L-Argininamide improves the refolding more effectively than L-arginine

Hiroyuki Hamada and Kentaro Shiraki*

Institute of Applied Physics, University of Tsukuba, 1-1-1 Tennodai, Tsukuba, Ibaraki 305-8573, Japan

* Corresponding author. Tel.: +81-29-8535306; fax: +81-29-8535215.
Email address: shiraki@bk.tsukuba.ac.jp (K. Shiraki)

Total number of manuscript pages: 28
Tables: 2
Figures: 7
Supplementary material pages: 3
Supplementary Figures: 2
Filenames of supplementary materials: Hamada_SupplFig.pdf
Abstract

L-Arginine (Arg) is a widely used additive for suppressing protein aggregation during refolding. Systematic screening of Arg analogs provides superior additives that enhance the refolding yield more effectively than Arg. The refolding yield of hen egg lysozyme in the presence of 500 mM L-argininamide (ArgAd) increases 1.7-fold higher than Arg. Thermal unfolding experiments indicate that ArgAd has a greater denaturing effect than Arg, which positively relates to the net charge of Arg analogs. Moreover ArgAd was also effective for the refolding of bovine carbonic anhydrase. High potency to increase the refolding yield of ArgAd compared to Arg results from high positive net charge and the denaturing property.

Keywords: L-argininamide; L-arginine; aggregation; lysozyme; refolding; renaturation
1 **1. Introduction**

Protein aggregation during refolding is a serious problem in biotechnology. The high-level expression of heterologous protein in prokaryotic and eukaryotic hosts frequently leads to the formation of insoluble aggregates, referred to as inclusion bodies (Marston 1986; Lilie et al. 1998; Villaverde and Carrió 2003). In order for the recovery of biological active protein, solubilization of the inclusion bodies with a denaturing agent such as urea or guanidine (Gdn) and subsequent *in vitro* refolding by dialysis or direct dilution are required (Fischer 1994; Rudolph and Lilie 1996). However, the refolding yield of reactivation is usually lowered because correct folding *in vitro* competes with unproductive side reactions, e.g., the formation of misfolded species and the aggregation of denatured protein (Zettlmeissl et al. 1979; Goldberg et al. 1991; Kiefhaber et al. 1991).

During the refolding reaction, the hydrophobic interaction drives the unfolded protein to sequester their hydrophobic patches from water when the denaturant is removed (Dill 1990). The difference between protein folding and aggregation is described as an intramolecular reaction (folding) or an intermolecular reaction (aggregation) by the following reactions:

\[
U \rightarrow N \quad (1a)
\]

\[
U + U \rightarrow A_2 \quad (1b)
\]

where \(U\), \(N\), and \(A_2\) represent the unfolded protein, native protein, and a dimer, respectively (Baynes et al. 2005). The simple model suggests that the marginal balance from the unfolded state to the native structure or aggregates affects the refolding yields of unfolded proteins.

In order to improve the refolding yield, many types of additives have been used in the refolding buffer. There are two types of refolding additives, folding enhancers and aggregation suppressors (Tsumoto et al. 2003). The former is ammonium sulfate, polyols, sugars, and
certain amino acids such as glycine and proline (Maeda et al. 1996; Kumar et al. 1998; Samuel et al. 2000; Meng et al. 2001; Ou et al. 2002; Mishra et al. 2005). The folding enhancer stabilizes the native structure and enhances intermolecular interactions. The latter includes denaturants such as urea and Gdn (Orsini and Goldberg 1978), mild detergents (Zardeneta and Horowitz 1994; Wetlaufer and Xie 1995), polyethylene glycols (Cleland and Wang 1990; Cleland et al. 1992), and organic acids (Yang et al. 1996). Aggregation suppressors inhibit the folding reaction due to destabilization of the native structure. In most cases, these additives improve the refolding yield due to the deceleration of aggregation by weakening the intermolecular hydrophobic interactions.

