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Only the Reduced Conformer of α-Lactalbumin Is Inducible to Aggregation by Protein Aggregates[1]

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Reduced apo-α-lactalbumin (r-LA) in the pre-molten globule state is soluble in neutral and reduced buffer at 25°C but becomes aggregated when aggregates of various proteins are added. However, protein aggregates do not induce the aggregation of apo-α-lactalbumin in the molten globule state. The presence of the molecular chaperone protein disulfide isomerase or the "chemical chaperone" polyethyleneglycol inhibits the induced aggregation. Native proteins, aggregation-free folding intermediates, and soluble aggregates do not induce the aggregation. The interaction between r-LA and protein aggregates is hydrophobic in nature. These findings suggest that pre-molten globule state of LA is the target not only for chaperones but also for protein aggregates.

Key words: folding intermediate, induction of aggregation, pre-molten globule, protein aggregation, reduced apo-α-lactalbumin.

Unraveling the mechanism of protein folding represents one of the most challenging problems in biology today. Protein folding is also an important issue in producing recombinant proteins in *Escherichia coli* and yeast (*1*) and in tackling protein-folding diseases such as prion disease and Alzheimer's disease (*2*). In the former case, we usually have difficulties with correct refolding of over-expressed proteins in inclusion bodies with high efficiency after solubilization by denaturants (*1*). In the latter case, misfolded proteins with non-native conformation have been suggested to result in diseases. The key problem in the above two cases has been recognized to be protein aggregation leading to accumulation of insoluble deposits. Therefore, elucidation of the mechanism of formation of protein aggregates assumes the same importance as the formation of native proteins. A kinetic competition of two alternative pathways has been suggested during protein folding: an on-way folding into biologically active proteins with native conformation and an off-way folding into misfolded products followed by aggregation or degradation (*3*). Protein aggregation in cells has been ascribed to incorrect interactions between partially folded intermediates with transiently exposed hydrophobic surfaces and consequent escape from the quality control system (*4*). For protein-folding diseases, a proposed mechanism is the conversion of soluble proteins into aggregates under denaturing conditions induced by nuclei (*2*). Three models, sequential polymerization, multimeric polymerization and nucleation-growth, have been proposed to depict the mechanism of aggregation (*5*). However, the nature of folding intermediates prone to aggregation is still not clear.

Folding intermediates of many proteins have been characterized to be in the molten globule (MG) state (*6*). Holo-α-lactalbumin (h-LA) is a four-disulphide-bound calcium-binding protein (*7*). Calcium-depleted apo-α-lactalbumin (a-LA) has been reported to exist in the MG state (*8*), and reduced apo-α-lactalbumin (r-LA) in an even more relaxed and expanded structure (*9*), which is the target that molecular chaperones recognize and bind with (*9–12*). In this communication we have investigated the interactions between the above three LA conformers and aggregates of various proteins, and found that the presence of protein aggregates only induces the aggregation of r-LA, but not of h-LA and a-LA, through hydrophobic interaction. Protein disulfide isomerase (PDI) as a molecular chaperone and polyethyleneglycol 4000 (PEG) as a "chemical chaperone" protect r-LA from induced aggregation.

MATERIALS AND METHODS

Materials—Bovine milk h-LA and a-LA, BSA, chick egg lysozyme, 8-anilino-1-naphthalenesulfonic acid (ANS), and guanidine hydrochloride (GdnHCl) were Sigma products. DTT was from Promega. All other reagents were local products of analytical grade.

In all experiments, unless otherwise specified, 0.1 M potassium phosphate buffer (pH 7.5) containing 2.5 mM EDTA and 5 mM DTT was employed and is referred to as phosphate buffer. For a-LA, DTT was omitted; and for h-

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Abbreviations: ANS, 8-anilino-1-naphthalenesulfonic acid; B-P, spontaneous refolding product of denatured BSA; DHFR, dihydrofolate reductase; D-P, spontaneous refolding product of denatured DHFR; GAPDH, D-glyceraldehyde-3-phosphate dehydrogenase; GdnHCl, guanidine hydrochloride; G-P, spontaneous refolding product of denatured GAPDH; LA, α-lactalbumin; h-LA, holo-α-lactalbumin; a-LA, apo-α-lactalbumin; r-LA, reduced apo-α-lactalbumin; L-P, spontaneous refolding product of denatured and reduced lysozyme; MG, molten globule; PDI, protein disulfide isomerase; PEG, polyethyleneglycol 4000; P-P, spontaneous refolding product of denatured PDI.

