Dissolution of protein aggregation by small amine compounds

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Abstract

Proteins tend to form insoluble and inactive aggregation when they are exposed to extreme-environment such as high temperature. One of the major approaches for a new method to prevent undesirable aggregation formation is to explore new small molecular additives, such as ion, amino acid, and small amount of organic solvent and denaturant. Here we propose that polyamines, which is naturally occurring amine compounds, exhibited much stronger preventive effect on heat-induced protein aggregation formation than arginine, the most conventionally used additive. In addition, spermidine could even dissolve protein fibril, although the fibrils are resistant against glycine and arginine. These results implied that polyamines could function as a new class for aggregation suppressors to stabilize heat-labile proteins. Moreover, polyamines might be important precursors for drugs against folding-related diseases.

1. Introduction

Protein aggregation is a major problem both in laboratories and industries because many proteins are prone to form insoluble and inactive aggregation in vitro. Due to the advances in gene expression techniques, finding more general strategies to prevent the inactivation and aggregation of protein is expected. In order to prevent the protein aggregation formation in vitro, various techniques have been developed, including the molecular chaperone [1] and artificial chaperone using cyclodextrin-detergent system [2]. These excellent but complex systems contributed to overcome many difficulties in recovery of active proteins from insoluble inclusion body at laboratory scale. However, the complicated procedures and high costs are disadvantages to apply the method in industrial scale. Major approach to prevent aggregation formation is to add small molecular additives in protein solution. Many additives have been reported and in practical use. Addition of Pro [3], sugar [4], glycerol [5], or detergents [6] is very often used. These additives can partly prevent the aggregation formation of proteins, however, the effect is not completely satisfactory to solve problems of protein aggregation especially in industrial scale. In particular, the method to dissolve protein aggregation has not been established yet.

Recently, we reported that arginine (Arg) showed the best result among 15 kinds of amino acids [7]. Here we report that the amine group plays a key role as the aggregation suppressor. Moreover, we propose a new class of additives, polyamine, including putrescine, spermidine, and spermine, to prevent the aggregation formation of proteins. Previously it was reported that polyamins could bind and dissolve DNA and RNA molecules [8–10]. Polyamines can be found in hyperthermophilic microorganisms living in high-temperature environment [11,12], implying the possibility that polyamins may have a role for prevention of the aggregation formation of proteins.

2. Experimental procedure

Hen egg white lysozyme was purchased from Sigma Chemical Co. (St Louis, Missouri). All amino acids, putrescine, spermidine, spermine, and other chemicals were purchased from Wako Pure Chemical Industries (Osaka, Japan).

The amount of heat-induced aggregation of lysozyme was determined as follows. Lysozyme at 0.2 mg/ml in 50 mM Na–phosphate buffer (pH 6.5) with various concentrations
of Arg, glycine (Gly), putrescine, spermidine, spermine, guanidine hydrochloride (Gdn), urea, sodium chloride (NaCl), potassium chloride (KCl), 2-propanol, and dimethylsulfoxide (DMSO) were prepared. All additives were adjusted to pH 6.5 before preparation of the measured samples. After heat treatment at 98 °C for a specific time, the sample was centrifuged at 15,000 g for 20 min at 4 °C. The sample was monitored by a Jasco spectrophotometer, model V-550 (Japan Spectroscopic Company, Tokyo, Japan). The concentration of soluble fraction was determined by absorbance spectrum at around 280 nm intensity changes.

Amyloid-like fibril of lysozyme was prepared as follows. Lysozyme at 3 mg/ml in 20 mM HCl was boiled for 5 min, and then the sample was frozen in liquid nitrogen. The sample was incubated at 37 °C for 2 months. The solution with fibrillar aggregation was diluted two-fold with buffers containing 0.1 M spermidine, Arg, and Gly. The solution was incubated at 37 °C for 7 days and then the shape of fibril was observed under atomic force microscope (AFM). A portion of the sample was placed on freshly cleaved mica. The sample was dried up and rinsed by pure water. After rinsing for two times, AFM images were obtained under ambient conditions with SPA400 (Seiko Instruments, Inc. Chiba, Japan) operating in tapping mode.

