. Another striking contrast with $MgCl_2$ is its effect on the Trp solubility, which is in the same order of magnitude for the solubility of glycine; i.e., there is no favorable interaction between $MgCl_2$ and Trp, completely different from arginine and GdnHCl. Thus, the mechanism of salting-in effects of $MgCl_2$ must be different from the mechanism for arginine and GdnHCl, which operates in their salting-in effects on proteins. However, the amino acid solubility clearly demonstrates that $MgCl_2$ is not a salting-out salt, since a typical salting-out salt, $MgSO_4$, shows a strong unfavorable interaction with Leu and Trp (red bar), while the interactions for $MgCl_2$ are nearly neutral. These results demonstrate that arginine and GdnHCl interact favorably with the majority of amino acid side chains. Their interactions with peptide bond, i.e., diketopiperazine (DKP), are slightly favorable, consistent with the favorable interactions with GlyGly. Would these favorable interactions be manifested on binding to the protein surface? This topic is discussed in the next section.

## 4. Preferential interaction

Preferential interaction is the manifestation of widely different mode of bindings ranging from weak transient interactions to strong, stoichiometric interactions of solvent components with the protein surface in the native state and hence reflects the interactions of the additives with the amino acid side chains and peptide bonds as described in the previous section. Preferential interaction data have been successfully used to explain the effects of salting-out and salting-in additives or protein stabilizing and destabilizing additives [21–30]. Those with salting-out and stabilizing effects show that they are preferentially excluded from the surface of the native proteins. Those with salting-in and destabilizing effects are characterized by their preferential binding to the proteins. Does arginine follow this rule; i.e., does arginine show preferential binding? The preferential interaction data were assembled and reorganized from the published reports [25,28,30,38]. The preferential interactions of a neutral amino acid, α-alanine, and an acidic amino acid, sodium glutamate (NaGlu), were compared with the data for arginine. Fig. 3 shows the preferential hydration of bovine serum albumin (BSA) in aqueous solution containing sodium glutamate (NaGlu), α-alanine and arginine as a function of additive concentration. Before discussing the data, it is necessary to show how the preferential hydration is related to the preferential interaction (binding) of the additive. Eq. (1) shows a relationship between preferential hydration, $(\partial g_1/\partial g_2)$, and preferential additive binding, $(\partial g_3/\partial g_2)$.

$$(\partial g_1/\partial g_2) = -(1/g_3)(\partial g_3/\partial g_2) \quad (1)$$

where $g_1$, $g_2$ and $g_3$ are the concentration of water, protein and additive in grams per gram [39–42]. The constancy of temperature and pressure is omitted. Those additives, which are preferentially excluded from the protein surface and have a negative value of preferential additive interaction parameter, exhibit a positive value of preferential hydration; i.e., those with negative $(\partial g_3/\partial g_2)$ show a positive value of $(\partial g_1/\partial g_2)$. Fig. 3 plots the $(\partial g_1/\partial g_2)$, instead of binding parameter, $(\partial g_3/\partial g_2)$, against the additive concentration. This value for arginine is initially negative and gradually increases to a positive value as the concentration is increased. Fig. 3 also shows the data for NaGlu and α-alanine. In contrast to arginine, these two amino acids show a nearly constant preferential hydration for BSA. In addition, the preferential hydration is larger in these two amino acid solutions than the highest value achieved for arginine at 1.5 M. It is evident that the preferential hydration pattern is different between arginine and the other two amino acids.

Is such a difference specific to BSA? Fig. 4 shows preferential hydration of ribonuclease and lysozyme in arginine solutions. Both proteins are similarly preferentially hydrated in arginine solution. The results are in contrast to the concentration-dependent interaction of arginine with BSA in arginine solution; i.e., ribonuclease and lysozyme are always preferentially hydrated over the wide range of arginine concentration. The difference between BSA and ribonuclease/lysozyme may be due to the isoelectric point of the proteins; BSA is a slightly acidic protein and both ribonuclease and lysozyme are basic proteins. However, preferential hydration with ribonuclease and lysozyme in arginine is much smaller than in α-alanine or betaine, which are also plotted in Fig. 4. Betaine has a tertiary methyl group and a carboxyl group, but has no side chain. Both betaine and α-alanine are protein stabilizers.