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LA, both EDTA and DTT were omitted.

Preparation and Determination of Proteins—r-LA was prepared by incubation of a-LA in phosphate buffer containing 5 mM DTT for 30 min at 25°C (*8*). Rabbit muscle D-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was prepared and assayed according to Liang *et al.* (*13*), and bovine liver PDI, according to Lambert and Freedman (*14*). Chicken liver dihydrofolate reductase (DHFR) (*15*) was a kind gift from Dr. Ying Wu of this laboratory.

Protein concentrations were determined by measuring the absorbance at 280 nm with the following absorption coefficients ($A^{0.1\%}_{1cm}$): 2.01 for h-LA, 1.95 for a-LA and r-LA (*9*), 1.00 for GAPDH (*16*), 0.9 for PDI (*17*), 0.66 for BSA, 1.29 for DHFR (*15*), and 2.63 and 2.37 for native and denatured lysozyme respectively (*18*). Tetrameric GAPDH and dimeric PDI were both considered as protomers in the calculations of concentrations.

Characterization of h-LA, a-LA, and r-LA—CD spectra from 200 to 250 nm of 14 μM h-LA, a-LA, and r-LA were examined in a Jasco J720 spectropolarimeter (Tokyo) at 25°C. Intrinsic fluorescence spectra with an excitation at 295 nm and ANS fluorescence spectra with an excitation at 365 nm after addition of 200 μM ANS were recorded in a Hitachi F4010 spectrofluorimeter (Tokyo) at 25°C. The values of control samples containing no proteins were subtracted respectively.

Preparation of Refolding Products of Proteins—GAPDH was fully denatured with 3.0 M GdnHCl at 4°C overnight. PDI and DHFR were denatured with 6 M GdnHCl and BSA with 7.2 M GdnHCl at 25°C for 24 h. All the above proteins were denatured in phosphate buffer at 210 μM. Lysozyme was reduced and denatured according to Song *et al.* (*19*).

Refolding of denatured proteins was carried out by at least 50-fold rapid dilution in phosphate buffer at 25°C, and aggregation and reactivation reached maximum in 3.5 h. The spontaneous refolding products of denatured GAPDH, lysozyme, and BSA were composed largely of aggregates with low activity recovery. They were named G-P, L-P, and B-P respectively. Denatured PDI and DHFR were fully reactivated upon dilution with no aggregation detected, and the spontaneous refolding products were named P-P and D-P, respectively.

Induction of r-LA Aggregation—Two groups of experiments were designed to examine the induced aggregation of r-LA. In the first group, r-LA was rapidly mixed with G-P, L-P, B-P, P-P, D-P, or native proteins in phosphate buffer to specific concentrations. In the second group, denatured GAPDH, PDI, or DHFR was diluted quickly into phosphate buffer containing r-LA at different concentrations. Aggregation in the above systems was continuously monitored immediately after mixing or dilution at 25°C. The effect of G-P formed in the absence of DTT and EDTA on the solubility of h-LA or a-LA was also examined. In order to identify which fraction of the refolding product G-P acted upon to induce r-LA aggregation, the soluble part and precipitable aggregates of G-P were separated by centrifugation at 12,000 rpm for 10 min at room temperature. The precipitate was washed twice and then suspended in phosphate buffer by sonication. The concentration of GAPDH in the supernatant was determined by measuring absorbance at 280 nm, and the extent of association of soluble refolding products was analyzed by size-exclusion chromatography on a Bio-Rad SEC 250-5 column. The supernatant and the suspended aggregates were mixed respectively with r-LA or buffer only. The turbidity values in the mixture with buffer only were subtracted from that in the mixture with r-LA. The mixtures were then centrifuged at 12,000 rpm for 10 min at room temperature when the induced aggregation was complete in 2 h. The remnant r-LA in the supernatant was determined by absorbance at 280 nm.