L-Arginine (Arg) has the most basic side chain that possesses a similarity to Gdn. Arg has been found to increase the refolding yields of human tissue type plasminogen activator (Rudolph and Fischer 1990). Thereafter, Arg has been used for refolding various proteins, such as F_ab antibody fragments (Buchner and Rudolph 1991), single-chain immunotoxins (Buchner et al. 1992; Brinkmann et al. 1992), interleukin-6 receptor (Stoyan et al. 1993), interleukin-21 (Asano et al. 2002), human matrix metalloproteinase-7 (Oneda and Inouye 1999), and recombinant human neurotrophins (Suenaga et al. 1998; Rattenholl et al. 2001). Arg consists of four functional groups, i.e., guanidino-, amino-, carboxy-, and methylene-groups. Although Arg contains a guanidino group, it has only a minor effect on the thermodynamic stability of folded proteins (Taneja and Ahmad 1994; Lin and Timasheff 1996; Shiraki et al. 2002; Arakawa and Tsumoto 2003; Reddy et al. 2005). Arg does not accelerate the refolding kinetics, but increase the solubility of aggregate-prone molecules (Hevehan and De Bernardez Clark 1997; Reddy et al. 2005). Arg is the most effective suppressor for heat-induced aggregation among natural amino acids (Shiraki et al. 2002). In addition to the inexpensive and nontoxic
properties of Arg, it is expected to act as a solution additive in long term storage and affinity column chromatography (Arakawa et al. 2003, 2004; Ejima et al. 2005a,b). In spite of wide usage of Arg as an aggregation suppressor, a mechanistic explanation of the effects of Arg on protein refolding is rarely provided.

The aim of this study was to find a better additive among Arg analogs. We used hen egg white lysozyme as the model protein (Epstein and Goldberger 1963; Saxena and Wetlaufer 1970; Rudolph and Fischer 1990; Goldberg et al. 1991; Fischer et al. 1992, 1993). The results of this study show that L-argininamide (ArgAd) is more effective for oxidative refolding of lysozyme than Arg. Moreover ArgAd was effective for the refolding of bovine carbonic anhydrase (CA) which has lower pI than lysozyme. As discussed below, comparative analysis of additives provides information requiring superior refolding additives.
2. Materials and methods

2.1. Materials

Hen egg white lysozyme, Bovine carbonic anhydrase (CA), ethylenediaminetetraacetic acid disodium salt dihydrate (EDTA), p-nitrophényl acetate (p-NPA), L-arginine hydrochloride (Arg), L-homoarginine hydrochloride (HArg), L-2-amino-3-guanidinopropionic acid hydrochloride (AGPA), L-arginine ethylester dihydrochloride (ArgEE), L-argininamide dihydrochloride (ArgAd), L-citrulline (Cit), L-ornithine hydrochloride (Orn), guanidinopropionic acid (GPA), glycine (Gly), and glycaminamide hydrochloride (GlyAd) were purchased from Sigma (St. Louis, MO, USA). Guanidine hydrochloride (Gdn), Micrococcus lysodeikticus, dithiothreitol (DTT), and oxidized and reduced glutathione (GSSG and GSH) were purchased from Wako Pure Chemical Industries (Osaka, Japan). Tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl) and sodium phosphate were purchased from Nacalai Tesque (Kyoto, Japan). All the compounds were of the highest grade available commercially.

2.2. Protein concentration

Protein concentration was determined photometrically by measuring the absorbance at 280 nm with an appropriate blank, using extinction coefficients of 2.63 mL mg⁻¹ cm⁻¹ for native and 2.37 mL mg⁻¹ cm⁻¹ for denatured lysozyme (Saxena and Wetlaufer 1970) and 1.83 mL mg⁻¹ cm⁻¹ for CA (Pocker and Stone 1967). Absorbance was measured with a UV-Vis spectrophotometer model ND-1000 (NanoDrop Technologies, Inc., Wilmington, DE, USA).

2.3. Preparation of denatured protein
Lysozyme at 16-132 mg mL\(^{-1}\) was reduced and denatured in a solution containing 6 M Gdn, 40 mM DTT, 0.1 M Tris-HCl (pH 8.0), and 1 mM EDTA. Bovine CA was denatured in 6 M Gdn containing 0.1 M Tris-HCl (pH 8.0) at 44 mg mL\(^{-1}\). The resulting protein solutions were incubated for 2 h at 37°C. The denatured protein solution was stored at 4°C and used within 1 week.