Next, the preferential interaction of arginine was compared with a stronger salting-in additive GdnHCl. The preferential

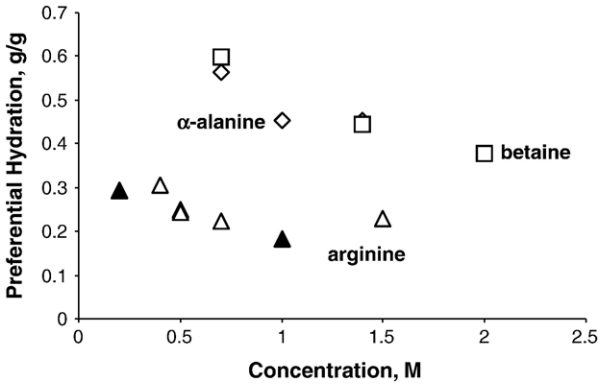


Fig. 4. Preferential hydration of lysozyme and ribonuclease in arginine, α-alanine and betaine solutions. Solid triangle, ribonuclease in arginine; Open triangle, lysozyme in arginine; open square, lysozyme in betaine; open diamond, lysozyme in α-alanine. Data taken from Refs. [25,34].

interaction of GdnHCl with lysozyme was determined at different GdnHCl concentrations by equilibrium dialysis and density measurement (Arakawa, T. and Timasheff, S.N., unpublished). Due to its solubility limit, such data for arginine is available only up to 1.5 M. In Fig. 5 is plotted the preferential binding parameters of GdnHCl and arginine with lysozyme. The results for GdnHCl and arginine were totally opposite. Above 1 M, preferential interaction of GdnHCl is positive and increases as the GdnHCl concentration is increased. Lysozyme unfolds at about 3–6 M GdnHCl, which parallels the increased preferential GdnHCl binding. The results clearly indicate that above 1 M, there are more GdnHCl molecules in the vicinity of protein surface relative to the GdnHCl concentration in the bulk phase, i.e., the protein molecule has higher affinity for GdnHCl than for water, in particular at the higher GdnHCl concentration where the protein is unfolded. This is consistent with the fact that all amino acid side chains and peptide groups interact favorably with GdnHCl and such favorable interactions are greater at higher GdnHCl concentration. Conversely, arginine exhibits negative preferential binding to lysozyme, i.e., the overall interaction between arginine and the native lysozyme is unfavorable. This shows that arginine interactions, if any, with amino acid side chains or peptide groups, are more limited on the protein level than the interactions observed for GdnHCl. It is interesting to note that on the amino acid level (i.e., amino acid solubility), these two additives are similar. Negative preferential binding of arginine does not, however, necessarily mean that arginine does not bind to lysozyme, as described above.

Preferential interactions are a balance between the interactions of the additive and water molecules to the protein surface, i.e., this difference reflects binding of arginine. Namely,

$$(\partial g_1/\partial g_2) = A_1 - (1/g_3)A_3 \tag{2}$$

$$(M_3/M_2)(\partial m_3/\partial m_2) = (\partial g_3/\partial g_2) = A_3 - g_3 A_1 \tag{3}$$

