J. Biochem. 132, 591–595 (2002)

Biophysical Effect of Amino Acids on the Prevention of Protein Aggregation[1]

Kentaro Shiraki,* Motonori Kudou,* Shinsuke Fujiwara,† Tadayuki Imanaka,‡ and Masahiro Takagi*,[2]

**School of Materials Science, Japan Advanced Institute of Science and Technology, 1-1 Asahidai, Tatsunokuchi, Ishikawa 923-1292; †Department of Bioscience, School of Science and Technology, Kwansei-Gakuin University, 2-1 Gakuen Sanda, Hyogo 669-1337; and ‡Department of Synthetic Chemistry and Biological Chemistry, Graduate School of Engineering, Kyoto University, Yoshida-honmachi, Sakyo-ku, Kyoto 606-8501*

Received June 17, 2002; accepted July 24, 2002

Each protein folds into a unique and native structure spontaneously. However, during the unfolding or refolding process, a protein often tends to form aggregates. To establish a method to prevent undesirable protein aggregation and to increase the stability of native protein structures under deterioration conditions, two types of aggregation conditions, thermal unfolding-induced aggregation and dilution-induced aggregation from denatured state, were studied in the presence of additional amino acids and ions using lysozyme as a model protein. Among 15 amino acids tested, arginine exhibited the best results in preventing the formation of aggregates in both cases. Further biophysical studies revealed that arginine did not change the thermal denaturation temperature (T_m) of the lysozyme. The preventive effect of arginine on aggregation was not dependent on the size or isoelectric point of eight kinds of proteins tested.

Key words: amino acid, dilution-induced aggregation from denaturant, heat-induced aggregation, protein aggregation.

Proteins in their functional native structure are engaged in promoting or controlling virtually every event on which life depend. We have learned a great deal about the manner in which native structures are attained through the complex process of protein folding. However, many proteins often form undesirable and uncontrollable aggregates during the unfolding or refolding processes, even in the natural environment of the living cell, despite the elaborate biological procedures to ensure that a protein folds correctly (*1–3*). Actually, a range of debilitating human diseases is associated with misfolded proteins, including non-disease-associated proteins, that result in the malfunctioning of the cellular machinery (*4*). Among these diseases, most attention has focused on a group of diseases in which proteins or fragments of proteins convert from their normally soluble forms to insoluble fibrils or aggregates that accumulate in a variety of organs.

During the protein folding process, the formation of aggregates competes with the correct folding pathway during *in vitro* folding (*5*). A simple protein folding scheme can be described as follows:

$$N \longleftrightarrow A \longrightarrow Agg \qquad \text{(Scheme 1)}$$

where N, A, and Agg represent a native state, a non-native state, and an aggregation form (*6*). Scheme 1 involves the first-order reversible unfolding of the protein from "N" to "A" and subsequent inter-molecular association with a higher order process. Aggregates are operationally defined by poor solubility in aqueous or detergent solvents (*2*). The process "A" → "Agg" is an irreversible intermolecular process. Scheme 1 has been confirmed for several model proteins (*7, 8*). The kinetics of Scheme 1 is also changed by various environmental factors, such as temperature, pH, pressure, ionic strength, and the coexistence of small molecule additives. When establishing a method to prevent protein aggregation by additions to a protein that aggregates *via* this folding pathway, it is important to apply a practical method of production because it is easy to remove a small molecule after refolding is completed. Accordingly, a small molecular additive is useful as an aggregation suppressor.

These additives may influence both the solubility and the stability of proteins in the unfolded and native states, respectively. They also may change the folding rate to prevent or accelerate the formation of non-specific aggregates. During refolding of human plasminogen activator, Arg is very effective in increasing the yield of the active, soluble form (*9*). The effect of Arg on the refolding yield was confirmed for immunotoxine (*10*) and antibody Fab fragment (*11*), while Arg slightly destabilizes cytochrome *c* (*12*). Other molecules also have been reported as refolding additives. Pro at 1 M, glycerol at 10%, and heparine at 25 μg/ml prevent the aggregation of creatine kinase (*13*). Pro prevents aggregation during the oxidative refolding of reduced and

[1] This work was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture of Japan (14350433, 14045229) and a grant from the Science and Technology Incubation Program in Advanced Region by JST (Japan Science and Technology Corporation).