2.4. Protein refolding

The reduced and denatured lysozyme solution was diluted by 40-fold into the refolding buffer containing 0.1 M Tris-HCl (pH 8.0), 1 mM EDTA, and refolding additives at an appropriate concentration. The final refolding solution has a volume of 200 µL containing 0.1 M Tris-HCl (pH 8.0), 1 mM EDTA, 5 mM GSH, 5 mM GSSG, 150 mM Gdn, 1 mM DTT, and 0.4-3.3 mg mL\(^{-1}\) lysozyme. The denatured CA was diluted by 40-fold into the refolding buffer containing 0.1 M Tris-HCl (pH 8.0) and refolding additives at the concentration of 250 mM. The final CA concentration was 1.1 mg mL\(^{-1}\). The diluted solution was mixed by a vibrating mixer for 2 s and then incubated at 25°C for 12 h without shaking.

2.5. Activity assay

Prior to measuring the enzymatic activity, refolding solutions were centrifuged at 15,000 g for 20 min to remove precipitates. The activity of lysozyme was assayed by bacteriolysis of \(M.\) lysodeikticus. Ten µL of the refolded lysozyme was added to 1490 µL of 0.5 mg mL\(^{-1}\) \(M.\) lysodeikticus solution containing 50 mM Na-phosphate buffer (pH 7.0). The decrease in the light scattering intensity at 600 nm of the solution was monitored using a UV-Vis spectropolarimeter model V-550 (Japan Spectroscopic Co., Tokyo, Japan) at room
temperature. The enzymatic activity was determined from the initial velocity of the substrate degradation. The activity of CA was determined by hydrolysis of p-NPA. A hundred µL of the refolded CA solution was diluted into the substrate solution containing 1 mM p-NPA, 50 mM Tris-HCl (pH 7.5) and 5 mM EDTA. The increase in absorbance at 348 nm was measured using the spectrophotometer. The refolding yield was determined as for lysozyme.

2.6. Alkylation of free sulphydryl groups

In order to quench the formation of disulfide bonds, free sulphydryl groups were alkylated with iodoacetic acid. The kinetics of the reactivation of lysozyme was monitored by removing 50 µL samples of refolding lysozyme at specific time intervals and quenching the reaction by addition 5 µL of 0.5 M iodoacetic acid dissolved in 1 M Tris-HCl (pH 7.0) and 1 M potassium hydroxide (Darby and Creighton 1995). The quenching procedure had no effect on the activity of native lysozyme.

2.7. Kinetic model

Figure 1 shows the refolding kinetic model as described previously (Hevehan and De Bernardez Clark 1997). Briefly, this model assumes the formation of the transient intermediate (I) from the unfolded protein (U) in the early phase of refolding. This is a rapid process with a millisecond time range, so that the rate constant of the formation of I ($k_I$) can be assumed to be infinite. Thus, this folding scheme is simplified to the parallel reaction, which consists of the formation of native structure (I -> N) and aggregates (I -> A). The folding is a unimolecular reaction, whereas aggregation is a higher order reaction. In the oxidative refolding of lysozyme, the aggregation can be best described by third-order kinetics (Hevehan and De Bernardez Clark 1997).
The refolding yield of lysozyme \(Y\) over time can be described by the following equations:

\[
Y = \phi \{\tan^{-1}[(1+\phi^2)\exp(2k_N t)-1]^{1/2} - \tan^{-1}\phi}\]  

(2a)

where \(\phi\) is defined as

\[
\phi = \left(\frac{k_N}{k_A U_0^2}\right)^{1/2}
\]

(2b)

where \(U_0\) represents the initial unfolded protein concentration, \(t\) the refolding time, \(k_N\) and \(k_A\) the folding and aggregation kinetic constants, respectively.

2.8. Circular dichroism spectroscopy

Circular dichroism (CD) spectroscopy was performed using a Jasco CD spectropolarimeter model J-720W with a Peltier cell holder with a temperature controller model PTC-348W (Japan Spectroscopic Co., Ltd., Tokyo, Japan). The near-UV CD spectra of lysozyme were monitored in the wavelength range of 265-310 nm. The heat-induced unfolding was monitored at 288.5 nm with a heating rate of 1°C min\(^{-1}\) using a 1 cm path-length cell. All spectra representing the native lysozyme were solubilized into the buffer containing 0.1 M Tris-HCl (pH 8.0), 1 mM EDTA, and 500 mM additives. The final protein concentration was adjusted to 0.2 mg mL\(^{-1}\).