where $A_3$ is the amount of additive bound and $A_1$ is the amount of water bound (or layer of water at the protein vicinity), both expressed as grams per gram protein [39–42]. $(\partial m_3/\partial m_2)$ is the preferential binding of additive in moles per mol and $M_3$ and $M_2$ are the molecular weights of the additive and protein. As Eq. (2) indicates, preferential interaction is the difference in concentration of additive at the protein surface and its concentration in the bulk phase. Thus, when $A_3$ is zero, i.e., no binding of the additives, the preferential hydration, $(\partial g_1/\partial g_2)$, becomes equal to $A_1$, meaning that a relatively constant value of $(\partial g_1/\partial g_2)$ can be observed, provided that the additive does not interfere with the hydration, $A_1$. From the Eq. (2),

$$A_3 = g_3 A_1 + (\partial g_3/\partial g_2) \tag{4}$$

and hence when there is hydration (or a layer of water around the protein surface) and $A_1 > 0$, it is possible that $A_3$ is positive even when $(\partial g_3/\partial g_2)$ is negative as is observed for arginine. Calculation of $A_3$ from the experimental value of $(\partial g_3/\partial g_2)$ requires knowledge of $A_1$. What is the reasonable value of $A_1$ for proteins?

## 5. Protein hydration

Determination of the absolute additive binding requires knowledge of protein hydration, which cannot be explicitly determined, as it depends on the definition of hydration. There are at least three definitions of protein hydration: 1) Hydration due to surface tension, 2) Excluded volume and 3) Water adsorption (binding). The first two mechanisms are not associated with the actual affinity of water for protein and occur only in the presence of the additives, which have tendency to stay away from the protein surface. As described in the previous section, those salts with the positive surface tension increment in water interact unfavorably with the interface (or protein surface). This unfavorable interaction must be overcome by binding of arginine or GdnHCl (solvation) to have salting-in or aggregation-suppressive effects on proteins, as proposed by Breslow and Guo [36]. Such unfavorable interaction due to the surface tension increase must have a consequence that they are excluded from the protein surface

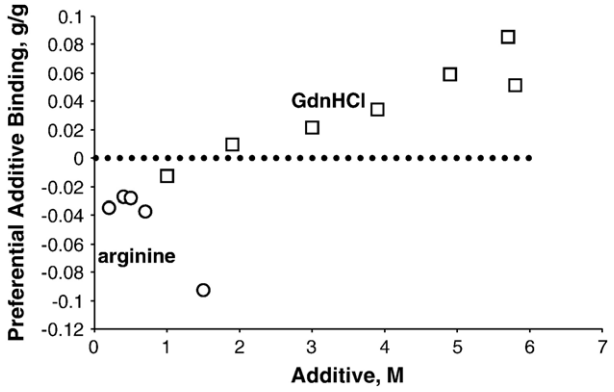


Fig. 5. Preferential interaction of arginine (circle) and GdnHCl (square) with lysozyme. Arginine data taken from Ref. [30]. The data for GdnHCl were determined by equilibrium dialysis and density measurements (Arakawa, T. and Timasheff, S.N., unpublished).

(Fig. 6A). Such relation can be shown by a Gibbs adsorption isotherm Eq. (3) [43]

$$A_1 = K(\partial\sigma/\partial g_3) \quad (5)$$

where $\sigma$ is the surface tension and $(\partial\sigma/\partial g_3)$ is the surface tension increment of water by the additive and $K$ is the constant related to the surface area of the protein (or water–air interface). Eq. (5) shows that those, which increase the surface tension of water, have an excess of water, $A_1$, around the protein surface. Opposite to this are the detergents, which decrease the surface tension of water and hence bind to the interface. In this case, hydration value, $A_1$, depends on the surface tension increment of the additive. Those additives, which raise the surface tension more effectively, should have higher hydration. In this case, arginine should have a larger hydration than GdnHCl.