3. Results and discussion

3.1. Preventive effect of small molecular additives on heat-induced aggregation of lysozyme

We showed earlier that Arg is the best additive for heat-induced aggregation of lysozyme and dilution-induced aggregation from denatured lysozyme among 15 kinds of amino acids [7]. The preventive effect of well-known additives in addition to amino acids on aggregation were compared to that of a new class of additives, polyamine. Lysozyme was used as a model protein because this protein is one of the most extensively studied in protein folding and misfolding [13–16] and was irreversibly unfolded by heat treatment at neutral pH [17]. Aggregation was initiated by incubation of lysozyme at a concentration of 0.2 mg/ml in 50 mM Na–phosphate buffer (pH 6.5) with various additives at 98 °C. After incubation for 30 min, the samples were immediately centrifuged at 15,000 g for 20 min at 4 °C. The supernatant of the sample was monitored by absorbance at 280 nm and the amount of aggregation was determined.

Fig. 1A shows the aggregation formation after heat treatment at 98 °C for 30 min with various concentrations of Arg and Gly. At concentrations lower than 0.4 M of
Arg, the amount of soluble lysozyme increased as the concentration of Arg increased. At Arg concentration higher than 0.4 M, most of the lysozyme remained in the soluble form. On the other hand, Gly did not prevent the aggregation formation at concentrations ranging from 0 to 0.5 M. The amount of aggregation decreased as NaCl concentration increased from 0 to 1 M (Fig. 1B). KCl showed similar profile to the case of NaCl (Fig. 1B). On the contrary, higher concentrations of NaCl and KCl than 1.0 M slightly rather accelerated the aggregation formation of lysozyme. In either case, these ions had low preventive effect on aggregation formation. Fig. 1C shows the amount of aggregation in the presence of Gdn and urea. The amount of aggregation decreased as Gdn concentration increased from 0 to 0.3 M. Above the Gdn concentrations of 0.4 M, aggregation was not observed. The curve of Gdn was almost identical to that of Arg. On the other hand, the amount of aggregation rather increased by addition of urea. Fig. 1D shows the amount of aggregation in the presence of 2-propanol and DMSO. The amount of heat-induced aggregation increased as the concentration of 2-propanol increased from 0 to 40%. On the other hand, the amount of heat-induced aggregation did not change as the concentration of DMSO increased from 0 to 40%. These data reconfirmed the idea that Arg has the best effect on heat-induced aggregation of lysozyme among amino acids, ions, organic solvents, and denaturants tested. The conclusion is the same as previously reported [7].

Fig. 2 shows the amount of aggregation in the presence of polyamines, putrescine (NH₂–C₃H₆–NH₂), spermidine (NH₂–C₃H₆–NH–C₄H₈–NH₂), and spermine (NH₂–C₃H₆–NH–C₄H₈–NH–C₃H₆–NH₂). Under the neutral pH, putrescine, spermidine, and spermine have positive charges of +2, +3, and +4, respectively. The curve for the smallest polyamine, putrescine, was almost identical to those of Arg and Gdn. Interestingly, spermidine and spermine showed higher preventive effect on prevention of heat-induced aggregation of lysozyme among amino acids, ions, organic solvents, and denaturants tested. The conclusion is the same as previously reported [7].

Fig. 2 shows the amount of aggregation in the presence of polyamines, putrescine (NH₂–C₃H₆–NH₂), spermidine (NH₂–C₃H₆–NH–C₄H₈–NH₂), and spermine (NH₂–C₃H₆–NH–C₄H₈–NH–C₃H₆–NH₂). Under the neutral pH, putrescine, spermidine, and spermine have positive charges of +2, +3, and +4, respectively. The curve for the smallest polyamine, putrescine, was almost identical to those of Arg and Gdn. Interestingly, spermidine and spermine showed higher preventive effect on prevention of heat-induced aggregation of lysozyme than Arg. The addition of 80–100 mM spermidine and spermine completely prevented the heat-induced aggregation formation of lysozyme. The concentration of 80–100 mM spermidine and spermine is a good data for practical application of additive because protein solution usually contains several molecules ranging from 10 to 100 mM when chromatography and spectroscopy were performed.