[2] To whom correspondence should be addressed. School of Materials Science, Japan Advanced Institute of Science and Technology, 1-1 Asahidai, Tatsunokuchi, Ishikawa 923-1292. Tel: +81-761-51-1650, Fax: +81-761-51-1655, E-mail: takagi@jaist.ac.jp

Abbreviations: A, a non-native state; Agg, aggregation form; DSC, differential scanning calorimetry; N, native state; RCM, reduced carboxymethyl.

denatured lysozyme, while sucrose, glycerol, Gly, and ethylene glycol do not prevent it (*3*). Polyethylene glycol improves the structure formation of bovine carbonic anhydrase B *in vitro* (*1*). Empirical data have been reported for the prevention of protein aggregation by small molecular additives, but systematic analysis has not been done.

In this paper, we focus on the mechanism of protein aggregation with the addition of amino acids and ions. Heat-induced aggregation and dilution-induced aggregation by denatured hen-egg lysozyme were systematically examined and compared. Findings about the protein aggregation process and additives for the prevention of protein aggregation will help to improve the *in vitro* folding process of proteins and provide clues for designing drugs to prevent protein misfolding *in vivo*.

MATERIALS AND METHODS

Materials—Hen-egg white lysozyme, *Candida rugosa* lipase, bovine trypsin, bovine α-chymotrypsinogen, bovine ribonuclease A, horse myoglobin, bovine cytochrome *c*, and concanavalin A were purchased from Sigma Chemical (St. Louis, Missouri). All amino acids and urea were purchased from Wako Pure Chemical Industries (Osaka). All other chemicals used were of high-quality analytical grades. Reduced carboxymethyl lysozyme (RCM-lysozyme) was prepared as described previously (*14*).

Heat-Induced Aggregation of Lysozyme—The amount of heat-induced aggregation of lysozyme was determined as follows. Lysozyme or other proteins at 0.2 mg/ml in 50 mM Na-phosphate buffer (pH 6.5) with various concentrations of amino acids, NaCl, and KCl were prepared. All additive preparations were adjusted to pH 6.5 before the preparation of samples. Samples were subjected to heat treatment at 98°C for the specified time, and centrifuged at 15,000 ×*g* for 20 min at 4°C. The samples were monitored by a Jasco spectrophotometer, model V-550 (Japan Spectroscopic Company, Tokyo). The concentration of the soluble fraction was determined by the absorbance spectrum, focusing on changes at 280 nm.

Dilution-Induced Aggregation from Denatured RCM-Lysozyme—The amount of dilution-induced aggregation from the denatured state was determined as follows. RCM-lysozyme at 2.0 mg/ml in 8 M urea was diluted with 10-fold sample buffer containing various concentrations of amino acids, NaCl, or KCl in 50 mM Na-phosphate buffer (pH 6.5). All additives were adjusted to pH 6.5 before preparation of the monitored samples. Protein stock solutions and sample buffers were preincubated at 30°C. After dilution, the refolded sample was centrifuged at 15,000 ×*g* for 20 min. The samples were monitored in a Jasco spectrophotometer, model V-550. The concentration of the soluble fraction was determined from the absorbance spectrum at around 280 nm.

Differential Scanning Calorimetry (DSC) Measurement—DSC scans for measuring the T_m of lysozyme with amino acid additives were obtained using a nano-DSCII Differential Scanning Calorimeter 6100 (Calorimetry Sciences Corporation, UT) with golden cells in a volume of 0.30 ml at scanning rates of 1°C/min. Degassing during the calorimetric experiments was prevented by maintaining an additional constant pressure of 2.5 bar over the liquid in the cells. The measurements were performed in 50 mM Na-phosphate buffer (pH 6.5) with and without Gly or Arg. The protein concentration was adjusted to 4.0 mg/ml.

