Reactions of denatured proteins with other cellular components to form insoluble aggregates and protection by lactoferrin

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Abstract Elucidation of the mechanism of formation of insoluble protein aggregates is essential to resolve problems such as protein folding diseases. In this study the effects of various types of biomolecules on the aggregation of denatured proteins were investigated. Denatured α-lactalbumin, an acidic protein, was found to be precipitated by lactoferricin, a basic peptide derived from lactoferrin. Denatured lysozyme, a basic protein, by itself showed aggregation, which was promoted by addition of native α-lactalbumin. Heparin and nucleic acids caused almost instant aggregation of denatured lysozyme. Native lactoferricin was also found to aggregate with heparin or nucleic acids. The results show that denatured/misfolded proteins as well as peptides are highly reactive with other cellular components to form insoluble aggregates and suggest a possible mechanism by which protein folding diseases progress. Most of the above aggregation reactions were inhibited by lactoferrin, which could form soluble complexes with denatured α-lactalbumin, heparin, and nucleic acids.

Key words: Aggregation; Denatured protein; Lactoferrin; Misfolding; Protein folding disease

1. Introduction

Protein folding is an important issue both in the production of recombinant proteins in bacteria such as *Escherichia coli* [1] and in tackling protein folding diseases such as prion and Alzheimer's diseases [2]. In the former case one often encounters inclusion bodies, which must be solubilized by denaturants and then refolded into their native conformation efficiently. In the latter misfolded proteins are thought to be causative agents of the diseases. Both cases involve aggregation of proteins leading to accumulation of insoluble deposits. Thus, elucidation of the mechanism of formation of insoluble protein aggregates is essential to resolve these problems. Most previous studies on protein folding and aggregation have dealt with the self-association of pure proteins without interacting ligands except molecular chaperones. In the course of screening ligands that might promote protein folding, I have found that some natural substances have adverse effects, causing precipitation of denatured proteins and thus preventing the proper folding. Since significant precipitation was observed at a physiological ionic strength and at low ligand concentrations, it is postulated that this kind of aggregation reaction may be significant in vivo if unfolded proteins exist. A possible link of this finding with protein folding diseases is discussed.

Lactoferrin is an iron-binding glycoprotein present in milk and other secretions [3]. It is thought to be involved in host defense by acting as an antimicrobial agent or by stimulating the immune system. The bactericidal domain is located at the N-terminal portion, which can be released by pepsin digestion of the 80-kDa protein [4]. The bactericidal peptide is named lactoferricin and is rich in arginine and lysine; HLFcin corresponds to the N-terminal residues 1–47 having two intramolecular disulfide bonds, BLFcin to residues 17–41 having one intramolecular disulfide bond [5]. I have tested these cognate peptides and proteins for interactions with denatured proteins and found mutually opposing effects. Lactoferrin was found to protect denatured proteins from aggregation induced with lactoferricin and other ligands. The result is discussed in relation to the diseases caused by peptides derived from precursor proteins.

2. Materials and methods

2.1. Materials

Bovine α-lactalbumin, CM-α-lactalbumin, human lactoferrin, bovine lactoferrin, ribonuclease A, bovine serum albumin, low molecular weight heparin from porcine intestinal mucosa (sodium salt; average molecular weight ∼3000) (heparin fragment), poly[C], and poly[C]·poly[G] were purchased from Sigma. Hen egg-white lysozyme (6× crystallized) was from Seikagaku. R-lysozyme and heparin (sodium salt) were from Wako. Urea (biochemical grade) and dithiothreitol (biochemical grade) were from Nacalai Tesque. tRNA from brewer's yeast was from Boehringer Mannheim. λDNA was from Takara. OligoDNA was custom-synthesized. BLFcin and HLFcin were kindly provided by Morinaga Milk Industry, Japan. All of the above reagents were used without further purification. Nucleic acids were dissolved in sterile water or buffer and stored at −80°C until use. Urea solution was prepared fresh each time. Either dithiothreitol was dissolved just before use or a 0.2 M solution was stored at −20°C for a single use.

2.2. Refolding experiments and turbidity measurements

Unless otherwise noted, the experiments were done in 20 mM Tris-HCl+0.15 M NaCl (pH 7.5). α-Lactalbumin and lysozyme were used as model proteins. The two proteins are structurally homologous with four disulfide bonds, but have different isoelectric points: ∼4.5 for α-lactalbumin and ∼11 for lysozyme [6].

