Amyloid Fibril Formation by Bovine Milk κ-Casein and Its Inhibition by the Molecular Chaperones α_S- and β-Casein†

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ABSTRACT: Caseins are a unique and diverse group of proteins present in bovine milk. While their function is presumed to be primarily nutritional, caseins have a remarkable ability to stabilize proteins, i.e., to inhibit protein aggregation and precipitation, that is comparable to molecular chaperones of the small heat-shock protein (sHsp) family. Additionally, sHsps have been shown to inhibit the formation of amyloid fibrils. This study investigated (i) the fibril-forming propensities of casein proteins and their mixture, sodium caseinate, and (ii) the ability of caseins to prevent *in vitro* fibril formation by κ-casein. Transmission electron microscopy (TEM) and X-ray fiber diffraction data demonstrated that κ-casein readily forms amyloid fibrils at 37 °C particularly following reduction of its disulfide bonds. The time-dependent increase in thioflavin T fluorescence observed for reduced and nonreduced κ-casein at 37 °C was suppressed by stoichiometric amounts of α_S- and β-casein and by the hydrophobic dye 8-anilino-1-naphthalene sulfonate; the inhibition of κ-casein fibril formation under these conditions was verified by TEM. Our findings suggest that α_S- and β-casein are potent inhibitors of κ-casein fibril formation and may prevent large-scale fibril formation *in vivo*. Casein proteins may therefore play a preventative role in the development of corpora amylacea, a disorder associated with the accumulation of amyloid deposits in mammary tissue.

The opacity of milk is accredited to the light-scattering properties of large colloidal aggregates known as casein micelles. The micelles are composed primarily of casein, a heterogeneous phosphoprotein comprising four distinct gene products, α_{S1}-, α_{S2}-, β-, and κ-casein. The caseins have been classified as intrinsically disordered proteins (*1*, *2*), because they are extremely flexible, essentially unfolded, and have relatively little secondary or tertiary structure under physiological conditions (*3*). They have an amphiphilic character arising from a separation between distinct hydrophobic and negatively charged regions along the polypeptide chain (*4*). Although the caseins themselves are relatively small proteins, ranging in molecular mass from about 19 to 25 kDa, they are not usually found as individual molecular species. They each exhibit a strong tendency to associate with themselves and with each other through hydrophobic and electrostatic interactions (*5*). It is this tendency that, in the presence of calcium and other ions present in milk, leads ultimately to the formation of casein micelles having molecular masses from 10^3 to 3×10^6 kDa (*6*).

Caseins have been shown to act as molecular chaperones in a manner very similar to intracellular small heat-shock proteins (sHsps)[1] and the extracellular protein, clusterin (*7*), whereby they protect a variety of proteins, including whey proteins, against thermal-, chemical-, and UV-light-induced aggregation (*8*−*10*). sHsps play a particularly important role in stabilizing proteins under conditions of cellular stress (e.g., oxidative stress, heat shock, pH extremes), where proteins partially unfold, expose hydrophobic surfaces, and potentially aggregate (*11*). sHsp expression is often elevated in disease states that are characterized by the accumulation of misfolded and aggregated proteins (*12*, *13*). In some protein-misfolding diseases, referred to as amyloidoses, the aggregated material comprises a highly structured array of β-sheets, arranged into rope-like assemblies known as amyloid fibrils (*14*). Consistent with their ability to prevent protein aggregation and

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[1] Abbreviations: sHsp, small heat-shock protein; CA, corpora amylacea; BSA, bovine serum albumin; ThT, thioflavin T; ANS, 8-anilino-1-naphthalene sulphonate; DTT, 1,4-dithiothreitol; TEM, transmission electron microscopy; AU, arbitrary units.

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precipitation, *in vitro* studies demonstrate that sHsps suppress amyloid fibril formation by β-amyloid peptide (*15*, *16*), apolipoprotein C-II (apoC-II) (*17*), and α-synuclein (*18*). Likewise, clusterin can inhibit apoC-II fibril formation (*19*). One of the primary objectives of the study described in this paper was to establish whether caseins, like sHsps and other chaperones, are able to inhibit amyloid fibril formation.

Amyloid-like plaques have been identified in bovine, rat, and canine mammary glands within calcified stones known as corpora amylacea (CA) (*20*−*22*). It has been postulated that CA can cause complications during late lactation by accumulating in luminal spaces and blocking small ducts, leading to a reduction in milk secretion and flow (*23*). The identity of the protein(s) involved in plaque formation has not been determined conclusively, but immunoblotting and sequence analysis of peptides obtained from mammary CA has suggested that several milk proteins, including the caseins, are present (*24*).