The aggregation curves were fitted by the exponential function $A = A_\infty (1 - e^{-kt})$, using Microcal Origin 5.0 software. The time range employed for fitting was from 0 to 40 min. The values of kinetic parameters were shown as averages of three independent measurements.

SDS-PAGE Analysis of Induced Aggregates of r-LA—Aggregates of r-LA induced by protein aggregates were collected by centrifugation at 12,000 rpm for 10 min, washed twice with water, dried in vacuum, then analyzed by SDS-PAGE on 15% polyacrylamide gel.

Effect of Additives on the Induced Aggregation of r-LA—Aggregation of r-LA induced by G-P was monitored in the presence of different additives: ethyleneglycol, glycerol, PDI, PEG, or BSA in the phosphate buffer containing 0.5 M potassium phosphate.

Aggregation of proteins in all experiments were monitored by measuring turbidity at 488 nm in a Shimadzu UV-250 spectrophotometer (Tokyo).

RESULTS

Characterization of h-LA, a-LA, and r-LA—As shown in Fig. 1A, the CD spectrum of a-LA is very similar to that of h-LA with typical negative peaks at 208 and 222 nm, while that of r-LA is devoid of the two peaks completely. The intrinsic fluorescence emission maximum for h-LA at 328.4 nm red-shifts to 331.2 nm and further to 344.6 nm for a-LA and r-LA, respectively (Fig. 1B). h-LA shows little ANS binding, whereas a-LA and especially r-LA show markedly increased ANS fluorescence (Fig. 1C). All the above indicate a more relaxed 3D structure of r-LA with more hydrophobic groups exposed than that of h-LA and a-LA.

Induced Aggregation of r-LA—The solutions of h-LA, a-LA, and r-LA at 14 μM in phosphate buffer after standing for up to 15 days at 25°C show neither turbidity at 488 nm nor precipitates by centrifugation at 12,000 rpm for 10 min, indicating that these three forms of LA are stable at neutral pH and 25°C. As shown in Fig. 2A, strong aggregation occurs during spontaneous refolding of denatured BSA, GAPDH, and lysozyme, while PDI and DHFR refold with no aggregation and full activity recovery. Incubation of r-LA with G-P of different concentrations results in aggregation, which increases with increasing concentrations of r-LA and G-P and fitted well with the exponential function $A = A_\infty (1 - e^{-kt})$ (Fig. 2B and Table IA). However, G-P does not induce aggregation of h-LA or a-LA (Fig. 2B, curves 1 and 2). In the presence of 3.5 and 14 μM r-LA, the extent of the induced aggregation increases proportionally at 0.0507 and 0.0778 μM^{-1}, respectively, with the increasing concentration of G-P. The rate constant of aggregation also increases proportionally at 0.149 min^{-1} μM^{-1} with increasing the concentration of G-P, but does not depend on the concentration of r-LA. B-P and L-P also induce the aggregation of r-LA (Fig. 2C and Table IA). In contrast to G-P, B-P, and L-P, native proteins, P-P, and D-P all have no effect on the ag-

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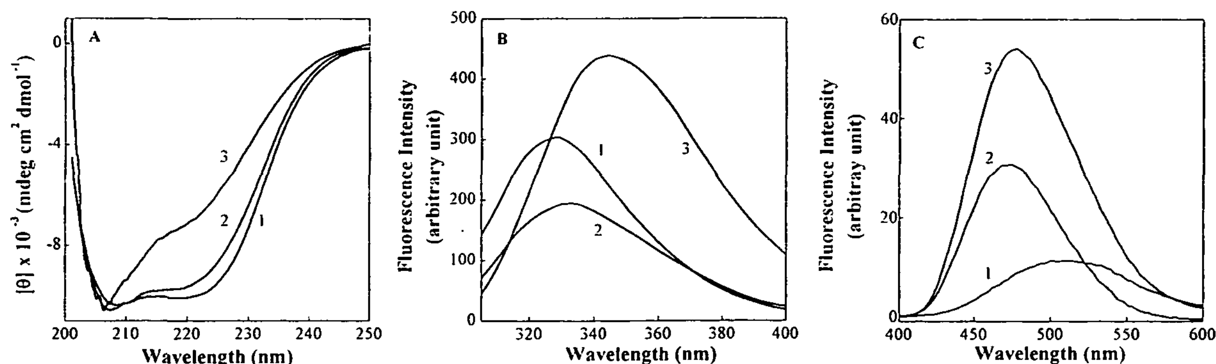