2.9. pH titration

In order to determine the charged state of additives at pH 8.0, the \(pK_a\) values of the amino group of additives were determined by pH titration. A small quantity of 1.0 M NaOH was added to 3 mL of 0.1 M additive solution. The change of pH was monitored with pH meter model TPX-90i (Toko Chemical Laboratories Co., Ltd., Tokyo, Japan) and equivalent points.
were calculated by derivation of titration curves.
3. Results

3.1. Screening of Arg analogs

In order to obtain superior additives to increase the refolding yields of protein, we investigated Arg analogs using oxidative refolding of lysozyme as a model. Figure 2 shows the 11 kinds of refolding additives tested in this paper. The compounds in class A are Arg and Gdn as well-known additives for refolding. The compounds in class B have no guanidino groups. Gly and GlyAd are selected to reveal the effect of the side chains and amidation of the carboxy group, respectively. In class C, ArgAd and ArgEE possess amide and carboxy ethylester groups substituted for the carboxy group of Arg, respectively. GPA has no amino group and two methylene groups shorter than Arg. In class D, HArg and AGPA have identical ionizable groups to Arg but different lengths of methylene groups.

The reduced and denatured lysozyme was diluted into refolding buffer in the presence of additives and then the refolding yield was compared by enzymatic activity. Figure 3 shows the refolding yields of lysozyme in the presence of 500 mM additives. As the refolding was accomplished by 40-fold dilution, 150 mM Gdn and 1 mM DTT remained in the final solution. The refolding yield was only about 8% in the absence of additives. In the presence of Arg and Gdn, the refolding yields were 37% and 40%, respectively. Orn, Gly, and GlyAd rather decreased the refolding yields below 7%. In the presence of Cit, which has an ureido group substituted for the guanidino group of Arg, the refolding yield slightly increased up to half in the presence of Arg. This result should be due to the ureido group sharing a common structure of urea, which is apparently similar to the weak chaotropic effect of Gdn. The additives that showed higher refolding yields than Arg were ArgAd, ArgEE, GPA, HArg, and Gdn, though they have a guanidino group. AGPA did not affect the refolding yield of lysozyme, while
HArg showed 1.3-fold higher yield than Arg. ArgAd showed the best data for the refolding yield of lysozyme ~ 63% among 11 additives tested. The substitution of amide group for the carboxy group of Arg is favorable for the refolding of lysozyme.

3.2. Comparative data of Arg, Gdn, and ArgAd for refolding additives

We found that ArgAd increased the refolding yield of lysozyme more effectively than Arg. In order to clarify the superior property of ArgAd, the oxidative refolding of lysozyme was performed at various concentrations of additives and protein.

Figure 4A shows the refolding yield of lysozyme in the presence of various concentrations of Arg, Gdn, and ArgAd at a protein concentration of 1.0 mg mL\(^{-1}\). The maximum refolding yields of lysozyme in the presence of ArgAd was 89% at around 0.8-1.0 M. The profile of Gdn was similar to that of ArgAd with a maximum yield of 79% at 1.0 M. However, the refolding yield in the presence of Arg was saturated above 1.3 M Arg. At 2.0 M Arg, the refolding yield was 72%. These data show that ArgAd is the best additive under a practical concentration for \textit{in vitro} refolding below 1 M.

Figure 4B shows the refolding yields of lysozyme depended on the protein concentration in the absence and presence of Arg, Gdn, and ArgAd. Reduced and denatured lysozyme at various concentrations (16–114 mg mL\(^{-1}\)) was diluted by 40-fold into the refolding buffer containing 500 mM additive and then the residual activity was measured. In the absence of additives, the refolding yields steeply decreased and nearly all the lysozyme could not refold above 1.6 mg mL\(^{-1}\). In the presence of 500 mM Arg, Gdn, and ArgAd, the profiles were clearly improved. At a high concentration of protein above 1.6 mg mL\(^{-1}\), ArgAd increased the refolding yield by 8-fold compared to absence of additive, while Arg and Gdn increased
5-6-fold (Fig. 4B inset). It is interesting to note that at the low protein concentration, the refolding yield is little improved by the additives, implying that preventing intermolecular interaction plays a key role in increasing the refolding yield.

