Review

Potential application of arginine in interaction analysis

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ABSTRACT: Aqueous solution of 0.1-2 M arginine at mildly acidic to neutral pH is widely used in biotechnology and protein research, including protein refolding, purification, and formulation. This is largely because of its ability to suppress non-specific protein-protein and protein-surface interactions. Here we propose potential applications of arginine in interaction analysis for proteins. One of the important goals of such analysis is discovery of small molecule antagonistic or agonistic ligands that bind to target proteins and thereby modulate their function. Such research is often hampered by the low solubility of the small molecules, the instability of target proteins and the non-specific protein-ligand interactions. Aqueous arginine solution increases the solubility of small molecules, which should give an alternative to conventional dissolution method of small molecules by organic solvents. Arginine may also directly impact on the analysis of protein-protein or protein-ligand interactions by suppressing weak non-specific interactions.

Keywords: Small molecule, solubility, arginine, interaction analysis, aggregation suppression

1. Introduction

One of the important goals of protein-ligand interaction studies is discovery of novel small molecule ligands that bind to the target proteins and affect their functions (*1*). Such small molecules should help understand *in vitro* and *in vivo* functional roles of the proteins and may be developed as a therapeutic drug. A major problem in the analysis of ligand-protein interaction is a poor aqueous solubility of small molecules, instability of target proteins and non-specific ligand-protein interactions. There have been a number of studies to increase the solubility of small molecule drug substances, *e.g.*, micronization (*2*), generation of crystalline formulation (*3*), salt form with hydrophilic counterions (*4*), or formulation with delivery vehicles (*5-7*). Solvent additives, such as arginine, have also been used to enhance the solubility of the drug substances that had been formulated with cyclodextrins (*8,9*).

Aqueous solution of 0.1-2 M arginine at mildly acidic to neutral pH is widely used in biotechnology field and protein research, including protein refolding, purification and formulation (*10-16*). While arginine is not a protein stabilizer, it suppresses aggregation of proteins and non-specific interaction of proteins with chromatographic resins (*17-21*). In addition, arginine has been observed to increase the solubility of small molecules (*22-24*). In this review we wish to propose that arginine may prevent non-specific interactions between the small molecules and proteins and aggregation of the target proteins during interaction analysis, and thereby find applications in the drug discovery research.

2. Effects of arginine on small molecules

There is no systematic study of the effects of arginine on the solubility of small molecules and hence we summarize below the data mostly from our laboratories. Coumarin is used as an anti-coagulant and has a poor aqueous solubility (*25,26*). It is a small organic compound with a molecular weight of 146.15 and contains an aromatic ring structure (see Figure 1 for chemical structure). Figure 2 plots the solubility of coumarin as a function of additive concentration in 50 mM citrate-phosphate buffer, pH 7.5. It is evident that arginine and guanidine hydrochloride (GdnHCl) increase the coumarin solubility concentration-dependently to a similar degree. Arginine as well as GdnHCl at 1.0 M increased the coumarin solubility by about 2-fold. Considering the non-denaturing property of arginine and its safety, 1.0 M arginine should be a

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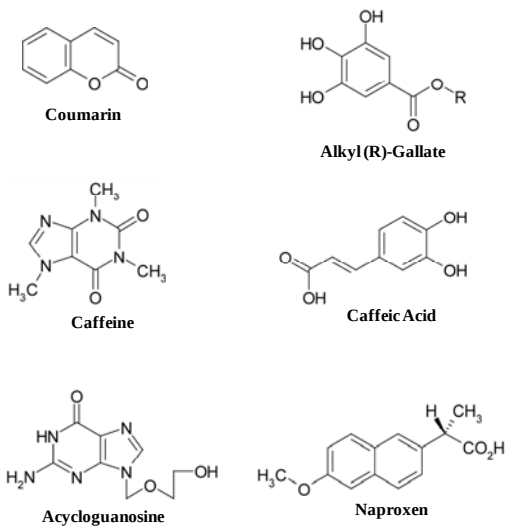

Figure 1. Chemical structure of small molecule drug substances.