Fig. 1. **Characterization of h-LA, a-LA, and r-LA.** CD spectra (A), intrinsic fluorescence spectra excited at 295 nm (B) and ANS fluorescence spectra excited at 365 nm with 200 μM ANS (C) of h-LA (curve 1), a-LA (curve 2), and r-LA (curve 3) at 14 μM in phosphate buffer were recorded at 25°C.

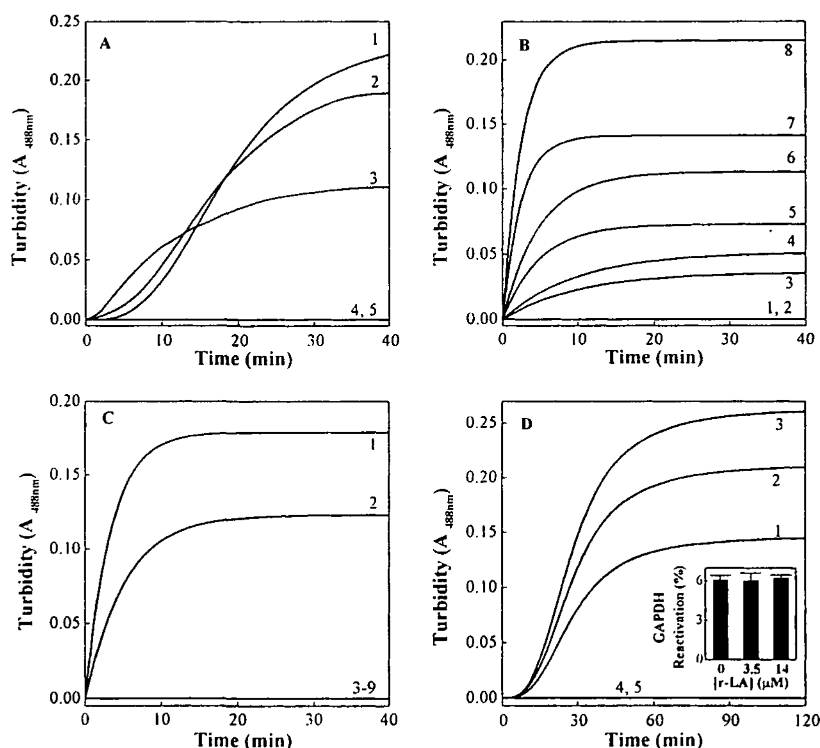

Fig. 2. **Aggregation of r-LA.** Induced aggregation of r-LA was examined at 25°C in phosphate buffer as described in the text. r-LA at different concentrations was quickly mixed with folding products of various proteins or native proteins. Denatured proteins were refolded upon dilution in the presence of r-LA of different concentrations. The turbidity at 488 nm was monitored immediately after mixing or dilution to measure aggregation. (A) Denatured BSA (curve 1), GAPDH (curve 2), lysozyme (curve 3), PDI (curve 4), and DHFR (curve 5) at 210 μM were spontaneously refolded upon dilution to 2.8 μM. (B) h-LA and a-LA at 14 μM were incubated respectively with G-P at 2.8 μM (curve 1 and curve 2); r-LA at 3.5 μM (curves 3, 5, and 7), and 14 μM (curves 4, 6, and 8) incubated with G-P at 0.7 μM (curves 3 and 4), 1.4 μM (curves 5 and 6), and 2.8 μM (curves 7 and 8) respectively. (C) r-LA at 14 μM was incubated with 2.8 μM B-P and L-P respectively (curve 1 and curve 2); or native GAPDH, BSA, lysozyme, PDI, and DHFR at 2.8 μM (curves 3–7); or P-P and D-P at 2.8 μM respectively (curve 8 and curve 9). (D) Denatured GAPDH (curves 1–3), PDI (curve 4), and DHFR (curve 5) were refolded upon dilution to 1.4, 2.8, and 2.8 μM respectively in the absence (curve 1) and presence of r-LA at 3.5 μM (curve 2) and 14 μM (curves 3–5). The inset showed the reactivation yields of GAPDH (data were expressed as mean ± SD, n = 3).