3.3. Kinetics for refolding in the presence of ArgAd, Arg, and Gdn

To further investigate the efficacy of ArgAd and its mechanism of action, refolding kinetics were measured in the absence or presence of 500 mM Arg, Gdn, and ArgAd. The refolding yields increased over time and reached plateau after 2-3 h, as shown in Supplementary Figure S1. The experimental data were well fitted to Eqs. (2a) and (2b). The deduced values of $k_N$, $k_A$, and $\phi$ are summarized in Table 1. As can be seen from the table, the values of $k_N$, which corresponds to the kinetic parameter from unfolded state to native, were slightly decreased when ArgAd, Arg, and Gdn were added at a concentration of 500 mM. In the presence of 500 mM additives, the values of $k_A$, which correspond to the kinetic parameter from aggregate-prone intermediate to aggregates, were 2 orders of magnitude lower than that in the absence of additives. Interestingly, the value of $k_A$ in the presence of ArgAd was about half of that in the presence of Gdn. The ratio of $k_N$ and $k_A$ or the value of $\phi$ defined by Eq. (2b) directly relates to the final refolding yield. As the $\phi$ value in the presence of ArgAd was higher than that of Arg and Gdn. These data indicate that ArgAd preferentially suppresses the aggregation process as compared to the folding process.

3.4. Thermodynamic stability of lysozyme in the presence of ArgAd, Arg, and Gdn

In order to clarify the denaturing effects of additives, thermal unfolding profiles of lysozyme were measured by CD spectroscopy. Figure 5A shows the near-UV CD spectra of
non-reduced lysozyme at 25°C in the absence or presence of Arg, Gdn, and ArgAd. There were no significant differences even in the presence of additives. Figure 5B shows the thermal unfolding curves of native lysozyme monitored at 288.5 nm. No aggregation was observed even at 98°C and thermal unfolding was irreversible even in the presence of additives. In the absence of additives, the apparent midpoint temperature of unfolding \( T_m \) was 74.9°C. In the presence of 500 mM Arg, the \( T_m \) value was 74.5°C, which is slightly lower than the absence of additives. On the other hand, in the presence of 500 mM Gdn and ArgAd, the \( T_m \) values were 70.4°C and 71.2°C, respectively. This indicates that Gdn and ArgAd possess denaturing properties. Interestingly, the denaturing effect of ArgAd was larger than that of Arg but smaller than that of Gdn. Thus, the improvement of the refolding yield in the presence of ArgAd cannot be explained by only the stabilization of aggregation-prone species relative to aggregates.

3.5. pH titration of Arg, Gdn, and ArgAd

The refolding process is generally dependent on the charged state of both the additive and the protein. Lysozyme was positively charged under the present experimental conditions due to the high isoelectric point (pI) from 9.3 (reduced form) to 10.8 (oxidized form). In order to determine the charged state of additives, we performed pH titration experiments and determined the \( pK_a \) value of the amino group responsible for these experimental conditions at pH 8.0. The pH titration curves of the solution at a 0.1 M concentration are shown in Supplementary Figure S2. The \( pK_a \) values of the amino group and the net charge of Arg, ArgAd, HArg, and AGPA, are summarized in Table 2. The \( pK_a \) value of the amino group of HArg was higher than that of Arg, while that of AGPA was lower. This would be due to the inductive effect depending on the number of the methylene group between the guanidino group
and α-carbon. Although the pK\textsubscript{a} of the amino group of ArgAd was the lowest value of all additives tested, the net charge of ArgAd was highest due to the substitution of the amide group for the carboxy group. The relationship between the net charge of Arg analogs and refolding yields suggests that the high net charge may contribute to the effect of ArgAd as discussed in the following section.