α-Lactalbumin was denatured in 7.2 M urea+2 mM dithiothreitol at room temperature for 30 min. Lysozyme was denatured in 8.1 M urea+2 mM dithiothreitol at 40°C for 2 h under nitrogen. The denatured proteins were subjected to refolding by 50-fold dilution into buffer with or without an additive(s). To monitor turbidity with time, 2 μl of the denatured protein solution was first placed in a microcuvette and then 98 μl of the refolding buffer was added and

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Abbreviations: BLFcin, bovine lactoferricin; CM-α-lactalbumin, *S*-carboxymethyl-α-lactalbumin; GAG, glycosaminoglycan; HLF, human lactoferrin; HLFcin, human lactoferricin; oligoDNA, a 24-mer oligodeoxyribonucleotide; poly[C], polycytidylic acid (5′); poly[C]·poly[G], polycytidylic-polyguanylic acid; R-lysozyme, *S*-3-(trimethylated amino)propylated lysozyme

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mixed immediately by pipetting in and out. About 2 min later, recording of absorbance at 550 nm was started and this continued for 20 min. A Pharmacia Ultrospec Plus spectrophotometer with a 70-μl ultramicrovolume black cell was used.

2.3. Gel filtration analysis

Protein-protein interactions were analyzed by gel filtration using a Superose 12 PC 3.2/30 column on the SMART system (Pharmacia). The column was equilibrated and run with 20 mM Tris-HCl+0.15 M NaCl (pH 7.5) at a flow rate of 40 μl/min at room temperature. The column was calibrated with a gel filtration calibration kit from Pharmacia. Before each run, the sample was mixed in the same buffer and filtered through an Ultrafree C3-GV (0.22 μm) filter (Millipore). A volume of 40 μl was applied. The apparent molecular weights for the protein peaks were evaluated from the plots of K_{av} vs. log(molecular weight), where $K_{av}=(V_e-V_o)/(V_t-V_o)$, where V_e is the elution volume, V_o the void volume, and V_t the total bed volume [7]. The molecular weights of lactoferrin and α-lactalbumin thus obtained were not quite compatible with the known values, ca. 80 000 [3] and 14 200 [6] for the two proteins, respectively. The reasons for the discrepancy were not pursued in this study.

3. Results

3.1. Aggregation of denatured proteins

Denatured and reduced α-lactalbumin did not show any significant aggregation leading to precipitation when refolded at 90 μg/ml in 20 mM Tris-HCl+0.15 M NaCl (pH 7.5) without any additives, although the reduced protein was expected to assume a denatured or misfolded conformation under the non-denaturing condition (the final concentrations of urea and dithiothreitol were 0.14 M and 40 μM, respectively) [8]. Addition of 20 μg/ml or more of BLFcin to this refolded solution caused a time-dependent precipitation. The same result was obtained when the refolding was conducted in the presence of BLFcin (Fig. 1A). HLFcin was found to have a similar effect. Refolded lysozyme (90 μg/ml) by itself showed aggregation, which was promoted by the addition of native α-lactalbumin (Fig. 1A). Addition of 10 μg/ml or more of heparin or heparin fragment caused rapid aggregation, the latter being more effective (Fig. 1A). These aggregation reactions could be observed at protein concentrations as low as 10–20 μg/ml (exception: without an additive, denatured lysozyme did not precipitate at 20 μg/ml). R-lysozyme [9], which did not by itself have a tendency to aggregate, was also aggregated with heparin or heparin fragment in a similar manner (data not shown). Various kinds of nucleic acids, double-stranded or single-stranded DNA, RNA, and oligonucleotide, had similar effects to those of heparin and heparin fragment on denatured lysozyme (Fig. 1B); similarly, tRNA and a sin-