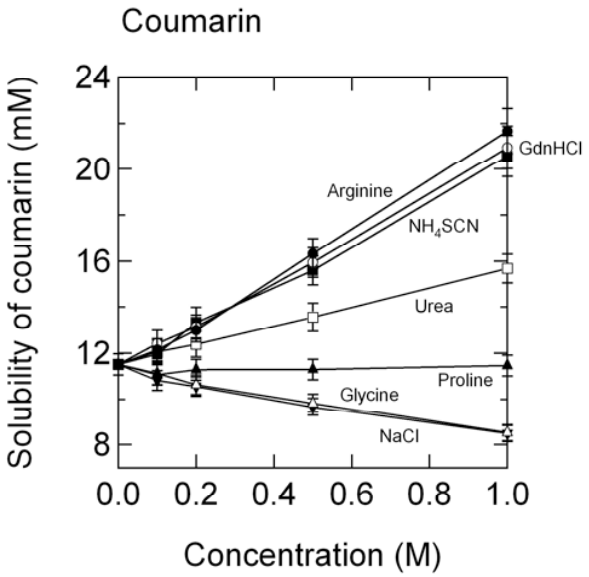

Figure 2. Solubility of coumarin in 50 mM citrate-phosphate buffer at pH 7.5. Closed circles, arginine; Open circles, GdnHCl; Closed squares, NH_4SCN; Open squares, urea; Closed triangles, proline; Open triangles, glycine; Closed inverse triangles, NaCl. The excess amounts of powder of coumarin were added into the stock aqueous additive solutions. Coumarin solutions were boiled at 100°C for 30 min, followed by incubation at 25°C for 2 h. After the incubation, the samples were centrifuged at 15,000 × g for 20 min at 20°C. The supernatants of the sample were diluted 20-fold with distilled water, and then the concentrations of soluble coumarin in the supernatant were measured by absorbance at 278 nm. Reformatted from *ref. 23*.

better additive, as GdnHCl may denature or destabilize the proteins when used in the interaction analysis. As shown in Figure 2, protein folding assisting osmolyte, proline (*27*), has essentially no effect on coumarin solubility at any concentration up to 1 M. Urea, a protein denaturant, is significantly less effective than GdnHCl at the same concentration, consistent with its weaker denaturing potency for proteins (*28*). Figure 2 also shows that another osmolyte, glycine, decreases the coumarin solubility. Other amino acids tested, *i.e.*, alanine, serine, and lysine, also decreased the coumarin solubility (data not shown). These four amino acids are similar, within experimental errors, to each other, decreasing the coumarin solubility by about 0.7-0.8-fold (data not shown). It is interesting to point out that a basic amino acid, lysine, decreased the coumarin solubility, in contrast to the effect of basic arginine. This demonstrates that the basic nature is not the factor responsible for the observed effectiveness of arginine.

Conversely, thiocyantates, salting-in salts, effectively increased the coumarin solubility (see Figure 2 for ammonium thiocyanate), ammonium salt being more effective than sodium salt (data not shown), consistent with their salting-in effects on proteins (*29-31*). Iodide salts were also slightly effective, with the magnitude similar to the effect observed for urea (data not shown). All the salting-out salts tested, *i.e.*, sulfate salts and chloride salts, decreased the coumarin solubility (data not shown), consistent with their known effects on protein solubility (*29-31*). Sulfate salts were most effective, consistent with the Hofmeister series of salts (*32,33*). Within the group of chloride salts, the salting-out effect increased in the order of $NH_4 < K \sim Li < Na$.

Gallic acid (3,4,5-trihydroxybenzoic acid) is obtained from nutgalls or other plants and fruits or by alkaline or acid hydrolysis of the tannins (see Figure 1 for chemical structure of alkyl-gallates) (*34-36*). Some of gallate derivatives have been extensively characterized in terms of physiological activities and cytotoxicities (*34-42*). Gallates are used as antioxidants in fats and oils and also used in cosmetics and, as food additives, in shortening, baked goods, candy and dried milk (*43*). Solubility of four different alkyl-gallates, *i.e.*, methyl-, ethy-, propyl-, and butyl-gallate, was measured in water as a function of arginine concentration and, for comparison, lysine concentration (*Hirano A, Kameda T, Arakawa T, Shiraki K, unpublished results*). Figure 3 plots the solubility ratio of these gallate derivatives as a function of arginine or lysine concentration. Arginine increased the solubility ratio of all 4 alkyl-gallates concentration-dependently (four closed symbols). It appears that the solubility enhancement by arginine is higher for more water-soluble methyl- and ethyl-gallate than the less soluble propyl- and butyl-gallate. As the alkyl-chain length increases in this order (*i.e.*, from methyl to butyl), the above results suggest that the solubility-enhancing effect of arginine is reduced with the increasing proportion of alkyl-chain length in the gallate-derivatives. In other words, arginine appears to interact with the aromatic structure in the gallates, leading to the enhanced solubility more effectively for shorter chain gallates, as has been observed (*44,45*). Conversely, the solubility-enhancing effect of lysine is marginal (four open symbols). Lysine increased the solubility of methyl- and ethyl-gallate only slightly, at