3.6. The effect of ArgAd on CA refolding

In order to confirm versatility of ArgAd as a refolding additive, we compared the additives on the refolding yields of CA, which is a monomeric protein with the molecular mass of 30 kDa with neutral pI. As shown in Figure 7, the refolding yield of CA in the absence of additives was about 13%. In the presence of 250 mM Arg and Gdn, the refolding yields were 23% and 36%, respectively. On the other hand, the refolding yield in the presence of 250 mM ArgAd was 1.4-fold higher than that in the presence of Arg. This result suggests the possibility of ArgAd as a versatile additive for protein refolding.
4. Discussion

The systematic screening of Arg analogs for oxidative refolding of lysozyme revealed that ArgAd is the most effective among the additives tested. We discuss why ArgAd is superior as a refolding additive as follows.

The simple finding is that guanidino compounds improve the oxidative refolding of lysozyme (Fig. 3). Gdn or the guanidino group binds to the aromatic side chains, as well as the peptide backbone and negatively charged side chains, resulting in stabilization of aggregation-prone species or folding intermediates to prevent aggregation (Nozaki and Tanford 1970; Arakawa and Timasheff 1984; Timasheff and Arakawa 1988; Lin and Timasheff 1996; Tsumoto et al. 2004). These facts suggest that the guanidino group should be an indispensable functional group in a refolding additive.

Early studies on refolding additives have shown that protein denaturants, typically Gdn, at a nondenaturing concentration improve correct refolding (Orsini and Goldberg 1978). The low concentration of the denaturant inhibits the intermolecular hydrophobic interactions, which lead to the suppression of the aggregation and improvement in the correct folding. In fact, Arg as well as Gdn decrease the melting temperature and perturb the spectroscopic properties of a certain protein (Ishibashi et al. 2005). These data suggest that the denaturing effect plays a key role in suppression of aggregation. In our data, ArgAd decreased the refolding yield of lysozyme at high concentrations (above 1.3 M) as shown in the case of Gdn (Fig. 4A). This phenomenon is consistent with the abilities of these chemicals to destabilize the native state (Fig. 5). These facts prompted us to the hypothesis that the amide group of ArgAd mainly contributed to the denaturing effect. Amide compounds, such as acetamide, are effective additives on the refolding of lysozyme (Yasuda et al. 1998; Dong et al. 2004).
Moreover, acetamide is a weak protein denaturant (Gordon and Jencks 1963; Warren and Gordon 1970). The amide group on ArgAd acts as both hydrogen bond donors and acceptors, as ArgAd shows a higher denaturing effect than Arg. However, Gdn is also a stronger protein denaturant than ArgAd. Although the protein-denaturing effect is key factor to understanding the refolding additives, not all the properties of additives could be described.

One of the finding of our study is that ArgAd preferentially suppressed intermolecular aggregation comparing to intramolecular folding (Table 1). This phenomenon is apparently similar to the thermal unfolding of lysozyme under the acidic condition; i.e., lysozyme does not aggregate but it reversibly refolds at acidic pH (Ueda et al. 2000). ArgAd has a higher positive net charge than Arg due to the unionizable amide group (Table 2). ArgAd may tend to interact with folding intermediates and neutralize local negative charges leading to enhanced electrostatic repulsion between aggregation-prone species. This idea is supported by the data that polyamine (Kudou et al. 2003) and ArgEE (Shiraki et al. 2004) suppress the heat-induced aggregation of lysozyme. In the case of ArgEE, the hydrophobic end of the ethyl group interacts with the hydrophobic patch on the unfolded polypeptide, leading to enhanced electrostatic repulsion between aggregation-prone unfolded lysozyme. Binding of ArgAd on the aggregation-prone species is related to the increase of net charge substituting the unionizable amide group for the carboxy group leads to improved refolding yield.

There is a positive relation between the refolding yield and the positive charge of Arg analogs (Fig. 6). The refolding yields in the presence of Arg, HArg, and AGPA were clearly different although they have the same ionizable groups. This difference would be due to the charged state of the Cα-amino group of additives (Table 2). Moreover, ArgAd has the highest net charge of all additives tested. These results support the idea that positively charged additive
increases the refolding yield.