RESULTS

Effect of Amino Acids on Aggregation—Two types of aggregation, heat-induced and dilution-induced, were investigated using lysozyme as a model protein. As intact lysozyme shows full reversibility from the denaturant-induced unfolded state, reduced and carboxymethylated (RCM) lysozyme was used to monitor dilution-induced aggregation.

The effect of amino acids on aggregation was investigated using the heat-induced aggregation method. Figure 1A shows the time course of aggregation lysozyme with and without Arg. Without amino acid, the amount of aggregation increased with longer incubation times at 98°C. The data can be described well by a single exponential function with a time lag of 4 min. The rate constant of aggregation was 9.3×10^{-3}/min. With 0.1 M Arg, the rate constant of aggregation decreased by about 40% (5.4×10^{-3}/min) compared to that without Arg. Figure 1B shows the dependence of cooling time on aggregation after heat treatment at 98°C for 30 min. No time dependence was detected from 0 to 25 min with or without Arg.

Figure 2A shows the concentration of soluble lysozyme remaining in the supernatant after heat-induced aggregation with various concentrations of Arg. In the case of heat treatment at 98°C for 30 min, Arg concentrations lower

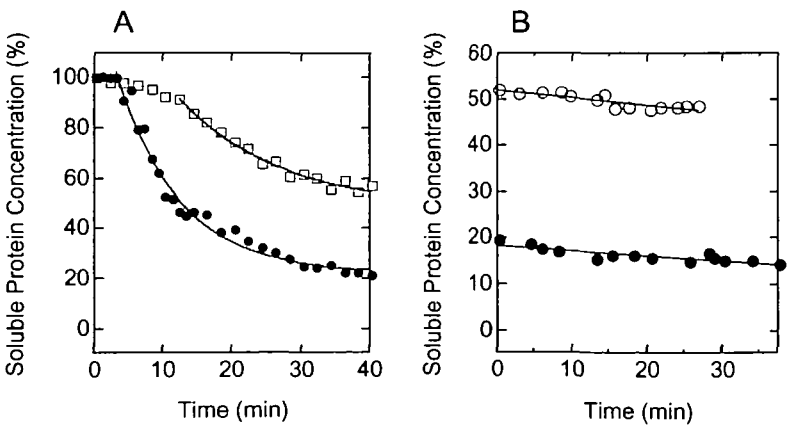


Fig. 1. **(A) Time course for the aggregation of lysozyme after heat-induced denaturation in the presence of 100 mM Arg (open squares) and without Arg (closed circles).** The continuous curves show the best fit to a single-exponential equation. **(B) Cooling time dependence of the aggregation of lysozyme after heat-treatment at 98°C for 30 min.** Horizontal axis is defined as the cooling time from 98 to 50°C. Closed and open circles show the data without Arg and with 100 mM Arg, respectively.