Most additives used for protein solution have a molecular size greater than water molecule and hence excluded from the protein surface. Even water molecule is considered to be excluded from the protein surface due to its size [44], although different radius of water molecules has been estimated (Table 1). This exclusion of water molecule reduces the volume of Brownian moment and hence entropy, which was proposed to cause protein folding; protein folding reduces the volume of water exclusion and hence increases the translational entropy [44]. As shown in Fig. 6B, there is a layer around the protein surface, from which water is excluded (dotted line), and the layer of additive exclusion (solid line), from which the additive is excluded. From the difference in the radius between water and additive molecules, the excluded volume, i.e., $A_1$, can be calculated. In this case, hydration, $A_1$, depends on the radius of the additive. Arginine (3.6 Å) has a larger radius than GdnHCl (3 Å) and NaGlu (3.3 Å)(see Table 1). This means that arginine should have a larger $A_1$ value than these latter additives. It should be noted here that these radii are calculated as a neutral salt form. What is excluded from the protein surface is an ionic form and hence the excluded volume of the salts, including arginine, may not be well defined.

The third mechanism of hydration is actual water binding. Proteins are hydrated in aqueous solution. Hydration studies of protein powders indicate that such biochemical properties as enzyme activities are regained when they absorb 0.2–0.3 g water per g protein [45,46]. Frozen NMR also indicated about 0.3 g of unfreezable water, which cannot form ice crystal due to binding to the protein surface [47,48]. There is no absolute evidence that such a level of hydration is maintained in the presence of additives at high concentration, although frozen NMR in urea suggested the similar water binding [48]. Assuming hydration of 0.3 g/g, none of the structure-stabilizing additives shown here (glycine, alanine, betaine and NaGlu) bind to the proteins; they have $(\partial g_1/\partial g_2)>0.3$ and hence $A_3<0$. In other words, these additives do not bind to the proteins, rather they are excluded from the protein surface, perhaps due to the excluded volume or the surface tension effect. Conversely, arginine and GdnHCl have $(\partial g_1/\partial g_2)$ smaller than 0.3 g/g and hence bind to the proteins. As has been described here, we do not know what would be the value of hydration to be used to calculate the additive binding using Eq. (4).

## 6. Protein–protein interactions and its suppression

How can we put together these data to explain the effect of arginine on protein–protein, protein–surface interactions and protein aggregation? First, it should be realized that the protein–protein or protein–surface interactions are complex. There are multitudes of interaction forces between the two proteins or between the protein and solid surface; hydrogen bonding, ionic

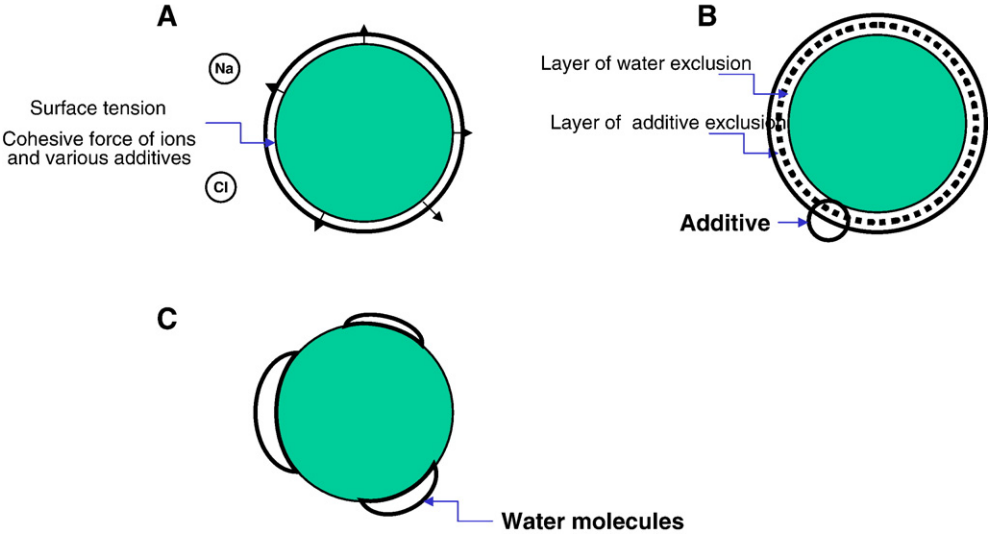


Fig. 6. Various sources of protein hydration. A. Hydration depends on the surface tension of additive in water. Ions move away from the protein surface to maximize hydration. B. Hydration depends on the radius of additive. PEG is excluded via this mechanism. C. Hydration depends on the amount of water bound: 0.2–0.3 g/g protein is observed by various techniques.

