Molecular Chaperone-like Properties of an Unfolded Protein, $\alpha_s$-Casein*

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All molecular chaperones known to date are well organized, folded protein molecules whose three-dimensional structure are believed to play a key role in the mechanism of substrate recognition and subsequent assistance to folding. A common feature of all protein and nonprotein molecular chaperones is the propensity to form aggregates very similar to the micellar aggregates. In this paper we show that $\alpha_s$-casein, abundant in mammalian milk, which has no well defined secondary and tertiary structure but exits in nature as a micellar aggregate, can prevent a variety of unrelated proteins/enzymes against thermal-, chemical-, or light-induced aggregation. It also prevents aggregation of its natural substrates, the whey proteins. $\alpha_s$-Casein interacts with partially unfolded proteins through its solvent-exposed hydrophobic surfaces. The absence of disulfide bridge or free thiol groups in its sequence plays important role in preventing thermal aggregation of whey proteins caused by thiol-disulfide interchange reactions. Our results indicate that $\alpha_s$-casein not only prevents the formation of huge insoluble aggregates but it can also inhibit accumulation of soluble aggregates of appreciable size. Unlike other molecular chaperones, this protein can solubilize hydrophobically aggregated proteins. This protein seems to have some characteristics of cold shock protein, and its chaperone-like activity increases with decrease of temperature.

From the time of synthesis through their entire lifetime in the cell, proteins are under constant threat to structural destabilization because of misfolding, stress, or other unfavorable interactions. Molecular chaperones recognize these unstable nonnative conformers of proteins and bind to them instantly, preventing their aggregation in the cell (1–8). Molecular chaperones comprise several structurally unrelated protein families and assist not only in folding of other proteins but also in subcellular transport, oligomeric assembly, and degradation of undesirable proteins (1–2, 4, 7–8). It is not understood at present whether the chaperone function of a protein is because of the presence of a particular sequence or three-dimensional structure, although the role of higher levels of organization of GroEL, TriC, $\alpha$-crystallin, etc. have been emphasized for their chaperone function (9–12). Although no common sequence has been identified among chaperones of different family of proteins, some common features emerges. Chaperones have distinct hydrophilic and hydrophobic domains to enhance solubility and to bind lipophilic molecules (13–15). Many of them have a characteristic micelle-like-associate structure. GroEL exists as an associated 14-mer (7, 9). TriC has a ring-like structure of 8 to 9 subunits (10). Tubulin, reported recently by us (16) to act like a chaperone, associates to form microtubules. $\alpha$-Crystallin, which belongs to small heat shock protein (sHSP) family (17), has been proposed to have a micellar architecture (11, 12). Even nonprotein biological molecules such as ribosomal RNA (18, 19) and phospholipid (20), which can form micelle-type aggregates, were shown to function as molecular chaperones.

It is long known that casein in bovine skim milk remains as large (40–300 nm) stable micelles (21). Bovine milk contains about 78% casein, of which 65% is $\alpha_s$-casein. More than 75% of $\alpha$-casein is Ca$^{2+}$-insensitive and is mainly $\kappa$-casein. $\alpha_s$-Casein has a molecular mass of 23.6 kDa (23). It is present in the milk of all mammals as a random coil protein (24, 25) and is the major protein constituent of casein micelle (21). In the absence of Ca$^{2+}$ ions, it is a highly soluble protein. Despite its unorganized secondary and tertiary structure, there are similarities between $\alpha_s$-casein and other known chaperones in their tendency to self-associate into micelle-like aggregate. This prompted us to test if $\alpha_s$-casein possessed any chaperone-like behavior. In this paper we report for the first time that a random coil protein $\alpha_s$-casein can prevent in vitro the thermal aggregation of whey proteins from milk as well as the aggregation of a variety of unrelated proteins/enzymes caused by thermal-, chemical-, and light-induced stress. We also show that unlike other chaperones, $\alpha_s$-casein can solubilize hydrophobically aggregated proteins and possesses some features similar to the cold shock proteins (CSP).1

**EXPERIMENTAL PROCEDURES**

Materials—$\alpha_s$-Casein, bovine serum albumin (BSA), rhodanese, insulin, carbonic anhydrase, alcohol dehydrogenase from equine liver, and citrate synthase were purchased from Sigma. Whey protein isolate (WPI) was obtained from Le Sueur Isolates (Le Sueur, MN). Dithiothreitol (DTT) was purchased from Sisco Research Laboratory, India. Reagents used for SDS-polyacrylamide gel electrophoresis were from Fisher. All reagents used for making buffer solutions were of analytical grade.

Preparation of $\beta$- and $\gamma$-Crystallin—Freshly excised bovine eyes were obtained from a local slaughterhouse. The lenses were surgically removed and homogenized in 10 mM Tris-HCl buffer, pH 8.0, containing 0.1 M NaCl and 0.02% w/v Na$_2$EDTA. The homogenate was centrifuged at 15,000 x $g$ for 20 min at 4 °C. The supernatant was then loaded to a Sephacryl S-300 column (1.5 cm x 90 cm). Five distinct peaks corresponding to high molecular weight $\alpha$-crystallin, low molecular weight $\alpha$-crystallin, high molecular weight $\beta$-crystallin, low molecular weight $\beta$-crystallin, and $\gamma$-crystallin were observed.

