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The effects of arginine on refolding of aggregated proteins: not facilitate refolding, but suppress aggregation☆

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Abstract

Arginine is one of the universal reagents that are effective in assisting refolding of recombinant proteins from inclusion bodies. The mechanism of the effects of arginine on refolding has remained, however, to be elucidated. Here we show that arginine does not stabilize proteins against heat treatment, as demonstrated by little change in melting temperature. It does increase reversibility of thermal melting and reduce aggregation under thermal stress. The observations suggest that arginine may not facilitate refolding, but may suppress aggregation of the proteins during refolding.

Keywords: Arginine; Inclusion body; Refolding; Reversibility; Aggregation

With a growing expansion of genomic data, a strong demand for purified proteins is increased. *Escherichia coli* is the most attractive host for expression of proteins due to its rapid growth and convenient handling. The overproduction of proteins, however, often leads to formation of insoluble aggregates, referred to as inclusion bodies. Refolding of recombinant proteins expressed as insoluble inclusion bodies is not straightforward [1–4].

Among many small molecule additives tested, arginine is one of the most commonly used additives effective in assisting refolding of recombinant proteins from inclusion bodies [5–12]. However, the mechanism of action of arginine during refolding is not well understood. In order to address the mechanism, thermal unfolding of three model proteins, bovine ribonuclease A (RNaseA), and hen lysozyme with and without arginine, has been investigated. On the basis of results obtained, we could conclude that arginine does not stabilize proteins, but it reduces aggregation and increases reversibility of thermal unfolding.

Materials and methods

RNaseA and hen lysozyme were used for this study. RNaseA, and hen lysozyme, and BSA were dissolved in 40 mM Tris–HCl, pH 7.5. RNaseA and hen lysozyme were also dissolved in 1 M arginine or 50 mM glycine–NaOH, pH 10. RNaseA concentration was adjusted to 2.0 mg/ml for absorbance and to 1.7 mg/ml for circular dichroism (CD) measurements. Hen lysozyme concentration was adjusted to 0.4 mg/ml for absorbance measurements. Hen lysozyme concentration for CD measurements was 0.295 mg/ml. Protein concentrations were spectrophotometrically determined using extinction coefficients of 2.74 for hen lysozyme and 0.738 for RNaseA for 0.1% protein solution [13].

Gilford response II spectrophotometer was used for thermal melting measurements. It can measure five samples simultaneously. A wavelength at 287 nm was used to monitor the thermal melting, expressed as a change in absorbance as the temperature is increased at an indicated rate of thermal scan. After the completion of thermal scan, the sample was cooled to 30 °C and incubated for 30 min before scanning for the second time. Jasco J-710 spectropolarimeter equipped with a Peltier cell holder and a temperature controller, PTC-348WI, was used for CD measurements. Only near UV CD was measured due to high concentration of arginine. For technical reasons, thermal melting at neutral pH was done with Gilford UV spectrophotometer, while that at pH 10 was done with Jasco CD.

☆ *Abbreviations:* RNaseA, bovine ribonuclease A; CD, circular dichroism.

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Results

RNaseA

Thermal unfolding was monitored by a decrease in absorbance with time. Table 1 shows the mid-temperature, T_m, of the thermal unfolding in the absence or presence of 0.1–0.4 M arginine at a scan rate of 0.1 or 0.5 °C/min. In all cases above, the values of T_m are essentially independent of the scan rate, indicating that thermal unfolding of RNaseA is a reversible process and is in equilibrium under the conditions used. The reversibility was also confirmed by the second thermal scan, which was done by cooling the sample to 30 °C and incubating it for 30 min at 30 °C. Identical transition temperatures were observed in the second scan. At the arginine concentrations of 0.1–0.4 M, there is little change in T_m, indicating that arginine does not stabilize nor does it destabilize the protein against thermal stress.