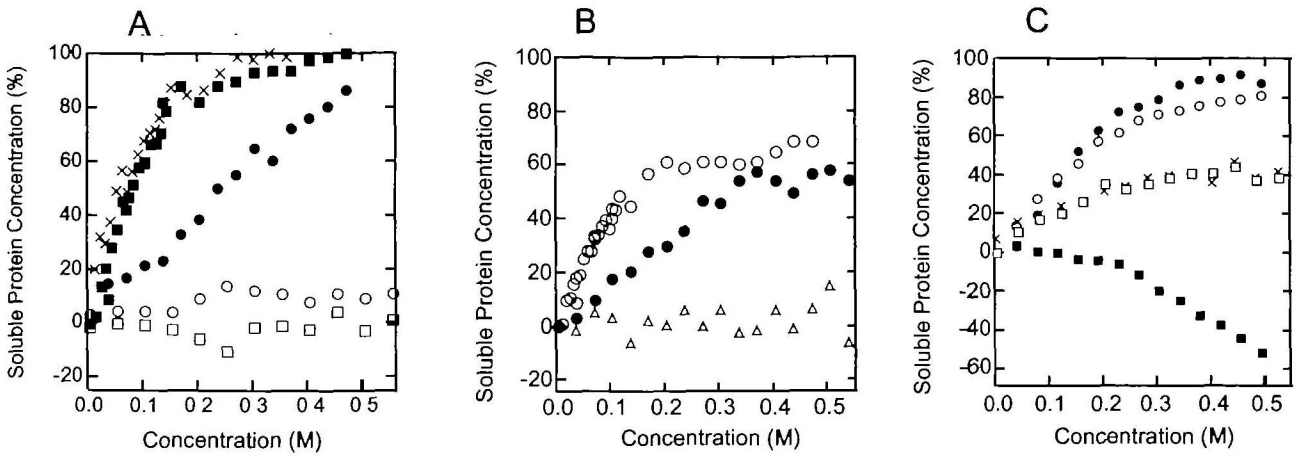


Fig. 2. **(A) Soluble lysozyme concentration after heat treatment at 98°C for 30 min with L-Arg (closed squares), 30 min with D-Arg (crosses), and 60 min with L-Arg (closed circles) at different Arg concentrations.** Open circles and open squares represent the concentration of soluble lysozyme after heat-induced denaturation at 98°C for 30 min with different concentrations of NaCl and KCl, respectively. **(B) Concentration of soluble lysozyme after heat-induced denaturation in the presence of Lys or Gly.** Soluble lysozyme concentration after heat treatment at 98°C for 30 min (open circles) or 60 min (closed circles) with various concentrations of Lys. Open triangles show the soluble lysozyme concentration after heat treatment at 98°C for 30 min in the presence of various concentrations of Gly. **(C) Prevention of RCM-lysozyme aggregation induced by dilution from the denatured state.** RCM-lysozyme in 8.0 M urea was diluted 10-fold in the presence of the respective concentrations of amino acids dissolved in 50 mM Na-phosphate buffer (pH 6.5). Closed circles, Arg; open circles, Lys; closed squares, Gly; crosses, NaCl; open squares, KCl.

than 200 mM, the amount of soluble lysozyme increased as the concentration of Arg increased (Fig. 2A). At Arg concentrations greater than 200 mM, almost 100% of the lysozyme remained in the soluble form. The preventive effect of D-Arg was identical to that of L-Arg. The titration curve of L-Arg monohydrochloride was identical to that of L-Arg (data not shown). When the heat-treatment time was extended to 60 min, the overall concentrations of soluble lysozyme decreased in comparison with the shorter heat-treatment time (30 min) and higher concentrations of Arg (>500 mM) were needed to prevent aggregation almost completely (>90%). It was clearly shown that 200 mM Arg could significantly prevent the formation of insoluble aggregates. Although ions have been considered to have a preventive effect against protein aggregation, NaCl and KCl did not prevent aggregation at concentrations of 500 mM (Fig. 2A). Figure 2B shows the concentration of soluble lysozyme in the presence of various concentrations of Lys and Gly. With increasing concentrations of Lys, the concentration of soluble lysozyme became higher. As in the case of Arg, after extended heat treatment (60 min), the concentration of soluble lysozyme was lower than after the shorter heat-treatment time (30 min). However, Gly did not prevent aggregation even at high concentrations (>500 mM), indicating that the side-chains of charged amino acids might be very important for preventing aggregation. Moreover, even among charged amino acids, the slight difference in the side-chain structures between Arg and Lys was crucial for the effective prevention of aggregation.