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gregation of r-LA (Fig. 2C, curves 3–9). As shown in Fig. 2D the aggregation during the refolding of GAPDH increases with increasing concentration of r-LA present in the refolding buffer (curves 1–3), but the refolded yields of GAPDH remain constant (Fig. 2D, inset). The presence of r-LA in the refolding buffer does not induce the aggregation during the refolding of PDI or DHFR (Fig. 2D, curves 4 and 5) or affect their full renaturation.

Large-Scale Aggregates in G-P Function to Induce Aggregation of r-LA—The supernatant of G-P of 2.8 μM contains 50.7% protein, which is composed of oligomeric forms with varied extent of association in the range of ~80 to ~1,000 kDa determined by size-exclusion chromatography on a Bio-Rad SEC 250-5 column (data not shown), and shows 6% enzyme activity. These results indicate that G-P contains 6% correctly folded molecules, 45% soluble but inactive forms, and 49% large-scale precipitable aggregates after centrifugation at 12,000 rpm for 10 min. Very similar results have been obtained with G-P of 0.7 and 1.4 μM (data not shown). As shown in Fig. 3 the supernatant of G-P does not induce the aggregation of r-LA (Fig. 3, curve 1), but the suspended aggregates do (Fig. 3, curves 2–4). With increasing amount of added G-P aggregates, the induced aggregation of r-LA is enhanced and the concentration of remnant soluble r-LA is reduced (Fig. 3, inset).

Composition of the Induced Aggregates of r-LA—As shown in Fig. 4, all the aggregates of r-LA induced by G-P, B-P, and L-P (lanes 5, 6, and 7 respectively) and the aggregates formed during GAPDH refolding in the presence of r-LA (lane 8) show both r-LA and the other proteins in SDS-PAGE, indicating the induced aggregation of r-LA by protein aggregates.

TABLE I. **Kinetic parameters, k and A_∞, of the induced aggregation of r-LA in the presence of protein aggregates.** The experiments in (A) and (B) were carried out as described in the legends to Fig. 2, B and C, and Fig. 5 respectively. The data were averaged from three independent measurements and shown as mean ± SD. The time range employed for fitting was from 0 to 40 min.

A

[r-LA] (μM)	k (min^{-1})	A_∞
3.5		
+ 0.7 μM G-P	0.103 ± 0.012	0.035 ± 0.002
+ 1.4 μM G-P	0.212 ± 0.016	0.073 ± 0.004
+ 2.8 μM G-P	0.415 ± 0.023	0.140 ± 0.014
14		
+ 0.7 μM G-P	0.105 ± 0.008	0.051 ± 0.011
+ 1.4 μM G-P	0.207 ± 0.015	0.113 ± 0.006
+ 2.8 μM G-P	0.411 ± 0.017	0.215 ± 0.018
+ 2.8 μM B-P	0.313 ± 0.011	0.179 ± 0.012
+ 2.8 μM L-P	0.198 ± 0.006	0.123 ± 0.010

B

[Potassium phosphate] (M)	k (min^{-1})	A_∞
0.1	0.207 ± 0.012	0.113 ± 0.009
0.5	0.531 ± 0.025	0.302 ± 0.015
+ 10% glycerol	0.254 ± 0.021	0.197 ± 0.022
+ 10% ethyleneglycol	0.326 ± 0.016	0.223 ± 0.018
+ 6-fold PDI	0.051 ± 0.004	0.010 ± 0.001
+ 5% PEG	0.070 ± 0.001	0.012 ± 0.002
+ 10-fold BSA	0.515 ± 0.033	0.299 ± 0.031

