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Minireview

Is arginine a protein-denaturant?

Matsujiro Ishibashi [a], Kouhei Tsumoto [b], Masao Tokunaga [a], Daisuke Ejima [c], Yoshiko Kita [d], Tsutomu Arakawa [e,*]

[a] *Laboratory of Applied and Molecular Microbiology, Faculty of Agriculture, Kagoshima University, Korimoto, Kagoshima, Japan*
[b] *Department of Medical Genome Sciences, Graduate School of Frontier Sciences, The University of Tokyo, Kashiwa 277-8562, Japan*
[c] *Central Research Laboratories, Ajinomoto, Inc., Kawasaki, Japan*
[d] *Department of Pharmacology, KEIO University School of Medicine, Tokyo, Japan*
[e] *Alliance Protein Laboratories, Thousand Oaks, CA 91360, USA*

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Abstract

Arginine is a useful solvent additive for many applications, including refolding and solubilization of proteins from insoluble pellets, and suppression of protein aggregation and non-specific adsorption during formulation and purification. However, there is a concern that arginine may be a protein-denaturant, which may limit the expansion of its applications. Such concern arises from the facts that arginine decreases melting temperature and perturbs the spectroscopic properties of certain proteins and contains a guanidinium group, which is a critical chemical structure for denaturing activity of guanidine hydrochloride. Here, we show that although arginine does lower the melting temperatures of certain proteins, the extent is insufficient to cause denaturation of proteins at or below room temperature. The proteins described here show enzymatic activity and folded structure in the presence of arginine, although the local structure around aromatic amino acids is perturbed by arginine. Arginine differs from guandinine hydrochloride in the mode of interactions with proteins, which may be a primary reason why arginine is not a protein-denaturant.

Keywords: Arginine; Protein-denaturant; Amino acid solubility; Transfer free energy; Preferential interaction

Arginine is a useful solvent additive for many applications in the biotechnology and pharmaceutical industries. It assists refolding of recombinant proteins, solubilizes proteins from certain inclusion bodies (IBs)[1] [1–10], facilitates elution of antibodies from Protein-A columns [11], reduces non-specific interactions with solid surface (D. Ejima, R. Yumioka, T. Arakawa, and K. Tsumoto, manuscript submitted), and reduces aggregation of proteins during storage [12]. However, there is a concern, based on the following observations, that arginine may be a protein-denaturant, which limits the expansion of its applications. In their elegant work on osmolytes, Yancey et al. [13] showed that the activity and stability of certain enzymes were perturbed by arginine and concluded that arginine is a protein-destabilizer and hence is not used by nature as an osmolyte. Xie et al. [14] have made a similar observation that fluorescence properties of aminoacylase are perturbed by arginine. They interpreted the observed effects of arginine in terms of denaturing property of the guanidinium group, which makes guanidine hydrochloride (GdnHCl) a strong denaturant. Here, we will summarize the effects of arginine on the stability, enzymatic activity, and spectroscopic property of proteins and show that arginine affects the melting temperature of certain proteins and their conformation, but not to such an extent that it

* Corresponding author. Fax: +1 805 388 7252.
E-mail address: tarakawa2@aol.com (T. Arakawa).
[1] *Abbreviations used:* IBs, inclusion bodies; GdnHCl, guanidine hydrochloride; RNase, ribonuclease; BSA, bovine serum albumin.

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causes protein denaturation and that arginine differs from GdnHCl in the mode of interactions with proteins.

Arginine is a weak protein-destabilizer

A clear evidence exists that arginine decreases the melting temperature of certain proteins, but to the extent much less than that by GdnHCl. For example, bovine pancreatic ribonuclease (RNase) showed a ~1 °C decrease in melting temperature by the addition of 0.2 M arginine [13], while it showed a ~2.5 °C decrease by 0.2 M GdnHCl [15]. Table 1 shows the changes in melting temperature of RNase at higher arginine concentration. The melting temperature decreases at most 3 °C in 1–2 M arginine. In another case, melting temperature of lysozyme decreased by 13–17 °C in the presence of 1.2 M GdnHCl [16–18], while it changed little in 0–2.0 M arginine (Table 1), consistent with the results of Shiraki et al. [19] and Kudoh et al. [20].