most 1.3-fold at 1 M and was ineffective for propyl- and butyl-gallate.

Arginine is also effective against a longer alkyl-chain gallate, *i.e.*, octyl-gallate, whose aqueous solubility is only ~ 0.072 mg/mL. The solubility increased by about 1.6- and 2.2-fold by the addition of 0.5 and 1 M arginine. On the contrary, NaCl decreased the solubility by ~ 30 and 50% at 0.5 and 1 M.

Hot water extracts of coffee exhibit strong antiviral and virucidal activities (*46*). Caffeine and caffeic acid are the components of coffee, which both also exhibit antiviral activities (*46-49*). Caffeic acid plays a role as an antioxidant (*50,51*) and exhibits various pharmacological activities as well as antiviral activities (*46,52-54*). Different from the compounds described above, caffeic acid is a weak acid (see Figure 1) and hence its solubility is not only solvent-dependent, but also pH-dependent. The deprotonated, charged state of caffeic acid is much more soluble than the protonated, uncharged state. Thus, the effects of 1 and 2 M arginine were examined in the pH range where the caffeic acid is mostly protonated, *i.e.*, between pH 3.5 and 5.0. At a given pH, 1 and 2 M arginine increased the solubility of caffeic acid by about 5- and 10-fold (data not shown) (*Hirano A, Shiraki K, Uozaki M, Koyama AH, Arakawa T, unpublished results*). Conversely, 1 M NaCl showed negligible effect and 2 M NaCl significantly decreased the solubility, indicating that ionic strength of arginine plays no major role in observed increased solubility. On the other hand, the solubility of caffeine was not affected by arginine (*Hirano A, Shiraki K, Uozaki M, Koyama AH, Arakawa T, unpublished results*), which may be due to its high aqueous solubility, namely, water may already be a good solvent for caffeine.

Acycloguanosine contains a partial nucleoside structure (see Figure 1) and is a potent antiherpetic agent, one of the most commonly used antiviral agents (*55-57*). It has a moderate aqueous solubility (1.5 mg/mL in 50 mM phosphate, pH 7.0). As acycloguanosine is a base (Figure 1), 50 mM phosphate was used to maintain the pH constant. The solubility of acycloguanosine in arginine was expressed as the solubility ratio, namely against the solubility in buffer. The solubility ratio is plotted in Figure 4 as a function of arginine concentration (*24*). Arginine up to 0.1 M showed no effect on the solubility of acycloguanosine, but significantly increased its solubility above 0.5 M. The solubility increased by 1.9- and 2.6-fold at 1 and 2 M.

We have described above that arginine by itself increases the solubility of small molecules in aqueous solution. The following example shows that arginine synergizes with other solubility enhancing additives. One of such solubility-enhancing additives uses the compound that binds or entraps the small molecule