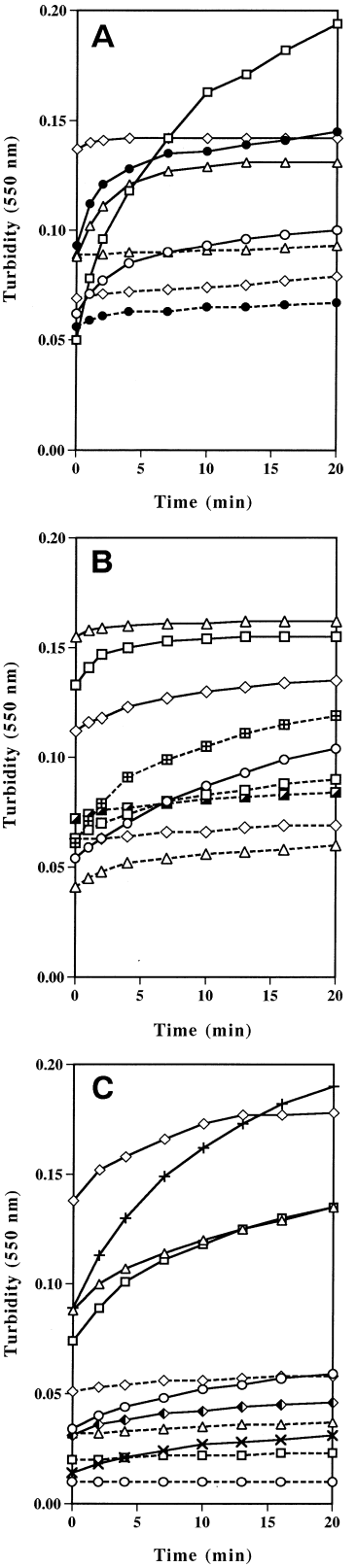

Fig. 1. Time course of aggregation. The effects of various additives on 90 μg/ml denatured α-lactalbumin (A, filled circles), 90 μg/ml denatured lysozyme (A, open symbols; B), and 100 μg/ml BLFcin (C) in 20 mM Tris-HCl+0.15 M NaCl (pH 7.5). Although the absorbance at 550 nm was recorded continuously, each curve is plotted with a specific symbol at certain time intervals to distinguish them from each other. The dashed lines indicate that the additives contained HLF. The additives (the numbers denote concentrations in mg/ml) are: in A, —●—, BLFcin 0.04; —○—, none; —□—, α-lactalbumin 0.05; —△—, heparin 0.1; —◇—, heparin fragment 0.1; --●--, BLFcin 0.04+HLF 1.0; -△-, heparin 0.1+HLF 2.0; --◇--, heparin fragment 0.1+HLF 1.0. In B, —○—, none; —△—, tRNA 0.01; —□—, λDNA 0.01 (oligoDNA 0.01 and poly[C] 0.01 almost overlap this curve); —◇—, poly[C]·poly[G] 0.01; --△--, tRNA 0.01+HLF 0.2; --□--, λDNA 0.01+HLF 1.0; plus-in-square, oligoDNA 0.01+HLF 1.0; black-and-white square, poly[C] 0.01+HLF 0.2; --◇--, poly[C]·poly[G] 0.01+HLF 1.0. In C, —○—, heparin 0.05; —△—, heparin fragment 0.1; —□—, poly[C] 0.01; —◇—, oligoDNA 0.01; —ı—, tRNA 0.01; black-and-white diamond, λDNA 0.01; —×—, poly[C]·poly[G] 0.01; --○--, heparin 0.05+HLF 2.0; --△--, heparin fragment 0.1+HLF 2.0; --□--, poly[C] 0.01+HLF 0.2; --◇--, oligoDNA 0.01+HLF 1.0.

gle-stranded poly[C] had prominent effects on R-lysozyme, but the effects of double-stranded λDNA and poly[C]·poly[G] were small, though significant (data not shown). Urea-denatured α-lactalbumin and lysozyme without dithiothreitol treatment, as well as the native proteins, did not show any aggregation behaviors under the same conditions as above (native lysozyme at 0.4 and 1.5 mg/ml or greater formed precipitates with 0.1 mg/ml heparin and heparin fragment, respectively). All of the above aggregations, except for the self-aggregation of lysozyme and the effect of α-lactalbumin, could be prevented effectively by the presence of lactoferrin; usually, 1 mg/ml lactoferrin was required for the effect, but 0.2 mg/ml was effective against tRNA and poly[C] (Fig. 1A,B). Both human and bovine lactoferrin were effective. Ribonuclease A, bovine serum albumin, and lysozyme did not have such protective effects as lactoferrin.

3.2. Reaction of BLFcin with heparin and nucleic acids

Native BLFcin, as well as denatured and reduced BLFcin, was found to react with heparin and nucleic acids, leading to precipitation (Fig. 1C). As was the case with denatured lysozyme, the aggregation was significant at 10 μg/ml heparin (fragment) or nucleic acids (BLFcin 0.1 mg/ml). Heparin fragment was more effective than heparin. The effects of tRNA, poly[C], and oligoDNA were quite prominent, while those of λDNA and poly[C]·poly[G] were small. Lactoferrin was effective in preventing these aggregation reactions (Fig. 1C).