Consistent with little changes in protein stability, a prolonged incubation at 4 °C in 2 M arginine of an enzyme, HsNDK (*Halobacterium salinus* nucleoside diphosphate kinase) used as a model protein, resulted in no apparent decrease in the activity [21]. Namely, the enzyme was incubated in 2 M arginine for 24 h and diluted into the assay buffer containing no arginine, which showed a comparable activity of the sample incubated similarly without arginine. A similar stability of the enzyme activity was observed in 2 M, but not 6 M, GdnHCl at 4 °C. However, the HsNDK stability was compromised in 2 M GdnHCl at elevated temperature. Table 2 shows the activity of HsNDK after incubation at 40 °C for 19 h. While it retains 100% activity in 1 M NaCl, a marginal activity was observed in 1 M GdnHCl. A majority of activity was retained in 1 M arginine.

Table 1
Changes in melting temperature of RNase and lysozyme as a function of arginine concentration[a]

Arginine (M)	ΔMelting temperature	
	RNase	Lysozyme
0.1	0	0
0.2	−1	
0.5	−1	−1
1.0	−3	−1
2.0	−3	0

[a] Data from [18].

Table 2
Stability of HsNDK at 40 °C[a]

Solvent condition	Activity (%)
1 M NaCl	100
1 M arginine	87
1 M GdnHCl	15

[a] HsNDK was incubated at 40 °C for 19 h [21].

Arginine perturbs aromatic environments

As described above, arginine does decrease, although only slightly, the stability of some proteins. Does it affect the structure of the native protein? A few examples exist that the addition of arginine alters the surface properties of proteins as shown below. α-Crystallin, a major lens protein, has been shown to suppress aggregation of proteins, presumably through hydrophobic surface present on the native α-crystallin [22–26]. The ability of α-crystallin to suppress aggregation is enhanced at elevated temperatures or by the addition of 5–200 mM arginine, suggesting that arginine increases exposure of hydrophobic surface on α-crystallin, as does the increased temperature [26]. On the contrary, the intrinsic tryptophan fluorescence of α-crystallin showed little change upon addition of arginine, indicating no apparent changes in the tryptophan environment of α-crystallin. The near UV circular dichroic spectra showed, however, significant changes in the presence of 0.1–0.3 M arginine. Interestingly, the signals above 290 nm (due do tryptophan) and below 270 nm (due to phenylalanine) were little affected. Significant changes occurred between 270 and 290 nm, suggesting that arginine altered environments for tyrosine residues. Arginine resulted in little changes in the secondary structure of α-crystallin. These spectroscopic results indicate that the overall fold of α-crystallin is unchanged in the presence of arginine, but the local structures, specifically surrounding tyrosine residues, are affected. The changes in local structure seem limited, since the fluorescent tryptophan residues are not affected by 0.3 M arginine. However, arginine does induce dissociation of multimeric α-crystallin, perhaps reflecting the effects of arginine on the structure surrounding tyrosine residues.

On the contrary, the following examples indicate the effects of arginine on tryptophan residues. Aminoacylase is a dimeric enzyme containing one Zn^{2+} per subunit [14]. The enzyme aggregates as the protein concentration is increased. At neutral pH, the addition of 0.4 M arginine (data obtained using chloride salt of arginine) suppresses aggregation and interferes with the enzyme activity. There is no dissociation of the dimer in the presence of arginine as determined by native gel electrophoresis [14]. Tryptophan fluorescence gradually changes in both intensity and emission wavelength as the arginine concentration is increased from 0.3 to 2.7 M at neutral pH. The intensity change is ~5% in 2.7 M arginine relative to its absence, while the emission wavelength shifts from 333 nm in the absence to 340 nm in 2.7 M arginine. Gel filtration experiments showed an

earlier elution of the enzyme as the arginine concentration was increased from 0 to 1.6 M. Since the dimer structure is intact in the presence of arginine, the slight decrease in elution volume may indicate an expansion of the molecule, perhaps consistent with fluorescence changes. It is evident that the structure of aminoacylase is altered locally, on the surface surrounding fluorescent tryptophan residues. However, both native gel and gel filtration analyses indicate that the overall structure is not altered, similar to the results of α-crystallin.