interactions and hydrophobic interactions are all involved in these types of protein interactions. High ionic strength can weaken hydrogen bonding and ionic interactions, but may enhance hydrophobic interactions. Conversely, organic solvents can weaken hydrophobic interactions, but may strengthen hydrogen bonding and ionic interactions. The solubility measurements of amino acids in 1 M arginine and GdnHCl showed that these additives favorably interact with almost all the amino acids side chains (both hydrophobic and hydrophilic) and peptide bonds, meaning that they can reduce both electrostatic (hydrogen bonding and ionic interactions) and hydrophobic interactions.

It is evident that the surface tension effect cannot explain the salting-in effects of arginine (and GdnHCl), which increases the surface tension of water. They should be excluded from the protein surface, meaning that their interactions with the protein surface are unfavorable. As Breslow and Guo [36] pointed out, these additives must have affinity for protein surface, which overcomes the unfavorable surface tension effect. In this regard, GdnHCl could be stronger than arginine in salting-in or suppressing protein interactions, since GdnHCl with lower surface tension increment requires less favorable affinity for the protein surface than does arginine. In the same token, the excluded volume effect also points the unfavorable interaction of arginine and GdnHCl with the protein surface ($A_1>0$), which is also greater for arginine than for GdnHCl. The amino acid solubility measurements showed that the interactions of arginine and GdnHCl in 1 M solution with most of amino acid side chains are highly favorable and of similar magnitude. Such high affinity for amino acid side chains renders arginine and GdnHCl bind to the protein surface, overcoming unfavorable factor due to the surface tension and excluded volume effects. It is most likely the difference in these unfavorable interaction factors that makes GdnHCl stronger than arginine in suppressing protein–protein interactions, since both the surface tension and excluded volume effects are smaller for GdnHCl.

Preferential interaction measurements also indicated binding of these additives to the protein surface. How does arginine binding translate into its effect on protein dissociation and aggregation suppression? How does arginine differ from GdnHCl? As described above, calculation of additive binding requires knowledge of hydration, $A_1$, which depends on definition. It is evident that the protein-stabilizing additives shown here, α-alanine, betaine and NaGlu, do not bind to the proteins, independent of the definition. Conversely, arginine and GdnHCl bind to the proteins, the magnitude of which depends on the value of $A_1$ used. Here we decided to use hydration value. Using $A_1=0.3$, the binding of arginine to BSA was calculated from the preferential interactions of Eq. (3). The calculation leads to an estimate of about 6–9 molecules of arginine bound per BSA molecule. Lysozyme and ribonuclease, which were always preferentially hydrated in arginine solution (Fig. 4), were calculated to bind only a few arginine molecules. This argument, of course, depends on the definition of $A_1$: an assumption of larger $A_1$ value leads to greater additive bindings.

How does such binding correlate with the effects of arginine? Binding of additives is related to the free energy change of the protein by Eq. (6) as [42,49]

$$(\partial\mu_2/\partial m_3) = -(RT/m_3)(\partial m_3/\partial m_2) \quad (6)$$

where the $(\partial\mu_2/\partial m_3)$ is the free energy due to preferential interaction, $R$ is the gas constant, $T$ is the absolute temperature, and $m_3$ is the molar concentration of the additive. Such free energy change of protein can be related to the equilibrium reaction between the two states, $A$ and $B$, as