3.3. Complex formation between denatured protein and lactoferrin

The interactions of lactoferrin with native and denatured α-lactalbumin were studied by gel filtration (Fig. 2). As a model of the denatured protein, CM-α-lactalbumin was used because

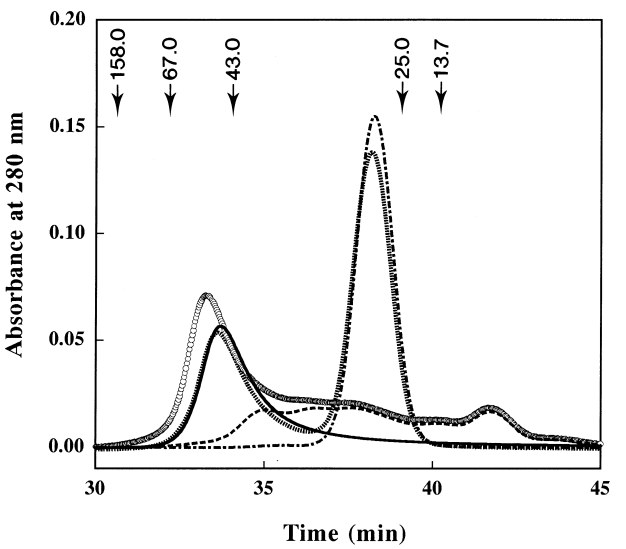

Fig. 2. Gel filtration of human lactoferrin (0.7 mg/ml) in the presence and absence of CM-α-lactalbumin (0.5 mg/ml) or native α-lactalbumin (0.5 mg/ml) in 20 mM Tris-HCl+0.15 M NaCl (pH 7.5). solid line, lactoferrin; dashed line, CM-α-lactalbumin; dot-dashed line, α-lactalbumin; circle line, lactoferrin+CM-α-lactalbumin; hatched line, lactoferrin+α-lactalbumin. The elution positions of the following standard proteins are indicated by arrows with molecular weights in kDa: aldolase (158.0 kDa), bovine serum albumin (67.0 kDa), ovalbumin (43.0 kDa), chymotrypsinogen A (25.0 kDa), and ribonuclease A (13.7 kDa).

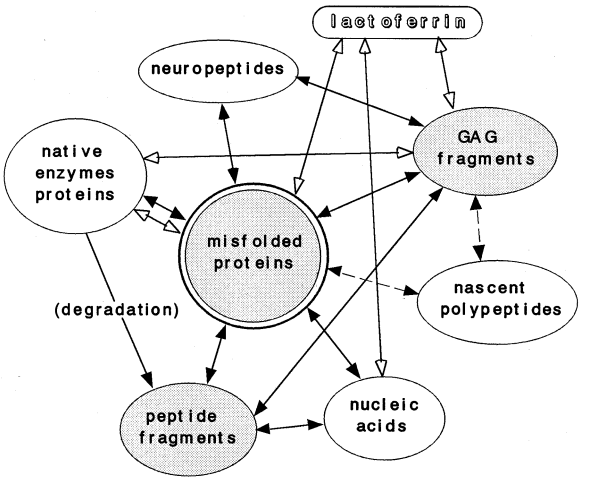

Fig. 3. Interactions involving misfolded proteins. The unfavorable (aggregate-forming; closed two-headed arrows) and favorable (protective; open two-headed arrows) interactions proposed on the basis of the present results are shown. The disease-causing unwanted species are tinted. Closed one-headed: degradation. The dashed line indicates that the interaction is merely suspected. The category 'neuropeptides' represents any biologically active peptides. The aggregation caused by a peptide fragment could be prevented by its parent protein, as in the case of lactoferricin and lactoferrin. This might also hold for amyloid β-peptide and amyloid precursor protein or its secreted form containing a C-terminal heparin-binding domain [20].

it did not require a reducing buffer condition to avoid possible precipitation arising from intermolecular disulfide bond formation. CM-α-lactalbumin did form a precipitate in the presence of BLFcin, though at much higher concentrations of BLFcin (≥ 0.2 mg/ml) than for urea-denatured reduced α-lactalbumin (data not shown). The precipitation could be effectively prevented by inclusion of 0.5–1.0 mg/ml human lactoferrin. Thus, it is assumed that lactoferrin interacts with CM-α-lactalbumin in a similar manner to that with the urea-denatured reduced protein.