Consistent with the above observation, the fluorescence intensity of bovine serum albumin (BSA), but not lysozyme, changed slightly at 25 mM–0.2 M arginine with no change in emission wavelength (Fig. 1). There are no changes in fluorescence properties of lysozyme by arginine. The observed slight perturbation of tryptophan residues by arginine agrees with the recent observation that guanidinium group interacts with tryptophan residues [27]. Tsumoto et al. [27] have shown that CutA from hyperthermophilic archaeon *Pyrococcus horikoshi* maintains the native structure even in the presence of 8 M GdnHCl. Fig. 2 shows that the fluorescence spectrum changes by the addition of GdnHCl with fluorescence intensity increasing at higher GdnHCl concentration. Consistent with the above observation, examination of crystal structure of CutA showed the structural perturbation of solvent-exposed tryptophans in the presence of 3 M GdnHCl. There is no shift in emission wavelength (Fig. 2), suggesting no gross conformational changes, consistent with the crystal structure in 3 M GdnHCl.

A pioneering work of Nozaki and Tanford [28] on the solubility of amino acids in aqueous GdnHCl solution demonstrated favorable interactions of this denaturant with tryptophan. It may thus be concluded that arginine interacts favorably with tryptophan and perhaps tyrosine residues through its guanidinium group.

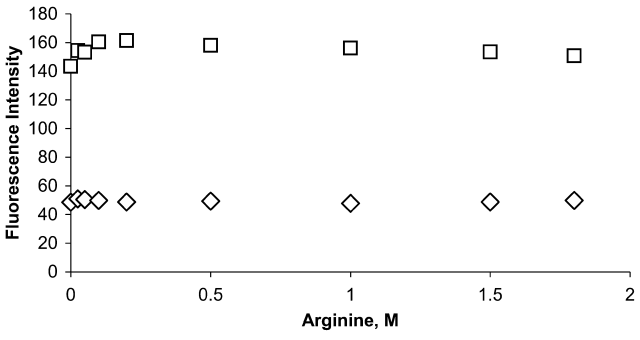

Fig. 1. Fluorescence intensity of lysozyme and BSA as a function of arginine concentration. Fluorescence intensity (arbitrary unit) at the emission maximum (~341 nm for lysozyme and 342 nm for BSA) is plotted. Open square, BSA; open diamond, lysozyme. The emission wavelength for lysozyme and BSA was nearly constant at different arginine concentrations.

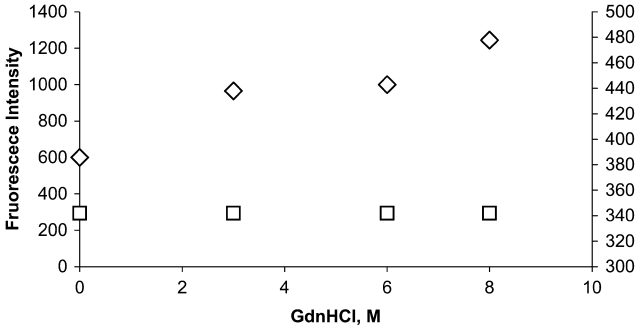

Fig. 2. Fluorescence properties of CutA as a function of GdnHCl concentration. Left side ordinate corresponds to fluorescence intensity (arbitrary unit) and right side ordinate corresponds to wavelength (nm). Open diamond, fluorescence intensity (arbitrary unit) at emission maximum; open square, wavelength of emission maximum.

Arginine interacts with a protein differently from GdnHCl

If arginine is different from GdnHCl, arginine is expected to interact with proteins differently from GdnHCl. There are two studies on this topic, i.e., preferential interaction and amino acid solubility. Preferential interaction is a measure of additive concentration in the vicinity of the protein surface relative to that in the bulk phase and hence an indication of binding of the additives to the entire surface of the native or unfolded proteins. Amino acid solubility in aqueous solution of additives gives information on how the additives interact with each amino acid and its side chain. There are no solubility data of amino acids in aqueous arginine solution, while Nozaki and Tanford [28] have shown that GdnHCl interacts favorably with all amino acid side chains and the peptide group. This is the reason why GdnHCl denatures proteins. Whether the interactions of arginine with amino acids and peptide group are more limited or not awaits the solubility measurements in aqueous arginine solutions.