$$A \Leftrightarrow B$$

where $K=[B]/[A]$ is the equilibrium constant. Let's examine the dissociation reaction, e.g., of antibody from Protein-A, in which an antibody molecule is either in dissociated state (state $B$) or bound (state $A$) to Protein-A. The free energy of both $A$ and $B$ will change upon transfer to aqueous arginine solution, depending on how these two states of the protein interact with arginine. The associated state is expected to have less arginine binding than the dissociated state. Assuming no change in hydration between the two states, it should have a consequence that $(\partial m_3/\partial m_2)^B>(\partial m_3/\partial m_2)^A$. This means that $(\partial\mu_2/\partial m_3)^A>(\partial\mu_2/\partial m_3)^B$ (i.e., the free energy change is more negative for the dissociated state than the associated state). Assuming that the dissociated antibody has one more arginine binding relative to the bound state at 0.5 M arginine concentration, the free energy change will be −1100 cal/mol of protein in each mole of arginine binding. This will translate dissociation constant increase of 5.4. If binding of five more arginine molecules occurs upon dissociation, then dissociation constant increases by 5400. This example calculation should apply to protein–protein or protein–surface interaction, as long as the dissociated state has more arginine binding sites. GdnHCl which showed preferential binding to the protein surface, should have much larger binding on the same assumption of $A_1=0.3$ (100–200 molecules per protein at 6 M GdnHCl).

## 7. Conclusion

Arginine is a versatile solvent additive for protein refolding, aggregation suppression and column chromatography. Arginine interacts favorably with a majority of amino acid side chains, similarly to GdnHCl. On the contrary, binding of arginine to the protein surface is limited, different from the binding of GdnHCl. Such limited binding of arginine plays a major role in its ability to suppress aggregation of, but not to destabilize the proteins.

## References

[1] J. Buchner, R. Rudolph, Renaturation, purification and characterization of recombinant Fab-fragments produced in *Escherichia coli*, Biotechnology 9 (1991) 157–162.

[2] U. Brinkman, J. Buchner, I. Pastan, Independent domain folding of *Pseudomonas* exotoxin and single-chain immunotoxins: influence of interdomain connections, Proc. Natl. Acad. Sci. U. S. A. 89 (1992) 3075–3079.