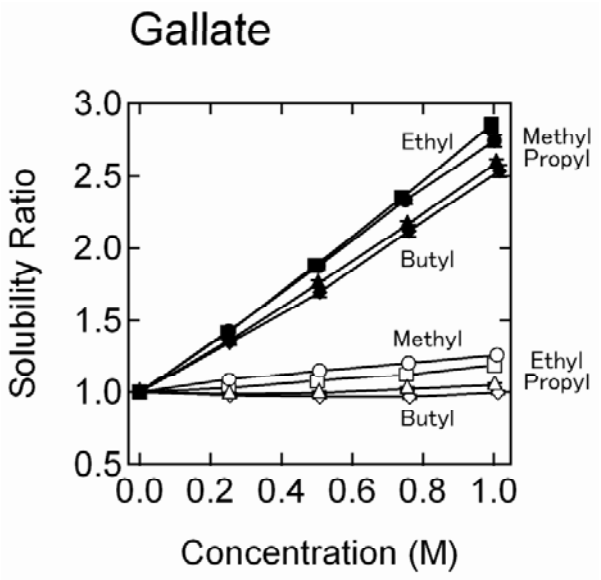

Figure 3. Solubility of alkyl gallates in aqueous lysine and arginine solutions. Ratio of the gallate solubility in the presence of additive to that in water is plotted. Closed symbols, solubility in aqueous arginine solutions; Open symbols, solubility in aqueous lysine solutions; Circle, methyl-gallate; Square, ethyl-gallate; Triangle, propyl-gallate; Diamond, butyl-gallate. Methyl-, ethyl-, propyl-, and butyl-galltes were dried in desiccators. Dried alkyl-galltes were transferred into test tubes, to which 0.5 mL of water or amino acid solutions were added. The suspension was heated at 40°C for 1 h with frequent vortex mixing, resulting in complete resolution of the gallate powders. The solution was then incubated at 25°C for 3 days with frequent vortex mixing, resulting in precipitation. The suspension was centrifuged at 25°C and 16,000 × g to obtain supernatant saturated with alkyl-gallate. After appropriate dilution with water, the absorbance of the supernatant was spectrophotometrically determined at 271 nm. The absorbance value was converted to the concentration based on the standard curve determined for each alkyl-gallate. The solubility thus determined was an average of triplicate experiments and the average and standard deviation were obtained. Reformatted from *Hirano A, Kameda T, Arakawa T, Shiraki K, unpublished results*.

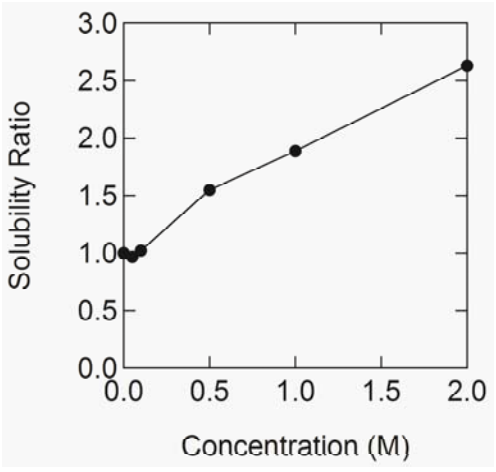

Figure 4. Solubility of acycloguanosine in aqueous arginine solution. About 5-10 mg of acycloguanosine were weighed into eppendolf tubes, into which 1 mL of test solvents in 50 mM phosphate, pH 7.0 was added. Such a strong buffer was required to minimize pH changes upon dissolution of basic acycloguanosine. The suspension was incubated at room temperature for 1 day with frequent vortexing and then centrifuged to separate supernatant. The supernatant was appropriately diluted with water for absorbance measurements. Concentration of acycloguanosine was spectrophotometrically determined at 252 nm. Reformatted from *ref. 24*.

drug. Cyclodextrin is often used as an additive for such purpose, but has limited application in pharmaceutical formulations due to low solubility and high molecular weight (*58*), although hydroxypropyl-β-cyclodextrin (HPC), a more water-soluble and low-cost derivative of cyclodextrin, has been developed. Mura and co-workers (*9,59*) have developed a novel system of multicomponent solution, termed ternary system that increases the solubility of a hydrophobic drug naproxen, a nonsteroidal anti-inflammatory drug (see Figure 1 for chemical structure), using cyclodextrin and amino acid, such as valine, lysine, and arginine, as solution additives. The solubility of naproxen is pH-dependent, due to the fact that naproxen is an acid with a carboxyl group (Figure 1). The solubility of naproxen as a protonated form is very low in acid (below 0.1 mg/mL) and increases with increasing pH by about 100-fold at neutral pH (data not shown), a similar observation for caffeic acid. Nevertheless, the solubility is still low at neutral pH in the absence of the additives (~ 2 mg/mL). When HPC and arginine were combined, both additives acted synergistically to the solubility of naproxen, leading to a 14-fold increase over the solubility of pH 6.9 control.