Results at higher arginine concentration are summarized in Table 2. Although not shown, the second scan also gave a similar transition temperature at any arginine concentrations used. It appears that higher arginine concentration slightly decreases the melting temperature. A drastic change was observed at pH 10. Fig. 1 shows the thermal transition of RNaseA in the absence and presence of 1 M arginine at pH 10. Thermal transition is slightly shifted to a lower temperature in the presence of 1 M arginine by about 4 °C, as shown in Table 2. Thermal unfolding of RNaseA at pH 10 is irreversible as the CD signal did not return to the preheating value. The CD value after heating and cooling was close to that of the thermally unfolded structure in the absence or presence of 1 M arginine. A large difference, however, was observed for the protein after heating and cooling. RNaseA in the absence of arginine developed turbidity (shown in the lower panel of Fig. 1) after heating and cooling, while the protein maintained a clear solution in the presence of 1 M arginine. Thus, at pH 10, arginine suppressed aggregation of RNaseA upon thermal unfolding, although irreversibility of the thermal unfolding could not be reversed.

Near UV CD spectra were compared at pH 10 in the absence and presence of 1 M arginine to assure that arginine did not alter the structure of RNaseA at this extreme pH. As shown in Fig. 2, the near UV CD spectra are identical, within experimental error, with

Table 1
Thermal transition temperature of RNaseA at 2 mg/ml in 40 mM Tris–HCl, pH 7.5

Arginine (M)	Transition temperature (°C)		
	First scan (T_m)		Second scan (T_m)
	0.5 °C/min	0.1 °C/min	0.1 °C/min
0	63	63	63
0.1	63	63	63
0.2	62	62	62
0.3	62	62	62
0.4	62	61	62

Table 2
Thermal transition of RNaseA at pH 7.5 at a scan rate of 0.1 °C/min

Arginine (M)	T_m (°C)
0	63
0.5	62
0.7	61
1.0	60
1.5	59
2.0	60
0 (pH 10)	63[a] (clear)
1.0 (pH 10)	59[a] (turbid)

[a] Determined using 0.265 mg/ml protein with CD at a scan rate of 1 °C/min.

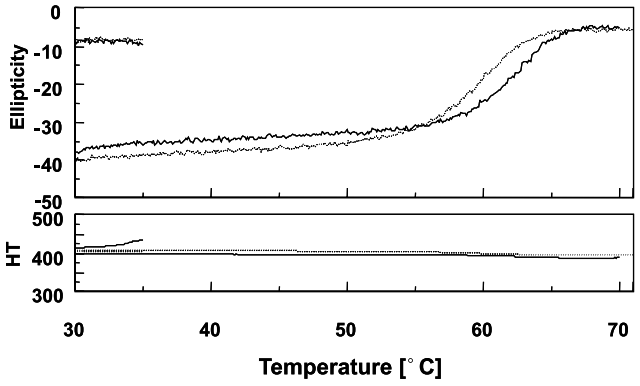

Fig. 1. Thermal unfolding of RNaseA monitored by CD. The upper panel corresponds to CD signal at 283 nm, while the lower panel corresponds to HT, high tension voltage (equivalent to absorbance or light scattering in this case). Short lines in both panels are signals of the second scan obtained after heating and cooling. Solid line, in 50 mM glycine–NaOH at pH 10; dotted line, in 1 M arginine at pH 10. Scan rate, 1 °C/min.

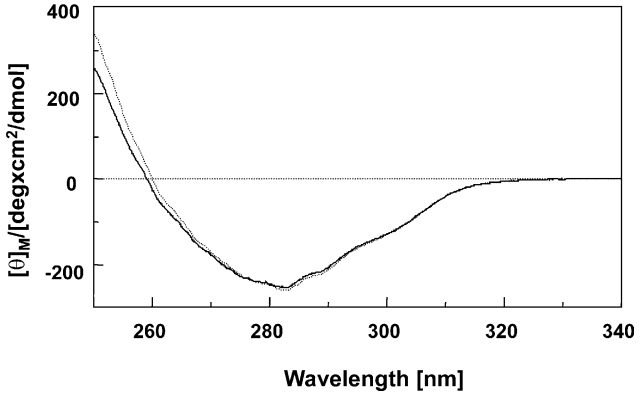

Fig. 2. Near UV CD spectra of RNaseA at pH 10. Solid line, in 50 mM glycine–NaOH at pH 10; dotted line, in 1 M arginine at pH 10.

and without arginine. The spectra are also similar to those published in the neutral solvent. It is, therefore, more likely that the effect of arginine on refolding of the protein is not in the native structure, but in the unfolded structure.