[3] D. Arora, N.J. Khanna, Method for increasing the yield of properly folded recombinant human gamma interferon from inclusion bodies, J. Biotechnol. 52 (1996) 127–133.
[4] K. Tsumoto, K. Shinoki, H. Kondo, M. Uchikawa, T. Juji, I. Kumagai, Highly efficient recovery of functional single-chain Fv fragments from inclusion bodies overexpressed in *Escherichia coli* by controlled introduction of oxidizing reagent-application to a human single-chain Fv fragment, J. Immunol. Methods 219 (1998) 119–129.
[5] M. Umentsu, K. Tsumoto, M. Hara, K. Ashish, S. Goda, T. Adschiri, I. Kumagai, How additives influence the refolding of immunoglobulin-folded proteins in a stepwise dialysis system. Spectroscopic evidence for highly efficient refolding of a single-chain Fv fragment, J. Biol. Chem. 279 (2003) 8979–8987.
[6] S. Bell, S. Hansen, J. Buchner, Refolding and structural characterization of the human p53 tumor suppressor protein, Biophys. Chemist. 96 (2002) 243–257.
[7] W.J. Lin, J.A. Traugh, Renaturation of casein kinase II from recombinant subunits produced in *Escherichia coli*: purification and characterization of the reconstituted holoenzyme, Protein Expr. Purif. 4 (1993) 256–264.
[8] M.H. Hsih, J.C. Kuo, H.J. Tsai, Optimization of the solubilization and renaturation of fish growth hormone produced by *Escherichia coli*, Appl. Microbiol. Biotechnol. 48 (1997) 66–72.
[9] E.D.B. Clark, E. Schwarz, R. Rudolph, Inhibition of aggregation side reactions during in vitro protein folding, Methods Enzymol. 309 (1999) 217–237.
[10] D.L. Havehan, E.D.B. Clark, Oxiditative renaturation of lysozyme at high concentrations, Biotechnol. Bioeng. 54 (1997) 221–230.
[11] T. Arakawa, K. Tsumoto, The effects of arginine on refolding of aggregated proteins: not facilitate refolding, but suppress aggregation, Biochem. Biophys. Res. Commun. 304 (2003) 148–152.
[12] K. Shiraki, M. Kudou, S. Fujiwara, T. Imanaka, M. Takagi, Biophysical effect of amino acids on the prevention of protein aggregation, J. Biochem. 132 (2002) 591–595.
[13] M. Kudou, K. Shiraki, S. Fujiwara, T. Imanaka, M. Takagi, Prevention of thermal inactivation and aggregation of lysozyme by polyamines, Eur. J. Biochem. 279 (2003) 4547–4554.
[14] K.R.C. Reddy, H. Lilie, R. Rudolph, C. Lange, L-arginine increases the solubility of unfolded species of hen egg white lysozyme, Protein Sci. 14 (2005).
[15] K. Tsumoto, M. Umetsu, I. Kumagai, J.S. Philo, T. Arakawa, Role of arginine in protein refolding, solubilization, and purification, Biotechnol. Prog. 20 (2004) 1301–1318.
[16] K. Tsumoto, M. Umetsu, I. Kumagai, D. Ejima, T. Arakawa, Solubilization of active green fluorescent protein from insoluble particles by guanidine and arginine, Biochem. Biophys. Res. Commun. 312 (2003) 1383–1386.
[17] M. Umetsu, K. Tsumoto, S. Nitta, T. Adschiri, D. Ejima, T. Arakawa, I. Kumagai, Non-denaturing solubilization of beta2 microglobulin from inclusion bodies by arginine, Biochem. Biophys. Res. Commun. 328 (2005) 189–197.
[18] T. Arakawa, J.S. Philo, K. Tsumoto, R. Yumioka, D. Ejima, Elution of antibodies from a Protein-A column by aqueous arginine solutions, Protein Expr. Purif. 36 (2004) 244–248.
[19] D. Ejima, R. Yumioka, K. Tsumoto, T. Arakawa, Effective elution of antibodies by arginine and arginine derivatives in affinity chromatography, Anal. Biochem. 345 (2005) 250–257.
[20] D. Ejima, R. Yumioka, T. Arakawa, K. Tsumoto, Arginine as an effective additive in gel permeation chromatography, J. Chromatogr. A. 1094 (2005) 49–55.
[21] J.C. Lee, S.N. Timasheff, Partial specific volumes and interactions with solvent components of proteins in guanidine hydrochloride, Biochemistry 13 (1974) 257–265.
[22] J.C. Lee, S.N. Timasheff, The stabilization of proteins by sucrose, J. Biol. Chem. 256 (1981) 7193–7201.
[23] K. Gekko, S.N. Timasheff, Mechanism of protein stabilization by glycerol: preferential hydration in glycerol–water mixtures, Biochemistry 20 (1981) 4667–4676.