3. Effects of arginine on proteins

The solubility of small molecules may be increased by arginine. Small molecules solubilized by arginine at high concentrations may be diluted into a solution containing a target protein as well as other macromolecules (*e.g.* in the cell-based analysis). Therefore, it is critical to know how arginine affects the properties of the proteins. Here we summarize our current knowledge on the effects of arginine on proteins.

First, arginine increases the solubility of proteins. In general, salting-in effects are difficult to measure, compared to the salting-out effects, as most globular proteins are highly soluble in aqueous solution. To our knowledge, three poorly water-soluble proteins have been used to assess the ability of arginine to increase the solubility of protein, wheat flour gluten (*22*), S-carboxymethylated lysozyme (*60*) and fibroblast growth factor-20 (*15*). These proteins are barely soluble in aqueous salt solutions. Arginine concentration-dependently increased the gluten solubility up to ~ 3-fold at 1-2 M (data not shown). Interestingly, the magnitude of the increase is similar to that observed for small molecules. S-carboxymethylated lysozyme has a solubility below ~ 0.1 mg/mL in neutral phosphate buffer (*60*). Addition of 0.2 M arginine increased the solubility by about 4-fold, more so than 0.2 M urea or GdnHCl. Other amino acids were either ineffective or less effective (data not shown). Fibroblast growth factor-20 has an extremely low solubility in aqueous solution (*15*). The solubility in 50 mM phosphate buffer is below 0.5 mg/mL at any pH, nearly zero between pH 6.0 and 6.5. The addition of 0.2 M arginine in the same buffer greatly increased the solubility of the fibroblast growth factor-20. The solubility increased to above 1 mg/mL between pH 7.0 and 8.5, reaching 8 mg/mL at pH 5.5 (data not shown). The ability of arginine to increase the solubility of proteins has been utilized to extract insoluble proteins from inclusion bodies (*61-63*). Certain inclusion bodies of recombinant proteins, when expressed in *Escherichia coli*, occur from aggregation of native or native-like structures and can be readily solubilized by 0.2-2 M arginine at neutral pH in the native protein structure.

The solubilizing effect of arginine is consistent with its ability to suppress protein aggregation (*17-19*). Proteins often aggregate during storage and experiment due to various stresses, including such physical stresses as shaking, surface adsorption, pH and temperature fluctuation, concentration, and freezing and such chemical stresses as UV light and metals (*64,65*). Arginine has been used to suppress aggregation of proteins induced by these stresses (*16-19,65*).

Similarly, arginine has been used to suppress protein interactions with surfaces or chromatographic resins (*20,21,66*). Size exclusion chromatography of protein often suffers loss or retention of the proteins due to non-specific binding to the resins. Addition of 0.2-0.5 M arginine in the elution buffer (mobile phase) reduced such non-specific protein binding, leading to normal elution of loaded proteins (*21*). Hydrophobic interaction chromatography uses mildly hydrophobic groups (ligands) conjugated to the resins. Many proteins can only bind to the column in the presence of salting-out salts and elute during descending concentration of the salt. Arginine has been found to facilitate elution of the proteins with both high recovery and earlier elution (*66*). Also, arginine reduced the protein binding when included in the loading sample containing salting-out salts. Thus, arginine reduces hydrophobic interaction.

Such suppression of protein interaction by arginine has been observed for small molecules. Octyl-gallate is highly insoluble in water as described earlier, most likely due to its hydrophobic property. Arginine not only increased the solubility of octyl-gallate, but also inhibited its binding to bovine serum albumin (*24*). In other words, the free concentration of octyl-gallate was reduced by bovine serum albumin and was enhanced when arginine was included. A similar observation was made with a protonated form of caffeic acid. Caffeic acid was shown to bind to the plastic surface and such binding was reduced by arginine (*Hirano A, Shiraki K, Uozaki M, Koyama AH, Arakawa T, unpublished results*).

4. Potential applications

An obvious application of arginine in small molecule-protein interaction analysis is dissolution of small molecules by arginine, instead of organic solvents. As organic solvents are generally stronger in the

solubilization effectiveness, the final concentration in the interaction analysis may be lower than the arginine concentration that would be required and hence may not be high enough to denature the protein of interest. However, the target protein will be exposed to a transient high organic solvent concentration, which may be sufficient to cause protein denaturation. In addition, certain organic solvents are strong protein precipitant, and hence such a transient high concentration may cause irreversible protein aggregation. On the contrary, arginine does not denature proteins nor does it cause aggregation. It even suppresses protein aggregation.