Previous *in vitro* studies have shown that reduced and carboxymethylated κ-casein forms long rodlike structures that display many of the characteristics of amyloid fibrils (*25*). We demonstrate here by X-ray fiber diffraction that reduced κ-casein forms fibrils having a cross-β core, a structural hallmark of amyloid fibrils (*26*). Furthermore, native (non-reduced) κ-casein also has a propensity to form fibrils, although at a significantly reduced level compared to the reduced protein. With the knowledge that caseins can act as molecular chaperones (*8*−*10*), we investigated the ability of individual caseins to prevent fibril formation by κ-casein. Having compared the extent of κ-casein fibril formation in the presence and absence of naturally occurring levels of α_S- and β-casein, it is concluded that the fibril-forming propensity of κ-casein is countered by the other components of whole casein. These findings highlight the possibility of κ-casein fibril formation *in vivo* and in the development of mammary CA and the likely role of the other casein proteins in preventing such fibril formation.

MATERIALS AND METHODS

Materials. Sodium caseinate, α_S-, β-, and κ-casein from bovine milk, and bovine serum albumin (BSA) were purchased from Sigma Chemical Co. (St. Louis, MO) and used without further purification. Thioflavin T (ThT), 8-anilino-1-naphthalene sulfonate (ANS), 1,4-dithiothreitol (DTT), and β-mercaptoethanol were also obtained from Sigma. Snakeskin dialysis tubing with a 3.5-kDa mass cutoff was obtained from Progen Biosciences (Australia). ThinSeal, an inert, translucent, adhesive tape used to seal microwell plates was purchased from Excel Scientific (Chicago, IL). All other reagents were of analytical grade, and all solutions were prepared with ultrapure MilliQ water. Proteins were incubated in a 50 mM phosphate buffer (pH 7.0, unless stated otherwise) containing 2 mM EDTA and 0.02% (w/v) sodium azide. Prior to use, proteins solutions were passed through 0.20 μm Supor syringe filters (Pall Corporation, New York) to remove pre-existing aggregates, and protein concentrations were determined spectrophotometrically using a Cary 300 UV/vis spectrometer (Varian) and extinction coefficients (A_{280}) of 1.09, 0.44, 1.05, and 0.66 mL mg^{-1} cm^{-1} for α_S-, β-, and κ-casein and BSA, respectively (*4*).

ThT Binding. Amyloid fibril formation was monitored using a fluorometric assay that relies on the enhanced fluorescence properties of ThT when bound to amyloid fibrils. ThT binds selectively to antiparallel β-pleated sheets, the major structural element of amyloid fibrils, and fibril formation is accompanied by an increase in the ThT fluorescence emission at approximately 490 nm (*27*). To follow the time-dependent formation of amyloid fibrils, two different methods were employed as outlined below.

Conventional ThT Assay. To generate amyloid fibrils, protein solutions were incubated without shaking at room temperature, 37 or 60 °C. Fibril formation is highly temperature-dependent and can usually be quenched by freezing the solutions in which it is present (*28*). Exploiting this fact, 10 μL aliquots were withdrawn from protein solutions at selected times and frozen at −20 °C. Immediately prior to analysis, the thawed samples were mixed with 1 mL of 5 μM ThT in 50 mM glycine-NaOH buffer (pH 9.0). The fluorescence emission intensity at 490 nm was then measured using a quartz cuvette (10 mm light path) and a Hitachi F-4500 spectrofluorimeter. The excitation wavelength was set at 442 nm, and bandwidths for excitation and emission were 5.0 and 10.0 nm, respectively. To minimize instrumental error, the sample was analyzed for 30 s, and the emission intensity was recorded each second. The averaged measurements were then plotted as a function of the incubation time, and where appropriate, data were fitted with linear, sigmoidal, or exponential-rise-to-maximum curves using SigmaPlot 8.0 software.

In Situ ThT Assay. For rapid time courses of fibril formation, ThT binding assays were performed *in situ* by a method adapted from Nielsen et al. (*29*). κ-Casein, at the approximate concentration that it is present in bovine milk [160 μM; 3.0 mg/mL (*30*)], was mixed with either α_S- or β-casein in the molar ratios of 2:1, 1:1, 1:2, and 1:3 [the κ/α_{S1} and κ/β molar ratios in milk are both in the order of 2:5 (*31*)]. Samples were prepared in duplicate and incubated with 10 μM ThT in Greiner black μClear 96-microwell plates (Interpath Services, Australia) using a sample volume of 200 μL in each well. As observed with insulin fibril formation (*29*), conventional ThT assays indicated that the presence of ThT in the protein solution, at the concentration used, did not affect the kinetics of κ-casein fibril formation, either in the presence or absence of other caseins. Plates were sealed with ThinSeal to prevent evaporation and incubated at 37 °C. The fluorescence intensity was measured at 10 min intervals for 40 h using a Fluostar Optima plate reader (BMG Labtechnologies, Australia) with a 440/490 nm excitation/emission filter set. During the early phase of incubation (i.e., the initial ∼30 min), fluorescence readings were affected by the change in temperature as solutions were heated from room temperature to 37 °C, resulting in an initial decrease in ThT fluorescence. This optical artifact was removed by extrapolating the initial ascending gradient of the aggregation curve back to the zero time point.