Lysozyme

In the absence of arginine, lysozyme developed turbidity at about 71 °C, shown in Table 3 as an onset temperature of unfolding. Thus, T_m of lysozyme could not be determined in the absence of arginine. Turbidity was not observed in the presence of arginine at 0.1–2 M. The case in the presence of arginine at 0.1 M has been shown in Fig. 3, and the T_m and the onset temperature are summarized in Table 3. The onset temperature of thermal unfolding in the presence of arginine is identical to that of turbidity development in the absence of arginine, indicating that arginine suppresses aggregation. The onset temperature was not affected by arginine, indicating no effect of arginine on the stability of lysozyme. In the presence of arginine, a similar transition temperature was observed between the first and second scans, indicating that arginine makes thermal unfolding of lysozyme reversible.

At pH 10, even in the presence of 1 M arginine, thermal unfolding has become irreversible (Table 3). Fig. 4 shows the change in CD signal at 289 nm as the temperature is increased. In the absence of arginine, turbidity developed (Fig. 4, lower panel) as the protein unfolded at about 63 °C, which made the T_m determination meaningless. In the presence of 1 M arginine, a clear thermal transition was observed with an onset temperature of 63 °C and a T_m of 70 °C. There was no turbidity development and the protein solution was clear at the end of the thermal scan. However, the CD signal did not return to the pre-heating value and the thermal unfolding was irreversible even in the presence of 1 M arginine at pH 10. Near UV CD spectra in Fig. 5 showed that 1 M arginine did not alter the conformation of lysozyme at pH 10. The spectra are similar to those published for the native protein at neutral pH.

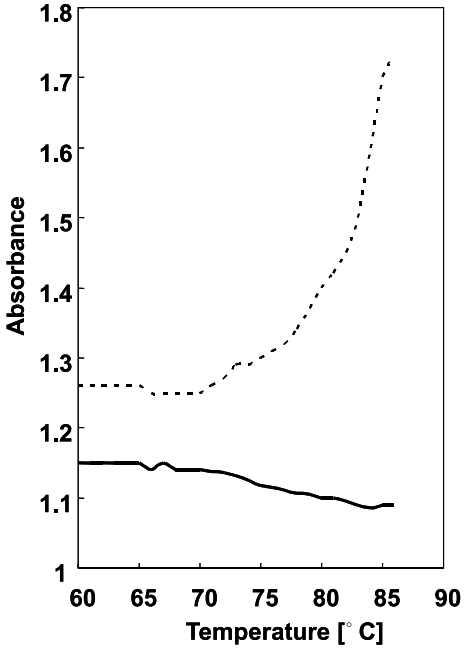

Fig. 3. Thermal transition of hen lysozyme at 0.4 mg/ml in 40 mM Tris–HCl, pH 7.5, at a scan rate of 0.5 °C/min. Solid and dotted lines are the absorbance at 287 nm in the presence and absence of 0.1 M arginine, respectively.

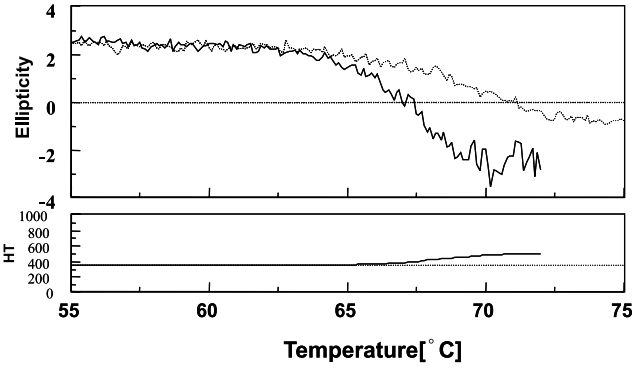

Fig. 4. Thermal transition of hen lysozyme monitored by CD. The upper panel corresponds to CD signal at 289 nm, while the lower panel corresponds to high tension voltage. Solid line, in 50 mM glycine–NaOH at pH 10; dotted line, in 1 M arginine at pH 10. Scan rate, 0.5 °C/min.