Preferential interaction data, although limited, are available using lysozyme. Fig. 3 shows the preferential binding of lysozyme with arginine [29] and GdnHCl (T. Arakawa and S.N. Timasheff, unpublished data). Because of solubility limit,[2] arginine data are available up to 1.5 M [29]. It is evident that preferential binding of arginine is negative and decreases as the arginine concentration is increased. It is opposite for GdnHCl, for which preferential interaction measurements were done similarly to those for arginine [29]. The preferential interaction is positive, except at 1 M (where the value is slightly negative), and increases with GdnHCl

[2] Arginine concentration can be increased to 3 M at neutral pH. However, it is safer to keep and use it below 2 M to avoid accidental crystallization, which can occur above 2 M.

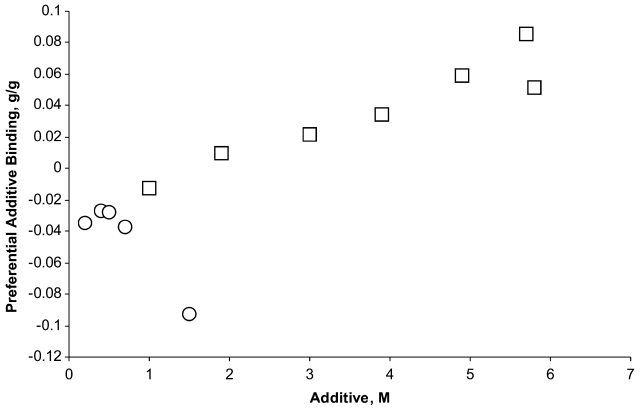

Fig. 3. Preferential interactions of GdnHCl (square) and arginine (circle) with lysozyme (T. Arakawa and S.N. Timasheff, unpublished data). Preferential interaction measurements were done at pH 5.5 as described in [29]. Namely, protein solution was equilibrated with aqueous GdnHCl solution and equilibrium concentration of GdnHCl was determined by density measurements.

concentration. This clearly indicates that arginine has little tendency for binding to lysozyme, while GdnHCl binds to lysozyme, more strongly at higher concentrations.

Preferential interaction is, however, not an absolute binding of solvent additives such as arginine and GdnHCl. It is a measure of both additive and water binding, since the preferential interaction parameter of additive (here expressed as component 3) with the protein (component 2), $(\partial g_3/\partial g_2)$, is expressed by Eq. (1) at constant pressure and chemical potentials of solvent components [30].

$$(\partial g_3/\partial g_2) = A_3 - g_3 A_1. \quad (1)$$

Namely, the preferential interaction parameter of the additive reflects both bindings of additive, A_3; and water (component 1), A_1; the bulk concentration of additive, g_3. Assuming that hydration is identical in arginine and GdnHCl solutions, one can conclude that GdnHCl binds more extensively with proteins. Since proteins are hydrated to some extent in arginine or GdnHCl solution, i.e., $A_1 > 0$, A_3 is not equal to, but less than, $(\partial g_3/\partial g_2)$.

As a final remark of this section, it should be noted that both arginine–base and arginine–HCl are currently commercially available. When making neutral or acidic solutions of arginine, it is important to select the right acid, since a choice of counter-ion can change the effects of arginine on proteins. For example, GdnHCl and the sulfate salt of Gdn are very different in their effects on proteins [31].