[24] T. Arakawa, S.N. Timasheff, Stabilization of protein structure by sugars, Biochemistry 21 (1982) 6536–6544.
[25] T. Arakawa, S.N. Timasheff, Preferential interactions of proteins with solvent components in aqueous amino acid solutions, Arch. Biochem. Biophys. 224 (1983) 169–177.
[26] T. Arakawa, S.N. Timasheff, Mechanism of protein salting in and salting out by divalent cation salts: balance between hydration and salt binding, Biochemistry 23 (1984) 5912–5923.
[27] T. Arakawa, T. Timasheff, Protein stabilization and destabilization by guanidinium salts, Biochemistry 23 (1984) 5924–5929.
[28] T. Arakawa, S.N. Timasheff, The stabilization of proteins by osmolytes, Biophys. J. 47 (1985) 411–414.
[29] T. Arakawa, R. Bhat, S.N. Timasheff, Why preferential hydration does not always stabilize the native structure of globular proteins, Biochemistry 29 (1990) 1924–1931.
[30] Y. Kita, T. Arakawa, T.Y. Lin, S.N. Timasheff, Contribution of the surface free energy perturbation to protein–solvent interactions, Biochemistry 33 (1994) 15178–15189.
[31] W. Melander, C. Horvath, Salt effect on hydrophobic interactions in precipitation and chromatography of proteins: an interpretation of the lyotropic series, Arch. Biochem. Biophys. 183 (1977) 200–215.
[32] J. Traube, Theory of attraction pressure, J. Phys. Chem. 14 (1910) 453–475.
[33] Y. Nozaki, C. Tanford, The solubility of amino acids, giglycine, and triglycine in aqueous guanidine hydrochloride solutions, J. Biol. Chem. 245 (1970) 1648–1652.
[34] T.Y. Lin, S.N. Timasheff, On the role of surface tension in the stabilization of globular proteins, Protein Sci. 5 (1996) 372–381.
[35] L.O. Narhi, Y. Kita, T. Arakawa, Hydrophobic interaction chromatography in alkaline pH, Anal. Biochem. 182 (1989) 266–270.
[36] R. Breslow, T. Guo, Surface tension measurements show that chaotropic salting-in denaturants are not just water structure breakers, Proc. Natl. Acad. Sci. U. S. A. 87 (1990) 167–169.
[37] K.D. Collins, Ion hydration: implications of cellular function, polyelectrolytes, and protein crystallization, Biophys. Chemist. 119 (2006) 271–281.
[38] T. Arakawa, S.N. Timasheff, The mechanism of Na glutamate, lysine HCl, and piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) in the stabilization of tubulin and microtubule assembly, J. Biol. Chem. 259 (1984) 4979–4986.
[39] H. Inoue, S.N. Timasheff, The interaction of beta-lactoglobulin with solvent components in mixed water–organic solvent systems, J. Am. Chem. Soc. 90 (1968) 1890–1898.
[40] S.N. Timasheff, H. Inoue, Preferential binding of solvent components to proteins in mixed water–organic solvent systems, Biochemistry 7 (1968) 2501–2513.
[41] H. Inoue, S.N. Timasheff, Preferential and absolute interactions of solvent components with proteins in mixed solvent systems, Biopolymers 11 (1972) 737–743.
[42] J.C. Lee, K. Gekko, S.N. Timasheff, Measurements of preferential solvent interactions by densimetric techniques, Methods Enzymol. 61 (1979) 26–49.
[43] J.W. Gibbs, On the equilibrium of heterogeneous substances, Conn. Acad. Arts Sci. 3 (1878) 343–524.
[44] Y. Hirano, M. Kinoshita, Large gain in translational entropy of water is a major driving force in protein folding, Chem. Phys. Lett. 399 (2004) 342–348.
[45] G. Careri, E. Gratton, P.H. Yang, J.A. Rupley, Correlation of IR spectroscopic, diamagnetic susceptibility and enzymatic measurements on lysozyme powder, Nature 284 (1980) 572–573.
[46] P. Yang, J.A. Rupley, Protein–water interactions. Heat capacity of the lysozyme–water system, Biochemistry 18 (1979) 2654–2661.
[47] I.D. Kuntz, T.S. Brassfield, G.D. Law, G.V. Purcell, Hydration of macromolecules, Science 163 (1969) 1329–1331.
[48] I.D. Kuntz Jr., T.S. Brassfield, Hydration of macromolecules. II. Effects of urea on protein hydration, Arch. Biochem. Biophys. 142 (1971) 660–664.
[49] J. Wyman, Linked functions and reciprocal effects in hemoglobin: a second look, Adv. Protein Chem. 19 (1964) 223–286.