3.2. Dissolution of fibrillar aggregation by additives

Hen egg white lysozyme is not a protein that causes misfolding-related disease, however, the solution at acidic pH formed fibrillar aggregate [18]. Several non-disease-related fibrils were reported, such as lyozyme in high concentration of ethanol [19], myoglobin at high temperatures [20], SH3 domain of the α-subunit of bovine phosphatidylinositol-3’-kinase at acidic pH [21], and β-lactogloblin in moderate concentration of urea [22].

Fibrils exhibited a height of ~10 nm and no significant height variation along their axis, while all fibrils reported possess almost the same width regardless of the original structure and size of polypeptides (e.g. all α-helix protein of myoglobin [20], all β-sheet protein of β-lactogloblin [22], and random-coil peptide of Aβ₁₋₂₈ fragment [23]). The lengths of lysozyme fibril induced by the condition without additives varied but were typically on the order of >10 μm. This fibril showed an X-ray diffraction pattern with the interstrand spacing at 4.7 Å and the intersheet spacing at about 10–11 Å (data not shown), suggesting that the lysozyme fibril possesses the same properties as those of amyloid-β peptide and other fibrils.

The solution with fibrillar aggregates was diluted two-fold with buffer containing 0 or 0.1 M spermidine. The solutions were incubated for 7 days and then the residual fibrillar aggregates were observed under AFM. The number of fibril significantly decreased by addition of spermidine and the length of fibril decreased with a typical length of <1 μm (Fig. 3B and D) comparing to control measurement without spermidine (Fig. 3A and C). In order to compare the effect of additives, the same experiment was performed in the presence of 0.1 M Gly and Arg. Gly and Arg were not effective to dissolve fibrils of lysozyme (Fig. 3E and F).

Although further analyses, such as time-dependent decrease in fluorescence intensity by thioflavin T, have to be done to conclude the role of polyamine in dissolving.
the amyloid fibril of proteins, these results indicate that spermine prevent the aggregation formation and can dissolve the fibril-like aggregation.

4. Conclusion

In this paper we showed the effect of polyamines on prevention and dissolutin of lysozyme aggregation. The following conclusions were made. (i) Spermidine and spermine prevented the heat-induced aggregation formation of lysozyme at a concentration of 80–100 mM. They are more effective than well-known conventionally used additives including two kinds of ions, denaturants, and organic solvents, as well as 15 kinds of amino acids. (ii) Spermidine and spermine dissolved even the fibril-like aggregation of lysozyme, while Gly and Arg did not. These data revealed that small amine molecules, polyamines, are a new class of molecular additives to prevent and dissolve protein aggregation.