The dilution-induced aggregation of denatured lysozyme was also attempted in the presence of various amino acids as additives. Figure 2C shows the preventive effects of Arg, Lys, and Gly on dilution-induced aggregation of the denatured state. As the Arg concentration increased from 0 to 300 mM, the concentration of soluble lysozyme in the supernatant became higher. At about 300 mM Arg, almost

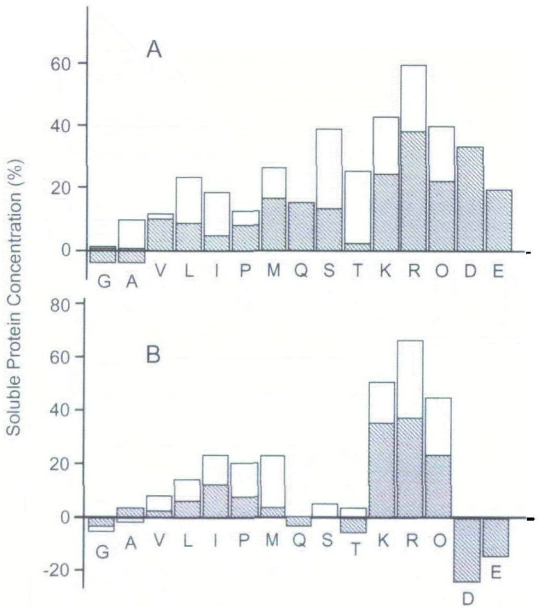


Fig. 3. **Preventive effect of amino acids on lysozyme aggregation by the heat-induced method (A) and the dilution method from denaturant (B).** Inserted letters show the single letter code for amino acids as additives. (A) Soluble lysozyme concentration after heat-induced denaturation at 98°C for 30 min with 200 mM (open bars) and 50 mM (shaded bars) of each amino acid. Data for Q, D, and E at 200 mM could not be determined because of the low solubility of the amino acids in the buffer. (B) Soluble RCM-lysozyme concentration after dilution from 8.0 M urea to buffer containing 200 mM (open bars) and 50 mM (shadow bars) amino acids. Q, D, and E at 200 mM could not be determined because of their low solubility.

all the lysozyme remained in a soluble form, even after the denaturing treatment. The profile for Lys was almost the

same as that for Arg, although the maximum effect of Lys on the prevention of aggregation was slightly weaker. On the other hand, as the concentration of Gly became higher, dilution-induced aggregation was accelerated. Ions showed less ability to prevent aggregation than Arg or Lys (Fig. 2C). By adding Arg or Lys as additives, dilution-induced aggregation of denatured lysozyme could be significantly prevented.

The effects of the side chain structure of amino acids on preventing lysozyme aggregation was carried out by studying the effects of various hydrophobic and charged amino acids, Ala, Val, Pro, Leu, Ile, Met, Gln, Ser, Thr, ornithine (Orn), Asp, and Glu. Because of low solubility in the buffer or strong noise while detecting absorbance around 280 nm, the effects of Trp, Phe, Tyr, His, Asn, and Cys on aggregation could not be examined. Figure 3 shows the ratio of soluble lysozyme concentration in the presence of 200 mM and 50 mM added amino acid in the cases of both heat-induced aggregation (Fig. 3A) and dilution-induced aggregation (Fig. 3B). In the case of heat-induced aggregation, charged amino acids (Lys, Arg, Asp, Glu, and Orn) exhibited high preventive effects on aggregation, but hydrophobic amino acids (Ala, Val, Leu, Ile, Pro, and Met) were less effective. In the case of dilution-induced aggregation (Fig. 3B), amino acids with a non-charged group (Ser, Thr) failed to prevent protein aggregation at all, although positively charged amino acids (Lys, Arg, and Orn) and hydrophobic amino acids prevented aggregation as in the case of heat-induced aggregation. Interestingly, in this case, negatively charged amino acids (Asp and Glu) promoted protein aggregation, unlike the case for heat-induced aggregation. The different effects of side-chain structures in heat-induced and dilution-induced aggregation must depend on the equilibrium condition between the unfolded state (U) and the native state (N) specifically found in the case of dilution-induced aggregation from the denatured protein. The data suggest that positively charged amino acids prevent the refolding rate of lysozyme because of their high isoelectric points. The results of these survey studies indicate that polarity rather than amphipathy of amino acids is important in preventing aggregation.