In summary, we showed that a new refolding additive ArgAd improves the refolding yield due to the suppression of aggregation. The comparative analysis of ArgAd to similar compounds reveals that the refolding additives require the guanidino group and some positive net charges. These experimental facts provide information for developing new protein aggregation suppressors and refolding additives. It should be noted that ArgAd is somehow toxic and too expensive to use the bioprocess. These properties can be overcome by searching the analogues of ArgAd.
Acknowledgments

This work was partly supported by Grant-in-Aid for Young Scientist (No. 18750140) from the MEXT of Japan, Special Research Project on Nanoscience, University of Tsukuba, and Tsukuba Industrial Liaison and Cooperative Research Center.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version.
References


Gordon, J.A. and Jencks, W.P. 1963. The relationship of structure to the effectiveness of


Kiefhaber, T., Rudolph, R., Kohler, H.H. and Buchner, J. 1991. Protein aggregation in vitro and
in vivo: a quantitative model of the kinetic competition between folding and aggregation.
Biotechnology (N Y) 9: 825-829.

inactivation and aggregation of lysozyme by polyamines. Eur. J. Biochem. 270:
4547-4554.

Kumar, T.K.S., Samuel, D., Jayaraman, G., Srimathi, T. and Yu, C. 1998. The role of proline in
the prevention of aggregation during protein folding in vitro. Biochem. Mol. Biol. Int. 46:
509-517.


Lin, T.Y. and Timasheff, S.N. 1996. On the role of surface tension in the stabilization of
globular proteins. Protein Sci. 5: 372-381.

Maeda, Y., Yamada, H., Ueda, T. and Imoto, T. 1996. Effect of additives on the renaturation of
reduced lysozyme in the presence of 4 M urea. Protein Eng. 9: 461-465.

Marston, F.A.O. 1986. The purification of eukaryotic polypeptides synthesized in Escherichia


Reddy, R.C., Lilie, H., Rudolph, R. and Lange, C. 2005. L-Arginine increases the solubility of...


Table 1

Kinetic constants of the folding \((k_N)\), aggregation \((k_A)\), and \(\phi\) value calculated by Eqs. (2a) and (2b)

<table>
<thead>
<tr>
<th>Additive</th>
<th>(k_N) (min(^{-1}))</th>
<th>(k_A) (mL(^2) mg(^{-2}) min(^{-1}))</th>
<th>(\phi) (-)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No Additive</td>
<td>0.0233±0.0019</td>
<td>23.4±2.1</td>
<td>0.03</td>
</tr>
<tr>
<td>Arg</td>
<td>0.0192±0.0006</td>
<td>0.498±0.026</td>
<td>0.20</td>
</tr>
<tr>
<td>Gdn</td>
<td>0.0150±0.0008</td>
<td>0.187±0.002</td>
<td>0.28</td>
</tr>
<tr>
<td>ArgAd</td>
<td>0.0140±0.0008</td>
<td>0.100±0.008</td>
<td>0.37</td>
</tr>
</tbody>
</table>

\(^a\) Triplicate experiments were performed and average values of \(k_N\) and \(k_A\) and their standard deviations are represented (Supplementary Figure S1).
Table 2

The $pK_a$ values and net charges of Arg analogs

<table>
<thead>
<tr>
<th>Additive</th>
<th>$pK_a$</th>
<th>Net Charge$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arg</td>
<td>9.04</td>
<td>+0.92</td>
</tr>
<tr>
<td>ArgAd</td>
<td>7.39</td>
<td>+1.20</td>
</tr>
<tr>
<td>HArg</td>
<td>9.33</td>
<td>+0.96</td>
</tr>
<tr>
<td>AGPA</td>
<td>7.66</td>
<td>+0.31</td>
</tr>
</tbody>
</table>

$^a$ The net charge of additives were measured at pH 8.0 (Supplementary Figure S2).
Figure legends

Figure 1. Simplified kinetic model represented by Hevehan and De Bernardez Clark. U, unfolded protein; I, folding intermediate; A, aggregates; N, native protein. \( k_I \), kinetic constant of formation of intermediates; \( k_N \), kinetic constant of folding; \( k_A \), kinetic constant of aggregation.

Figure 2. Chemical structure of additives. (A) Arginine and guanidine as standard additives. Arginine derivatives, (B) substituted for guanidino group, (C) modified in carboxy group, and (D) having shorter or longer methylene groups.