The CM-α-lactalbumin sample used in the present study had a broad molecular weight distribution (Fig. 2) possibly due to oligomerization of protein species with different degrees of modification (the manufacturer indicates at least 6 mol of *S*-carboxymethylcysteine per mol protein). Nevertheless, a shift of the peak position of lactoferrin to a higher molecular weight is evident when lactoferrin was mixed with CM-α-lactalbumin, the increase being about 6000 in molecular weight. Analysis by superposition of the curves suggested that it was the higher molecular weight species of CM-α-lactalbumin that reacted with lactoferrin (data not shown). On the other hand, native α-lactalbumin shifted the peak position of lactoferrin only slightly, the increase in molecular weight being about 2000. Essentially the same results were obtained at different mixing ratios of lactoferrin and CM- or native α-lactalbumin (molar ratios of 1:4 (Fig. 2); 1:2; 1:1), which demonstrates the formation of a complex between lactoferrin and CM-α-lactalbumin, a denatured protein.

4. Discussion

In the present study the interactions of denatured proteins with various types of biomolecules were investigated under non-denaturing conditions. It was found that at a physiolog-

ical ionic strength denatured/misfolded proteins tend to form insoluble aggregates by combining with other cellular components: native peptides, proteins, glycosaminoglycans (GAGs) such as heparin, and nucleic acids. The aggregating interactions required reduction of disulfide bonds and oppositely charged molecules, suggesting that they involve electrostatic interactions between a protein in an extended conformation and a ligand. Thus, the denatured state of an acidic protein α-lactalbumin reacted with a basic peptide BLFcin but not with negatively charged heparin or nucleic acids; conversely, the denatured state of a basic protein lysozyme reacted with heparin and nucleic acids but not with BLFcin. On the other hand, the disulfide-bonded small peptide, BLFcin, formed precipitates with heparin and nucleic acids. Most of these aggregation reactions were found to be prevented by lactoferrin, which could form soluble complexes with denatured α-lactalbumin (Fig. 2), heparin [10], and nucleic acids [11]. Lactoferrin has an isoelectric point of ~8.7 [12] and the protective effect of lactoferrin may be ascribed to the localized charge distribution throughout the molecule [13]; thus, lactoferricin, which was derived from the N-terminal basic region that constitutes the GAG-binding site [10], causes the aggregation of denatured α-lactalbumin, whereas the parent lactoferrin molecule affords protection. These effects were found to be highly sensitive to the ionic strength: in the absence of NaCl, substantial effects were observed at 20 μg/ml BLFcin and at 20–100 μg/ml lactoferrin (data not shown), again suggesting the importance of electrostatic interactions.

The proposed interactions involving misfolded proteins based on the present results are summarized in Fig. 3. If, by any mechanism, a misfolded protein happens to be formed, it may be deleterious for the cell since it could inactivate certain biologically active peptides such as neuropeptides and enzymes or inhibit protein synthesis by reacting with nucleic acids or nascent polypeptides. Peptide fragments produced by protein degradation may also react with the misfolded protein. These reactions produce aggregates that could act as a seed for triggering extensive aggregation, which eventually leads to the deposition of insoluble aggregates. Then, one of the consequences to occur in the cell would be to produce free radicals by a defense mechanism, which, at the same time, may damage the normal structural components such as GAGs; actually, amyloid β-peptide is reported to damage cells by producing free radicals [14,15]. The GAG fragments thus released might in turn complex with the misfolded protein, peptides, or even nascent polypeptides. The rapid association of heparin fragment with misfolded lysozyme observed in this study is noteworthy in this respect. Two human lysozyme variants are known to be amyloidogenic [16] and the amyloid deposits also contain GAGs [17]. It should also be noted that GAGs were found to be associated with amyloid plaques and neurofibrillary tangles in Alzheimer's disease [18,19]. The consumption of proteins and peptides as above might stimulate further protein synthesis, resulting in overexpression and misfolding. Thus, the present study suggests a new mechanism by which some kinds of protein folding diseases progress; it proposes how protein aggregation is provoked and how it leads to neuronal disorders and cellular dysfunction. Lactoferrin protects some misfolded proteins from aggregating by this mechanism. The same mechanism may also be worth consideration in understanding the inclusion body formation.

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