DSC Analysis—DSC analysis was performed to determine the melting temperature (T_m) of lysozyme in the presence of amino acids as additives (Fig. 4). The T_m value of lysozyme in 50 mM Na-phosphate buffer was 77.5°C. With 20 mM Arg and Gly, the T_m value of lysozyme in 50 mM Na-phosphate buffer was 77.8 and 77.7°C, respectively. With 60 mM Arg and Gly, the T_m value of lysozyme in 50 mM Na-phosphate buffer was 78.0 and 77.7°C, respectively. The results indicate that the prevention of aggregation by Arg is not due to a higher T_m, but that other factors are involved.

Prevention of Aggregation in Other Proteins—We further studied the preventive effects of Arg on protein aggregation using other target proteins with different sizes and p*I*s (Fig. 5). RNaseA (124 residues; p*I* 9.5), chymotrypsinogen A (245 residues; p*I* 9.5), concanavalin A (237 residues; p*I* 4.4), cytochrome *c* (103 residues; p*I* 10.1), myoglobin (153 residues; p*I* 8.1), trypsin (223 residues; p*I* 8.1), and lipase (534 residues; p*I* 4.5), as well as lysozyme (130 residues; p*I* 11.0), were tested. The proteins were heat treated at 98°C for 30 min in the presence of 50 mM amino acid, and the protein concentration in the soluble fraction was determined. Figure 5 shows the prevention of aggregation by Gly, Asp, Glu, Lys, and Arg. Protein aggregation was not prevented by the addition of Gly in the case of any of the tested proteins, indicating that the main chain of the amino acid does not contribute to the prevention of aggregation. Other charged amino acids prevented aggregation, except

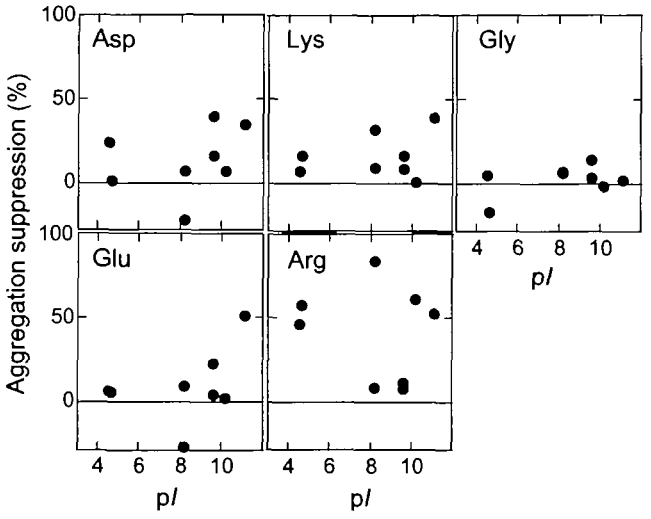
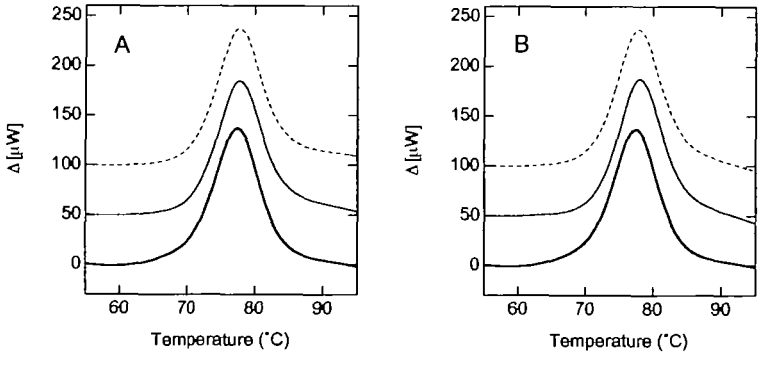