1 The abbreviations used are: CSP, cold shock proteins; BSA, bovine serum albumin; DTT, dithiothreitol; WPI, whey protein isolate.
Preparation of substrate proteins by molecular chaperones is commonly used method for in vitro assay of their activity. When solutions of substrate proteins such as alcohol dehydrogenase, carbonic anhydrase, and β-crystallin are heated at 40, 60, and 60 °C, respectively, the solutions get turbid because of the formation of large aggregates. Fig. 3 shows the kinetic traces of the apparent absorbance at 400 nm of these proteins in the presence and absence of αs-casein. In the absence of αs-casein, the substrates at the respective temperatures undergo denaturation followed by aggregation. However, in the presence of αs-casein, aggregation was suppressed. Approximately 92% protection was found at a 1:1 (w/w) ratio of alcohol dehydrogenase:αs-casein (Fig. 1A). Complete protection occurred at a 1:1.5 (w/w) ratio of alcohol dehydrogenase:αs-casein, corresponding to a molar ratio of 1:4. In the case of β-crystallin (Fig. 1B), complete suppression of aggregation required a β-crystallin:casein weight ratio 1:0.3 (1:0.6 molar ratio). At 1:3.5 weight ratio between carbonic anhydrase and αs-casein, 90% protection was obtained (Fig. 1C), and complete protection required approximately a 1:5 molar ratio (data not shown). αs-Casein was also effective in preventing thermal aggregation of a number of other proteins, including citrate synthase, γ-crystallin, and rhodanese (data not shown).

Milk contains a number of globular proteins such as β-lactoglobulin, α-lactalbumin, BSA, etc., collectively called whey proteins, which are highly sensitive to temperature, pH, and other conditions (22, 23, 29). WPI is a mixture of these proteins (about 20% of total milk proteins), which remain in the milk serum after removal of casein (29). When 0.5 mg/ml WPI solution in phosphate buffer, pH 6.6, is heated to 70 °C, the solution develops visible turbidity with time because of aggregation (Fig. 2A, trace 1). The presence of 0.4 mg/ml αs-casein completely prevents this aggregation (Fig. 2A, trace 2). Similarly, when 0.5 mg/ml BSA solution at pH 6.6 was heated at 70 °C, a low level of scattering was visible because of aggregated proteins (Fig. 2B, trace 1). The presence of 0.5 mg/ml completely prevents the formation of scattering BSA particles (Fig. 2B, trace 2). Our results show that αs-casein not only prevents the aggregation of unrelated proteins but also protects its natural substrates in vivo against thermal aggregation. Early work by Morr and coworkers (30, 31) also clearly demonstrated that whole casein prevented gross heat-induced aggregation of whey proteins through nonspecific interaction, even in calcium-containing systems.

Many investigators (32–38) have extensively studied the thermal aggregation properties of whey proteins. It is well known that the major whey proteins β-lactoglobulin, α-lact-
bumin, and BSA have disulfide bridges, and the former two have free thiol groups as well (21–23, 29). It was shown that thermal aggregation of whey protein was caused by a combination of hydrophobic as well as thiol-disulfide interchange reactions (34–38). αs-Casein, being a highly hydrophobic protein, interacts instantly with the exposed hydrophobic groups of denaturing proteins, preventing aggregation. However we feel that the lack of disulfide bridges and free thiol groups in \( \alpha_s \)-casein sequence (39) is another very important and unique feature that plays a significant role in inhibiting thiol-disulfide interchange reactions. These covalent reactions require close contact of appropriate residues, and \( \alpha_s \)-casein creates a nonre-active barrier by placing itself between the whey proteins.

Whey proteins under various conditions are also known to form soluble aggregates, which apparently cannot be detected by the light-scattering technique we have employed as the aggregation assay method. To check if \( \alpha_s \)-casein can prevent formation of soluble aggregates of appreciable size, we have employed a gel filtration assay using fast protein liquid chromatography. Although \( \alpha_l \)-lactalbumin does not aggregate on its own on heating, it is known to form soluble aggregates in the presence of \( \beta \)-lactoglobulin in the early stages of heat treatment (36, 37). A mixture of \( \alpha_l \)-lactalbumin (2 mg/ml) and \( \beta \)-lactoglobulin (2 mg/ml) at pH 7.0 was heated to 70 °C for 5 min and rapidly cooled to room temperature. The sample on gel filtration showed the presence of aggregated species of molecular mass in excess of 300 kDa eluting at the void volume, unreacted proteins corresponding to dimeric \( \beta \)-lactoglobulin (36.5 kDa), monomeric \( \alpha_l \)-lactalbumin (14.4 kDa), and some intermediate aggregates centered around molecular masses of approximately 100–120 kDa (Fig. 3, trace 1). In the presence of \( \alpha_s \)-casein (4 mg/ml), a considerable reduction in the high molecular mass species was observed, and most of the proteins was eluted in a relatively single peak centered around 60 kDa. In presence of 6 mg/ml casein, no trace of any species of more than ~80 kDa was observed. This clearly shows that in presence of sufficient quantity of \( \alpha_s \)-casein formation of soluble aggregates of any appreciable size is prevented.