Second potential application is inclusion of arginine to suppress non-specific protein-ligand interactions. Non-specific interactions of small molecules can cause the reduced effective concentration and false positives, as has been observed for benzofuran acyl-sulfonamide: this UDP-*N*-acetylmuramyl-L-alanine ligase inhibitor has been observed to bind to serum proteins (*67*). Arginine has been observed to suppress binding of octyl-gallate to bovine serum albumin (*24*). This raises a possibility that arginine may suppress weak, but specific, protein-ligand interactions, leaving only the strongly bound ligands in the protein-ligand complex.

Third potential application is to use arginine at 0.1-0.5 M level to prevent aggregation of target proteins or other proteins during the interaction analysis. Arginine is an effective suppressor of protein aggregation against various stresses as described above. This implies that care must be exercised for handling weakly associating oligomeric proteins. Arginine at 0.2 M has been found to cause dissociation of hemoglobin tetramer to the dimers (*Arakawa T, unpublished observation*).

Arginine may also prevent aggregation of small molecules. Small molecules have been observed to form a promiscuous cluster that presents non-polar surface, leading to non-specific binding of target proteins to the cluster (*68*). Inclusion of arginine in the interaction analysis may prevent such clustering of small molecules and also reduce non-specific interactions of the proteins with the cluster, if formed. Arginine has been observed to reduce non-specific binding of protein to chromatographic resins. In addition, arginine concentration-dependently prevented binding of caffeic acid to a plastic tube.

Finally, one of the ultimate goals of small molecule-protein interaction analysis may be the structure determination of the interacting complex. X-ray structure determination requires production of high quality crystals. Recently, arginine as well as some amino acids and their derivatives have been shown to assist production of high quality crystal of lysozyme, which otherwise tends to form amorphous precipitates. The effects of arginine to assist crystallization were ascribed to its suppressive effect on protein aggregation (*69,70*). Similarly, arginine assisted crystallization of hemoglobin (*71*).

5. Mechanism

Arginine increases the solubility of both small organic molecules and proteins as described above. The effects of arginine on protein solubility can be explained from its interaction with the proteins as determined by equilibrium dialysis and amino acid solubility measurements. Arginine shows an interaction pattern different from other solvent additives that enhance protein stability and decrease protein solubility (*72-75*). Salting-out salts are the typical additives, whose physical mechanisms can be explained by an increase in the surface tension of water. Namely, the higher the surface tension increment of water by the salts, the greater the decrease in the solubility of proteins is (*76*). Such correlation was also found in the solubility data for coumarin and caffeine as shown in Figure 5A (coumarin) and Figure 5B (caffeine). Although the data are somewhat scattering, there is a similar trend, *i.e.*, the more effectively the surface tension increase by the salts, the more they decrease the solubility of

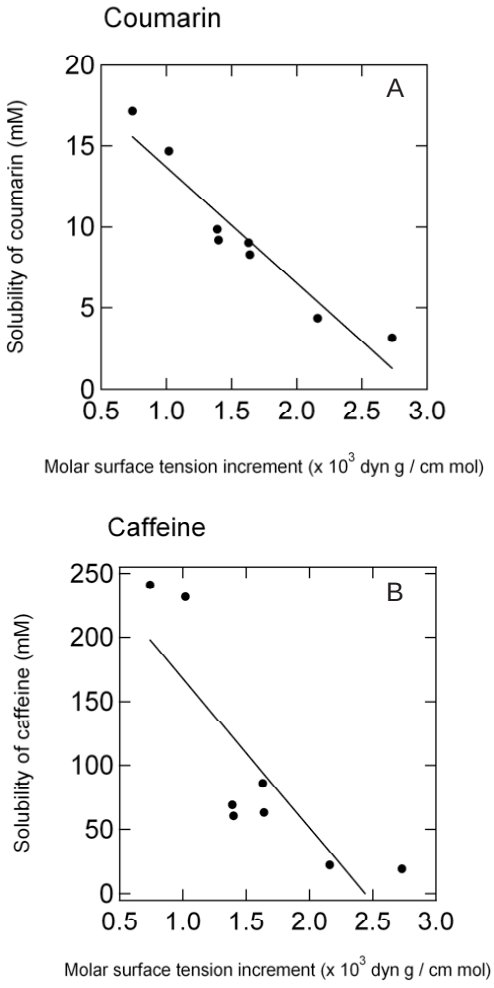

Figure 5. Correlation between the solubility of coumarin (A) or caffeine (B) and the molar surface tension increment. Linear lines indicate the least-square fitting of the line with correlation coefficients of –0.9573 ± 0.0006 (**A**, coumarin) and –0.8345 ± 0.0024 (**B**, caffeine).