Fig. 5. **Preventive effect of amino acids on heat-induced aggregation of different proteins.** Various proteins at a concentration of 0.2 mg/ml were incubated, respectively, with 50 mM Asp, Glu, Lys, Arg, and Gly at 98°C for 30 min. The concentration of soluble lysozyme was then determined. The isoelectric points of the proteins were calculated from their amino acid sequences. Chymotrypsinogen A, 9.5; concanavalin A, 4.4; cytochrome *c*, 10.1; lipase, 4.5; lysozyme, 11.0; myoglobin, 8.1; RNaseA, 9.5; trypsin, 8.1.

Fig. 4. **DSC measurements of lysozyme with Arg (thin solid line), with Gly (dotted line), and without amino acids (bold solid line).** (A) 20 mM additives. (B) 60 mM additives. DSC scan for each condition was obtained using a solution containing 4.0 mg/ml lysozyme in 20 mM Na-phosphate buffer (pH 6.5) at a scan rate of 1°C/min.



for Glu and Asp in the case of myoglobin. Interestingly, no clear relation could be found between the prevention of aggregation and the p*I* of the target protein. For example, the aggregation of an acidic protein (lipase: p*I* = 4.5) was prevented even by positively charged amino acids (Lys, 17%; Arg, 58%), while the aggregation of lipase could not be prevented by negatively charged amino acids (Asp, 1%; Glu, 6%). However, the aggregation of a neutral protein (myoglobin: p*I* = 8.1) showed a similar profile. The aggregation of myoglobin could be prevented by positively charged amino acids (Lys, 32%; Arg, 86%) but not by negatively charged amino acids (Asp, –22%; Glu, –27%). The averages of the preventive effects on aggregation by Asp, Glu, Arg, Lys, and Gly were 14, 9, 41, 16, and 3%, respectively. These results indicate that Arg has the most significant effect on the prevention of aggregation of various kinds of proteins despite differences in p*I* and molecular weight.

DISCUSSION

In this paper, two typical types of aggregation, heat-induced and dilution-induced, were chosen and the effects of amino acids as additives were systematically examined. From both the basic and practical points of view, protein aggregation could be improved by the following methods. (i) The addition of Arg at 200 mM improves heat-induced protein aggregation. When Arg cannot be used for some reason, charged amino acids can be used instead. However, Asp and Glu cannot be dissolved in buffers at high concentrations (upper limit 50–100 mM). (ii) The addition of Arg at 200 mM also improves dilution-induced aggregation from the denatured form. However, dilution-induced aggregation is related to the balance of folding competition. (iii) Ions can prevent protein aggregation, but at least for lysozyme, the efficiency of prevention is lower than in the case of Arg. (iv) Keeping the protein concentration low is one of the easiest ways to minimize protein aggregation. Previous reports have suggested that optimum refolding yields can be expected in the range of 10–50 μg/ml (*15*).

Protein–protein interactions relate to the refolding yield. Basic proteins enhance the refolding yield of other basic proteins (*16*), suggesting that the net charge of proteins plays a significant role in protein aggregation. However, aggregation of the basic protein lysozyme (p*I* = 11) was improved by Asp and Glu as well as by Arg, Lys, and Orn (Fig. 3). Our results indicate that hydrophilicity rather than hydrophobicity of the additive is the important factor for preventing aggregation. However, the results of a survey of different proteins, as shown in Fig. 5, suggest that Arg has the greatest preventive effect against aggregation for various kinds of proteins with different molecular weights and p*I*s. If the side-chain of Arg possesses properties identical to those of Gdn-HCl, incorrect inter- or intra-molecular hydrogen bonds may be cleaved and rearranged by Arg as in the case by Gdn-HCl (*16*).

We thank Dr. Robert B. Digiovanni (JAIST) for helpful comments and critical reading of the manuscript.