Activity in arginine

An ultimate demonstration whether or not arginine is a denaturant is the activity of proteins in the presence of arginine, which may be difficult to measure, since arginine may interfere with the most activity assays. Nevertheless, a few examples exist, demonstrating function of proteins in the presence of arginine. When green fluorescent protein was exposed to 0.5–2.0 M arginine, it was fluorescent, resembling the fluorescence observed in the absence of arginine [32]. The results indicate that the core structure, necessary for green fluorescence, is intact and stable. Another example was obtained using HsNDK described above [21,33,34], i.e., activity measurements were carried out in 1 and 2 M additive solutions. HsNDK is active in the presence of 1 and 2 M arginine, GdnHCl and NaCl, suggesting that the protein is in the active conformation (Table 3). It is evident that there are varying degree of either enhancement or inhibition of the enzyme activity by these additives. In all cases, 2 M additive results in at least partial inhibition of the activity relative to that in 1 M. It is interesting to note that 1 M GdnHCl showed a much higher activity than the level achieved in 1 M NaCl, suggesting that 1 M GdnHCl enhances either substrate binding or catalytic activity, or both. There is strong reduction of the enzyme activity by arginine, suggesting that arginine affects substrate binding or catalytic activity, or both. It is clear, however, that arginine does not unfold and inactivates the enzyme.

The observed changes in the enzyme activity in the presence of additives at high concentration are expected, since the additives at such high concentration can affect both the substrate binding and catalytic function as shown above. It is also possible that these additives alter surface properties of the enzymes. The effects of arginine on the activities of enzymes might also involve interactions between arginine and substrates or cofactors of enzymatic reactions. Bowlus and Somero [15] found that arginine causes a large increase in the Michaelis–Menten constants (K_m) for certain substrates and cofactors. For example, binding of phosphoenolpyruvate to pyruvate kinase was strongly inhibited by arginine; the K_m of phosphoenolpyruvate increased about threefold as arginine concentration was raised to 0.1 M. However, these effects may not be a consequence of perturbation of

Table 3
The activity of HsNDK in the presence of 1 and 2 M NaCl, arginine, and GdnHCl[a]

Solvent conditions	Activity (%)
1 M NaCl	100
2 M NaCl	69
1 M Arginine	70
2 M Arginine	36
1 M GdnHCl	154
2 M GdnHCl	94

[a] The activity is expressed as the percentage to the value in 1 M NaCl (from [21]).

pyruvate kinase structure by arginine. Similarly, arginine inhibited binding of the cofactor, NADH, to lactate dehydrogenase [15].

Arginine may be a unique compound

We have so far shown that the effect of arginine on protein structure is limited to local environments of aromatic amino acids. How do such effects translate to the observed effects of arginine on various properties of proteins? Arginine is widely used to assist refolding of the proteins. Based on its effects on protein stability, it does not enhance conversion of unfolded to folded structure [35]. It is now believed that arginine suppresses aggregation of the proteins during refolding by binding to the folding intermediates [35]. We have observed that arginine can solubilize certain proteins, including green fluorescent protein [32] and β2 microglobulin [36], which are trapped in the insoluble pellets, termed "loose IBs," of *Escherichia coli*. These proteins are active and folded after solubilization by arginine, indicating that their structure in the insoluble pellets is close to the native structure and hence that such insoluble pellets are products of either native protein or late-stage folding intermediates. Arginine is completely ineffective in solubilizing proteins when they form firm IBs, which are the products of unfolded form or early-stage folding intermediates. Such IBs can readily be dissolved by GdnHCl, consistent with the differences between GdnHCl and arginine with arginine acting only on the surface of the native or native-like proteins.

Antibody binds to protein-A specifically and tightly. Arginine cannot dissociate such tight association. However, when the association is weakened, for example, by lowering the pH, arginine is effective in dissociation of the complex [11]. This leads to elution of antibodies from protein-A columns under the condition at which decreased pH alone is insufficient to dissociate the complex. This observation may again be consistent with the inability of arginine to penetrate tightly packed structure as in the intact antibody/protein-A complex.

We have also observed that arginine is effective in suppressing non-specific binding of protein to solid surface (D. Ejima, R. Yumioka, T. Arakawa, and K. Tsumoto, manuscript submitted). Similarly to the protein–protein interaction and aggregation, arginine is effective only when the interaction is weak so that it can penetrate the surface of the native protein loosely binding to the solid surface.

Conclusion

It can be concluded that arginine is not a protein-denaturant, but does affect, although weakly, the local structure surrounding tryptophan (and tyrosine) residues. Arginine suppresses aggregation and facilitates protein refolding and solubilization or elution of antibodies from protein-A columns. The destabilization effects of arginine are much weaker than those of GdnHCl.

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