Table 3
Thermal transition temperature of hen lysozyme at 0.4 mg/ml in 40 mM Tris–HCl, pH 7.5, at a scan rate of 0.5 °C/min

Arginine (M)	First scan		Second scan	
	Onset-temperature (°C)	T_m (°C)	Onset-temperature (°C)	T_m (°C)
0	71	Turbidity	Turbidity	
0.1	71	74	70	74
0.5	70	74	70	74
1.0	70	74	70	73
2.0	71	74	70	74
0 (pH 10)	63[a]	Precipitate	Precipitate	
1 M (pH 10)	63[a]	70[a]	Irreversible	

[a] Determined using 0.295 mg/ml protein with CD at a scan rate of 0.5 °C/min.

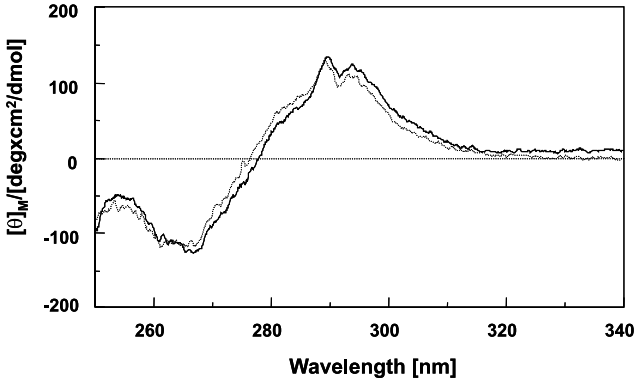

Fig. 5. Near UV CD spectra of hen lysozyme. At pH 10. Solid line, in 50 mM glycine–NaOH at pH 10; dotted line, in 1 M arginine at pH 10.

Discussion

We have shown here that arginine suppresses aggregation of two proteins, RNaseA, and hen lysozyme, different in chemical and physical properties. Suppression of aggregation was observed at a widely different arginine concentration from 0.1 to 2 M and at both neutral and high pHs. For hen lysozyme, arginine made the thermal unfolding reversible at neutral pH, while lysozyme developed turbidity without arginine. At both neutral and high pHs, addition of arginine to the protein did not lead to stabilization of the proteins. Melting temperature decreased only slightly at high arginine concentrations.

In the thermal unfolding, the native protein (N) unfolds to the denatured structure (U), which may aggregate to form (Agg).

The results reported here demonstrated that arginine must interact with (U) to affect its solubility. However, if such binding occurs to the unfolded state, then arginine must destabilize the proteins. By mass action law, preferential binding of any additives to (U) must lead to destabilization of the proteins, which was not observed here. This strongly suggests the possibility that arginine also binds to the native state.

We have shown before different contributions of arginine to the stability of proteins from other amino acids [14]. For instance, glycine, proline, serine, and alanine are protein stabilizers and increase the melting temperature of the proteins [15]. These amino acids are preferentially excluded from the protein surface. On the other hand, arginine was shown to preferentially bind to the proteins in the native state [15]. Putting together the results that arginine binds to the native state and solubilizes the unfolded state, binding of arginine to the unfolded state could be the mechanism of suppression of aggregations against thermal stress.

At neutral pH, arginine has two positive charges due to deprotonation of guanidino- and amino-groups, while at pH 10 it has only one positive, i.e., guanidino-group. The fact that arginine has aggregation-suppression effect at both neutral and high pHs suggests that guanidino-group, which retains the charged state at both pHs, is responsible for binding to proteins.

N ----------> U ----------> Agg

Scheme 1.

How does such binding relate to the effects of arginine on refolding? Since arginine does not stabilize the native state of proteins shown in Scheme 1, it is not likely that arginine facilitates refolding from U to N. A major problem of protein refolding is aggregation of an unfolded or an intermediate state during refolding. Arginine may work on refolding of proteins by suppressing aggregation of proteins, thereby allowing the unfolded or intermediate state to proceed to the native state [16]. Recently, Shiraki et al. [17] demonstrated that arginine suppresses aggregation during refolding of heat-denatured proteins. Taken together, it can be concluded that the effect of arginine on refolding of proteins is suppression of aggregation, not facilitation of refolding. How arginine suppresses the aggregation of unfolded proteins may be intriguing, and precise investigation of the interactions between side chains and/or main chains in proteins and arginine might give one the clues for elucidation of the mechanism.

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