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References

[1] F.U. Hartl, Molecular chaperones in cellular protein folding, Nature 381 (1996) 571–579.
[2] D. Rozema, S.H. Gellman, Artificial chaperones: protein refolding via sequential use of detergent and cyclodextrin, J. Am. Chem. Soc. 117 (1995) 2373–2374.
[3] D. Samuel, T.K. Kumar, G. Ganesh, G. Jayaraman, P.W. Yang, M.M. Chang, V.D. Trivedi, S.L. Wang, K.C. Hwang, D.K. Chang, C. Yu, Proline inhibits aggregation during protein refolding, Protein Sci. (2000) 344–352.
[4] T. Ueda, M. Nagata, T. Imoto, Aggregation and chemical reaction in hen lysozyme caused by heating at pH 6 are depressed by osmolytes, sucrose and trehalose, J. Biochem. (Tokyo) 130 (2001) 491–496.
[5] N. Borges, A. Ramos, N.D. Raven, R.J. Sharp, H. Santos, Comparative study of the thermostabilizing properties of mannosyl-glycerate and other compatible solutes on model enzymes, Extremophiles 6 (2002) 209–216.
[6] R. Rudolph, H. Lilie, In vitro folding of inclusion body proteins, FASEB J. 10 (1996) 49–56.
[7] K. Shiraki, M. Kudou, S. Fujiwara, T. Imanaka, M. Takagi, Biophysical effect of amino acids on the prevention of protein aggregation, J. Biochem. (Tokyo) 132 (2002) 591–595.
[8] J. Pelta, F. Livolant, J.L. Sikorav, DNA aggregation induced by polyamines and cobalthexamine, J. Biol. Chem. 271 (1996) 5656–5662.
[9] E. Raspaud, I. Chaperon, A. Leforestier, F. Livolant, Spermine-induced aggregation of DNA, nucleosome, and chromatin, Biophys. J. 77 (1999) 1547–1555.
[10] N.C. de la Pena, J.A. Sosa-Melgarejo, R.R. Ramos, J.D. Mendez, Inhibition of platelet aggregation by putrescine, spermidine, and spermine in hypercholesterolemic rabbits, Arch. Med. Res. 31 (2000) 546–550.
[11] T. Oshima, New polyamine, thermospermine, 1,12-diamino-4,8-diazadodecane, from an extreme thermophile, J. Biol. Chem. 254 (1979) 8720–8722.
[12] T. Oshima, A pentaamine is present in an extreme thermophile, J. Biol. Chem. 257 (1982) 9913–9914.
[13] Y. Maeda, H. Yamada, T. Ueda, T. Imoto, Effect of additives on the renaturation of reduced lysozyme in the presence of 4 M urea, Protein Engng 9 (1996) 461–465.
[14] D. Rozema, S.H. Gellman, Artificial chaperone-assisted refolding of denatured-reduced lysozyme: modulation of the competition between renaturation and aggregation, Biochemistry 35 (1996) 15760–15771.
[15] C.A. Summers, R.A. Flowers III, Protein renaturation by the liquid organic salt ethylammonium nitrate, Protein Sci. 9 (2000) 2001–2008.
[16] J. Klein-Seetharaman, M. Oikawa, S.B. Grimshaw, J. Wirmser, E. Duchhardt, T. Ueda, T. Imoto, L.J. Smith, C.M. Dobson, H. Schwalbe, Long-range interactions within a nonnative protein, Science 295 (2002) 1657–1658.
[17] H. Tomizawa, H. Yamada, K. Tanigawa, T. Imoto, Effects of additives on irreversible inactivation of lysozyme at neutral pH and 100 degrees C, J. Biochem. (Tokyo) 117 (1995) 369–373.
[18] M.R. Krebs, D.K. Wilkins, E.W. Chung, M.C. Pitkeathly, A.K. Chamberlain, J. Zurdo, C.V. Robinson, C.M. Dobson, Formation and seeding of amyloid fibrils from wild-type hen lysozyme and a peptide fragment from the beta-domain, J. Mol. Biol. 300 (2000) 541–549.
[19] S. Goda, K. Takano, Y. Yamagata, R. Nagata, H. Akutsu, S. Maki, K. Namba, K. Yutani, Amyloid protolillament formation of hen egg lysozyme in highly concentrated ethanol solution, Protein Sci. 9 (2000) 369–375.
[20] M. Fandrich, M.A. Fletcher, C.M. Dobson, Amyloid fibrils from muscle myoglobin, Nature 410 (2001) 165–166.
[21] J. Zurdo, J.I. Guijarro, J.L. Jimenez, H.R. Saibil, C.M. Dobson, Dependence on solution conditions of aggregation and amyloid formation by an SH3 domain, J. Mol. Biol. 311 (2001) 325–340.
[22] D. Hamada, C.M. Dobson, A kinetic study of beta-lactoglobulin amyloid fibril formation promoted by urea, Protein Sci. 11 (2002) 2417–2426.
[23] D.A. Kirschner, H. Inouye, L.K. Duffy, A. Sinclair, M. Lind, D.J. Selkoe, Synthetic peptide homologous to beta protein from Alzheimer disease forms amyloid-like fibrils in vitro, Proc. Nati Acad. Sci. USA 84 (1987) 6953–6957.