Figure 3. Refolding yield of lysozyme in the presence or absence of additives. Reduced and denatured lysozyme at 40 mg mL\(^{-1}\) were diluted 40-fold with buffer containing final concentration of 500 mM additives. Data represent the mean and error bars of three independent experiments.

Figure 4. Refolding yield of lysozyme in the presence of additives. (A) Reduced and denatured lysozyme at 40 mg mL\(^{-1}\) were diluted 40-fold into buffer containing various final concentrations of Arg (solid circles), Gdn (solid squares), and ArgAd (open circles). (B) The effects of lysozyme concentration on the refolding yield in the absence (cross) and presence of 500 mM Arg (solid circles), Gdn (solid squares), and ArgAd (open circles). (B, Inset) The relative refolding yield of lysozyme in the presence of additives. The vertical axis was divided by the refolding yields of no additives at respective concentrations. The final protein concentrations of 0.4-3.3 mg mL\(^{-1}\) were shown in the horizontal axis. Data represent the mean
and error bars of three independent experiments.

Figure 5. (A) Near-UV CD spectra of lysozyme in the presence of 500 mM additives, Arg (solid line), Gdn (short-dashed line), and ArgAd (long-dashed line), or in the absence of additives at 25°C (dotted line) and at 98°C (dash-dotted line). (B) Normalized near-UV thermal transitions of lysozyme monitored by CD in the absence (cross) and presence of additives, Arg (closed circle), Gdn (closed square), and ArgAd (open circle).

Figure 6. Relationship between refolding yield and net charge of additives. The additives are Arg, ArgAd, HArg, and AGPA. The refolding yields were referred to Figure 2. The net charges of additives were referred to Table 2. Data represent the mean and error bars of three independent experiments.

Figure 7. Refolding yield of CA in the presence or absence of additives. Denatured CA at 44 mg mL⁻¹ was diluted 40-fold with buffer containing final concentration of 250 mM additives. Data represent the mean and error bars of three independent experiments.
Hamada and Shiraki, Figure 1

\[
\begin{align*}
U & \xrightarrow{k_I} I \\
& \quad \downarrow{k_A} \\
& \quad \quad \quad A \\
I & \xrightarrow{k_N} N
\end{align*}
\]
Hamada and Shiraki, Figure 2

A  
\[
\begin{align*}
\text{L-Arginine (Arg)} & : & \text{Guanidine (Gdn)} \\
\end{align*}
\]

B  
\[
\begin{align*}
\text{L-Citrulline (Cit)} & : & \text{L-Ornitine (Orn)} & : & \text{Glycine (Gly)} & : & \text{Glycinamide (GlyAd)} \\
\end{align*}
\]

C  
\[
\begin{align*}
\text{L-Argininamide (ArgAd)} & : & \text{L-Arginine ethylester (ArgEE)} & : & \text{Guanidinopropionic acid (GPA)} \\
\end{align*}
\]

D  
\[
\begin{align*}
\text{L-Homoarginine (HArg)} & : & \text{L-2-Amino-3-guanidinopropionic acid (AGPA)} \\
\end{align*}
\]
Hamada and Shiraki, Figure 7

Refolding Yield (%)

- No additive
- Gdn
- Arg
- ArgAd
Electronic Supplementary Material for

L-Arginamidine improves the oxidative refolding more effectively than L-arginine

Hiroyuki Hamada and Kentaro Shiraki
Supplementary Figure S1

Kinetic profiles of lysozyme refolding.

The reduced and denatured lysozyme at 40 mg mL\textsuperscript{-1} were diluted 40-fold in the absence (cross) or presence of 500 mM Arg (solid circles), Gdn (solid squares), and ArgAd (open circles). After the incubation at respective periods, the residual activity was measured. Continuous curves show the nonlinear least-squares fitting to Eqs. (2a) and (2b).
Supplementary Figure S2

**pH titration curves in the presence of 100 mM additives.**

Determination of pKₐ values of amino groups of Arg analogs. The additives are Arg (closed circle), HArg (closed square), AGPA (closed triangle), and ArgAd (open circle).