REFERENCES

1. Cleland, J.L. and Wang, D.I.C. (1990) Refolding and aggregation of bovine carbonic anhydrase B: Quasi-elastic light scattering analysis. *Biochemistry* **29**, 11072–11078
2. Fink, A.L. (1998) Protein aggregation: folding aggregates, inclusion bodies and amyloid. *Fold. Design* **3**, R9-R23
3. Samuel, D., Kumar, T.K., Ganesh, G., Jayaraman, G., Yang, P.W., Chang, M.M., Trivedi, V.D., Wang, S.L., Hwang, K.C., Chang, D.K., and Yu, C. (2000) Proline inhibits aggregation during protein refolding. *Protein Sci.* **9**, 344–352
4. Bucciantini, M., Giannoni, E., Chiti, F., Baroni, F., Formigli, L., Zurdo, J., Taddei, N., Ramponi, G., Dobson, C.M., and Stefani, M. (2002) Inherent toxicity of aggregates implies a common mechanism for protein misfolding diseases. *Nature* **416**, 507–511
5. Zettlmeissl, G., Rudolph, R., and Jaenicke, R. (1979) Reconstitution of lactic dehydrogenase. Noncovalent aggregation vs. reactivation. 1. Physical properties and kinetics of aggregation. *Biochemistry* **18**, 5567–5571
6. Minton, K.W., Karman, P., Hahn, G.M., and Minton, A.P. (1982) Nonspecific stabilization of stress-susceptible proteins by stress-resistant proteins: a model for the biological role of heat shock proteins. *Proc. Natl. Acad. Sci. USA* **79**, 7107–7111
7. Winzor, C.L., Winzor, D.J., Paleg, L.G., Jones, G.P., and Naidu, B.P. (1992) Rationalization of the effects of compatible solutes on protein stability in terms of thermodynamic nonideality. *Arch. Biochem. Biophys.* **296**, 102–107
8. Georgiou, G., Valax, P., Ostermeier, M., and Horowitz, P.M. (1994) Folding and aggregation of TEM beta-lactamase: analogies with the formation of inclusion bodies in *Escherichia coli*. *Protein Sci.* **3**, 1953–1960
9. Rudolph, R., Boehn, G., Lilie, H., and Jaenicke, R. (1997) *A Practical Approach in Protein Function*, 2nd ed. (Creighton, T.E., ed.) pp. 57–99, IRL Press
10. Brinkmann, U., Buchner, J., and Pastan, I. (1992) Independent domain folding of *Pseudomonas* exotoxin and single-chain immunotoxins: influence of interdomain connections. *Proc. Natl. Acad. Sci. USA* **89**, 3075–3079
11. Buchner, J. and Rudolph, R. (1991) Renaturation, purification and characterization of recombinant Fab-fragments produced in *Escherichia coli*. *Biotechnology* **9**, 157–162
12. Taneja, S. and Ahmad, F. (1994) Increased thermal stability of proteins in the presence of amino acids. *Biochem. J.* **303**, 147–153
13. Meng, F., Park, Y., and Zhou, H. (2001) Role of proline, glycerol, and heparin as protein folding aids during refolding of rabbit muscle creatine kinase. *Int. J. Biochem. Cell Biol.* **33**, 701–709
14. Shiraki, K., Nishikawa, K., and Goto, Y. (1995) Trifluoroethanol-induced stabilization of the alpha-helical structure of beta-lactoglobulin: implication for non-hierarchical protein folding. *J. Mol. Biol.* **245**, 180–194
15. Rudolph, R. and Lilie, H. (1996) In vitro folding of inclusion body proteins. *FASEB J.* **10**, R49–R56
16. Trivedi, V.D., Raman, B., Rao, C.M., and Ramakrishna, T. (1997) Co-refolding denatured-reduced hen egg white lysozyme with acidic and basic proteins. *FEBS Lett.* **418**, 363–366