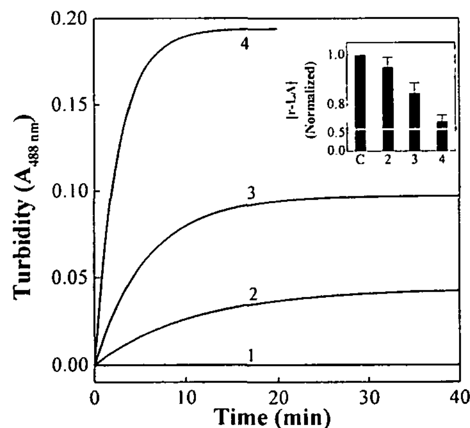

Fig. 3. **Induced aggregation of r-LA by soluble and aggregated part of G-P.** Experiments were carried out as described in the legend to Fig. 2. G-P was centrifuged at 12,000 rpm for 10 min, and the precipitate was suspended by sonication. r-LA at 14 μM was incubated with the supernatant of 2.8 μM G-P (curve 1) or the suspended aggregates of 0.7, 1.4, and 2.8 μM G-P respectively (curves 2, 3, and 4). The inset shows the concentrations of r-LA remaining soluble when aggregation was complete in 2 h. r-LA at 14 μM was taken as 100% for normalization of the concentration (lane C) (data are expressed as mean ± SD, n = 3).

Effect of Additives on the Aggregation of r-LA Induced by G-P—As shown in Fig. 5, the concentration of potassium phosphate in the phosphate buffer was increased from 0.1 to 0.5 M to increase the induced aggregation of r-LA (compare curve 1 to dashed curve), so as to make the effect of additives more marked. Addition of glycerol or ethyleneglycol reduces the aggregation (from curve 1 to 2 or 3). PDI and PEG suppress the aggregation (curves 4 and 5). BSA, as a non-specific protein, has no effect on the induced aggregation of r-LA (dotted curve).

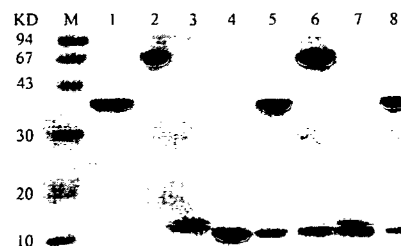

Fig. 4. **SDS-PAGE (15%) analysis of induced aggregates.** M, marker; lane 1, GAPDH; lane 2, BSA; lane 3, lysozyme; lane 4, r-LA; lanes 5–7, aggregates of r-LA at 14 μM induced by 2.8 μM G-P, B-P, and L-P respectively; lane 8, aggregates formed during the refolding of 1.4 μM GAPDH in the presence of 14 μM r-LA.

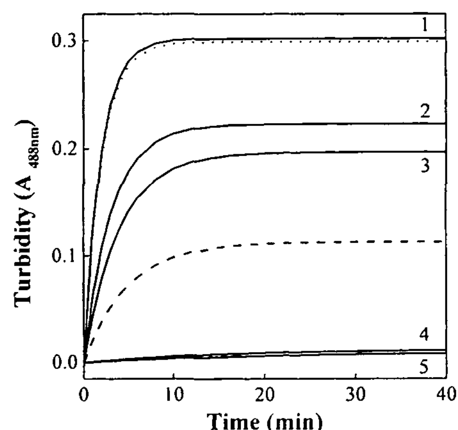

Fig. 5. **Effects of additives on the induced aggregation of r-LA by G-P.** Experiments were carried out as described in the legend to Fig. 2 except that the concentration of potassium phosphate in the buffer was increased to 0.5 M. Aggregation of 14 μM r-LA induced by 1.4 μM G-P was monitored in the absence (curve 1) or presence of 10% glycerol (curve 2), 10% ethyleneglycol (curve 3), 5% PEG (curve 4), 6-fold PDI (curve 5), or 10-fold BSA (dotted curve). Aggregation of 14 μM r-LA induced by 1.4 μM G-P in 0.1 M phosphate buffer is shown as a dashed curve.