For nonthermal aggregation of substrate proteins, insulin became a popular choice for as the assay system, because reduction of disulfide bond by DTT leads to aggregation of its B-chain at room temperature (16, 26, 40). Like other chaperones, \( \alpha_s \)-casein also can prevent disulfide cleavage-induced aggregation of insulin at 27 °C, requiring a 1:0.35 weight ratio between insulin and \( \alpha_s \)-casein for complete prevention of aggregation (Fig. 4, inset). Using this assay system, we investigated the effect of temperature on its chaperoning efficiency. Fig. 4 shows the bar diagram of the percentage protection of insulin aggregation with \( \alpha_s \)-casein (1:0.035 w/w ratio) at 37, 27, 22, and 18 °C. At 37, 27, and 22 °C, the suppression of aggregation is 39, 52, and 90%, respectively, whereas there was complete protection against insulin aggregation at 18 °C at the same ratio. This finding was in sharp contrast to the behavior of other known chaperones such as \( \alpha \)-crystallin, tubulin, etc., whose activity were generally found to increase with the increase of temperature (16, 26, 27). Also unlike other chaperones (16, 26, 27), preheating of \( \alpha_s \)-casein solution to 50 °C for 30 min and cooling back to 27 °C did not alter its chaperone efficiency.

\( \alpha_s \)-Casein can also prevent nonthermal aggregation of proteins as well such as those induced by UV light. The eye lens protein \( \gamma \)-crystallin in solution on being exposed to UV light (295 nm) becomes turbid because of aggregation (27, 28). Like the chaperone-like \( \alpha \)-crystallin, \( \alpha_s \)-casein also can prevent this aggregation (Fig. 5). Complete prevention requires a 1:2 weight ratio between \( \alpha \)-crystallin and \( \alpha_s \)-casein, corresponding to a molar ratio of ~1:0.7.

It is known that molecular chaperones bind only aggregation-prone conformers of the substrate protein but do not interact with native proteins or proteins that have already aggregated (41). To test if \( \alpha_s \)-casein acted similarly, we started an insulin aggregation reaction by adding DTT to it, and when nearly 50% aggregation occurred, we added \( \alpha_s \)-casein to the reaction mixture. Our results show that \( \alpha_s \)-casein not only prevented further aggregation of insulin, but unlike other
known chaperones, it also slowly solubilized the already-aggregated insulin (Fig. 6). It has also been observed that GroEL can prevent aggregation of substrate proteins both on the unfolding and refolding pathway (42, 43) (e.g. on dilution from 6 M guanidine hydrochloride solution). However, unlike GroEL but like α-crystallin (42), α-casein can effectively prevent aggregation in the unfolding pathway but fails to prevent aggregation completely on the refolding pathway (data not shown).

We have thus identified α-casein, which exits in nature as an unfolded random coil protein, as having chaperone-like functions. Like all other known chaperones, it can prevent irreversible aggregation of proteins induced by thermal as well as nonthermal stress by providing hydrophobic surfaces to unfolding proteins. The important features responsible for its chaperone-like activity are (i) its high hydrophobicity characterized by Bigelow’s parameter of 1170 (44) along with high estimated net negative charge of about 22 at pH 6.5 (39), (ii) its highly flexible nature because of the relatively high amount (8.5%) of proline residues distributed uniformly throughout the chain (29, 39), (iii) its lack of a cystine residue in the sequence (39). Interestingly α-casein possesses a few features similar to that of the CSP. Generally CSPs in bacteria have higher levels of expression following a cold shock (45) and play a major role in the activation (46) and physiology of adaptation to low temperature (45). Similarly greater amounts of α-casein are found in mammals of the low temperature zones such as reindeer (47), moose, yak, etc. compared with mammals living in other temperature zones (21). CSPs are known to be devoid of disulfide bonds in their sequence (48) and so is α-casein (39). These similarities between α-casein and CSPs of prokaryotes might suggest that α-casein act as a CSP in eukaryotes.

It is now recognized that molecular chaperones, all of which are known to date, are folded protein molecules and provide guidance to newly synthesized peptides through the folding process, and their presence is essential for cell survival (1–3, 5–8). How the chaperones themselves get folded, however, remains a mystery. It is too early to say if α-casein in vivo may help fold other proteins. It has very recently been shown that cyclophilin was able to refold properly from the unfolded state in the presence of α-casein (43). The discovery of RNA chaperones is considered to be of vital importance because their early appearance in the evolution is believed to have made the transition from RNA to a protein/RNA world by rescuing the RNA from possible kinetic trap (49). It has recently been hypothesized that proteins in the early stages of evolution of life were unfolded proteins, which through long evolutionary process ultimately became folded (50). Our observation for the first time that a commonly occurring unfolded protein α-casein can function as a molecular chaperone may be significant in understanding this aspect.

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