coumarin and caffeine. The mechanism of surface tension effect is due to interfacial free energy at the molecule surface. Surface tension of water creates the surface free energy at the interface between water and the compound. The free energy becomes greater with increasing surface tension, as schematically shown in Figure 6. The solubility of the compound is determined by the difference in the free energy between the liquid phase and the solid phase. Here it is assumed that the precipitate has a lower interfacial free energy than the soluble form, as the surface area per compound is smaller in precipitate than in the soluble form. Smaller interface of the precipitate also means that the increment of the interfacial free energy, upon transfer of the precipitate from water to salt solution, is smaller than that of the soluble form, as shown in Figure 6 (*lower panel*). Thus, the free energy difference between the soluble form and precipitate phase is larger in salting-out salt solution than in water, leading to decreased solubility of the compound.

This mechanism, however, does not apply for arginine, as it also increases the surface tension of water (*73*). This is exactly identical to the case of urea that also increases the surface tension of water, yet is a solubilizing agent (*76*). Urea binding has been used to explain its solubilizing and denaturing effects (*76-78*). Can binding also explain the arginine effects? Preferential interaction measurements suggest such possibility for proteins (*72-75*). Formation of arginine clusters and their binding to proteins were also suggested (*79*). A plenty of evidence exists for binding of arginine with π-electron of aromatic ring structures (*44,45,80*). The chemical structures of some of the compounds used here are shown in Figure 1. Such binding mechanism clearly applies to coumarin, alkyl-gallates, caffeic acid and naproxen. Due to this mechanism, solubilization could be most likely observed for small molecules containing one or more aromatic ring structures. It is not clear whether arginine can increase the solubility of non-aromatic compounds. It is possible that arginine may not increase the solubility of non-aromatic compounds, but may suppress their binding to proteins, if the protein contains aromatic groups involved in binding non-aromatic compounds. Although acycloguanosine and caffeine have a similar structure without aromatic ring structures, only acycloguanosine solubility increased in the presence of arginine. This may be due to the different electrostatic properties between the two compounds. It suggests, however, that aromatic ring structure may not be an absolute requirement for arginine to exert solubilizing effects. Thus, more studies are required to fully understand how arginine interacts with the small molecules. We have initiated molecular dynamic simulation to examine molecular detail of the interactions between gallates and arginine (*Hirano A, Kameda T, Arakawa T, Shiraki K, unpublished results*).

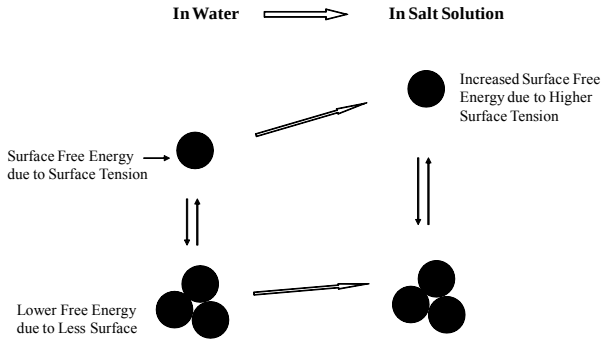

Figure 6. Schematic diagram of free energy of a compound in soluble form (*upper panel*) and in precipitate (*lower panel*) in the absence (*left panel*) and presence (*right panel*) of salting-out salt.

6. Conclusion

We have summarized the effects of arginine on small molecules and proteins. Arginine increases the solubility of proteins and small molecules and suppresses protein-protein and protein-surface interactions. These effects of arginine may find application in the analysis of drug-protein interactions and in solubilization of small molecules, and suppressing aggregation of target proteins and non-specific interactions.

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(Received June 27, 2010; Revised August 6, 2010; Accepted August 7, 2010)