DISCUSSION

Native LA changes its conformation to the MG state, a-LA, when the bound calcium is removed (*8*). Reduction of the three disulfide-bonds of a-LA to form r-LA relaxes the molecule further (*9*). The conformational differences among h-LA, a-LA, and r-LA have been commonly recognized, but different authors named the conformational state of r-LA differently. Ptitsyn (*20, 21*) and Huang *et al.* (*12*) designated r-LA as a "pre-molten globule (pre-MG) state," while Creighton (*6*) categorized the conformation of r-LA as a "molten globule state." We have also shown by CD, intrinsic and ANS fluorescence that the conformation of a-LA is more relaxed with less secondary structure and more hydrophobic surface than that of h-LA, while, like h-LA, a-LA does not aggregate in the presence of G-P. On the contrary, r-LA, although appearing stable and remaining soluble in neutral and reduced buffer at 25°C (also see Ref. *11*), does aggregate in response to G-P, B-P, and L-P, indicating that only the conformation of r-LA is inducible to aggregation, and protein species of aggregates are non-specific. In addition, only large-scale protein aggregates, but not native

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protein or soluble aggregates in G-P, are responsible for the induction. This is supported by the fact that the induced aggregation of r-LA is detected only after 5 min upon dilution of denatured GAPDH, when the GAPDH aggregates could be detected by monitoring turbidity. Native proteins and the aggregation-free folding intermediates of PDI and DHFR do not interact with r-LA to induce the aggregation. Speed *et al.* have proposed three models to depict the mechanism of protein aggregation (*5*): (a) sequential particle-cluster polymerization, which shows a plateau at the end when the monomeric intermediates become depleted; (b) multimeric cluster-cluster polymerization, which exhibits a linear increase with time; (c) nucleation-growth, which generates a sigmoidal-shaped curve. The induced aggregation curves of r-LA are fitted well by an exponential function, which is obviously inconsistent with multimeric polymerization and nucleation-growth, but probably in agreement with the sequential polymerization mechanism.

The induced aggregation results from interactions of r-LA with protein aggregates but not from a simple entrapment of r-LA by protein aggregates, since a-LA and h-LA do not interact with the protein aggregates and therefore remain soluble. Although the rate and extent of the induced aggregation increases proportionally with the increasing amounts of r-LA and protein aggregates, the rate constant is only dependent on the amount of protein aggregates, indicating that only the amount of protein aggregates, but not r-LA, is the determinant factor in the kinetics of the induced aggregation.

It has been suggested that electrostatic interaction is involved in the aggregation during the denaturation of LA and lysozyme (*22*). The interactions between r-LA and protein aggregates do not appear to be electrostatic: negatively charged BSA and positively charged GAPDH and lysozyme all induce the aggregation of r-LA in phosphate buffer (pH 7.5). Addition of organic solvents, glycerol or ethyleneglycol, increases the hydrophobicity of the buffer and also inhibits the induced aggregation. These facts indicate that hydrophobic interaction is the major driving force in the induced aggregation of r-LA.

PDI has been characterized as a molecular chaperone (*23, 24*), while PEG has been termed a "chemical chaperone" because of its ability to promote the renaturation of denatured carbonic anhydrase (*25*). The presence of either PDI at super-stoichiometric concentrations [as PDI exhibits its chaperone activity at super-stoichiometric concentrations (*23, 24*)] or PEG inhibits the induced aggregation of r-LA by G-P. It has been reported that molecular chaperones GroEL (*9, 10*), α-crystallin (*11*), and trigger factor (*12*) recognize only r-LA, not a-LA. Our results indicate that PDI and PEG also recognize r-LA, lending additional support for the suggestion by Okazaki *et al.* (*9*) that pre-MG is very likely the target conformer of chaperone action *in vivo.*

It is suggested that the pre-MG state is a key state for interaction with both chaperones and protein aggregates. The interactions with chaperones prevent proteins from aggregation and stimulate further folding, while the interaction with aggregates induces more aggregation. Pre-MG has been reported to be a kind of equilibrium intermediate between the MG and the unfolded state (*21*). Thus pre-MG may represent very early folding intermediates at the branch point of the folding pathway.

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