Transmission Electron Microscopy (TEM). Samples for TEM were prepared by adding 2 μL of protein solution, diluted to a concentration of ∼1 mg/mL, to Formvar and carbon-coated nickel grids (SPI Supplies, West Chester, PA). The grids were then washed 3 times with 10 μL of water and negatively stained with 10 μL of uranyl acetate [2% (w/v); Agar Scientific, U.K.]. The grids were dried with filter paper between each step. The samples were viewed under 25 000−64 000 magnifications at 80 kV excitation voltages

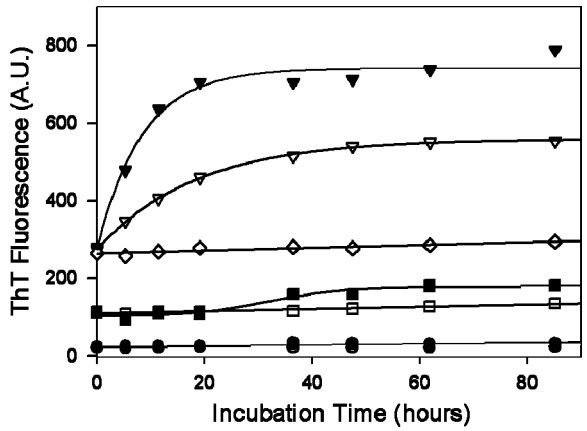


FIGURE 1: ThT fluorescence analysis of fibril formation by reduced and nonreduced casein proteins. In the presence of 20 mM DTT, α_S- (■), β- (●), and κ- (▼) casein (8 mg/mL) in 50 mM phosphate buffer (pH 7.0) were incubated at 37 °C. Under the same conditions and concentration, α_S- (□), β- (○), and κ- (▽) casein were incubated in the absence of DTT. Additionally, nonreduced κ-casein (◇) was incubated at room temperature. Data shown are single measurements and are representative of two experiments.

using a Philips CM100 transmission electron microscope (Philips, The Netherlands). Particle size measurements were performed using SIS Image Analysis software.

X-ray Fiber Diffraction. Samples for X-ray fiber diffraction were prepared by dialyzing the fibril solutions against water for 1 week at 4 °C to remove solvent ions and DTT. Droplets of the fibril-containing solution were suspended between the ends of two wax-filled capillaries and allowed to dry in air at room temperature. The capillaries were separated slowly while drying to enhance the alignment of amyloid fibrils (*32*), and a small stalk of fibrils protruding from the end of one of the capillaries was obtained. The sample was aligned in an X-ray beam, and diffraction patterns were collected using a Cu Kα Rigaku rotating anode source (wavelength of 1.5418 Å) and MARresearch image plate detector. Images were examined using Mosflm (A. Leslie, Laboratory of Molecular Biology, Cambridge, U.K.) enabling the intensities of the various reflections to be measured.

RESULTS

Investigating the Amyloidogenicity of Reduced Casein Proteins. In its native multimeric state, κ-casein contains extensive intermolecular disulfide bridging (*33*) and is therefore potentially susceptible to destabilization of its quaternary structure by reducing agents. In this study, κ-casein fibril formation was induced at neutral pH via reduction with either DTT or β-mercaptoethanol. The ability of κ-casein fibrils to bind ThT was used to monitor the time-dependent formation of fibrils. Figure 1 shows the time course of ThT fluorescence for nonreduced κ-casein incubated at room temperature and 37 °C and reduced κ-casein at 37 °C. Prior to incubation, both reduced and nonreduced κ-casein display a substantial capacity to bind ThT, as previously reported (*25*). When incubated in the absence of a reducing agent, at room temperature, the fluorescence emission intensity for κ-casein at 490 nm was unchanged with time, suggesting that κ-casein does not form amyloid fibrils under these conditions. When the temperature was raised to 37 °C, however, a time-dependent increase in fluorescence was observed (Figure 1). Higher temperatures of 60 °C promoted further increases in fluorescence (data not shown). At temperatures of 37 °C and above, fluorescence increases for κ-casein were noteworthy both in the presence and absence of DTT, although they were not as rapid or as large in magnitude when reducing agent was omitted (Figure 1). The kinetics of κ-casein fibril formation were rapid, particularly for the reduced protein, with ThT fluorescence rising sharply during the early periods of the incubation procedure and exhibiting no discernible lag phase. The propensity of α_S- and β-casein to form amyloid fibrils under identical destabilizing conditions was also investigated. The α_S-casein fraction comprises two distinct proteins, α_{S1}- and α_{S2}-casein, in the relative proportions of approximately 4:1, respectively (*31*). Only the minor component, α_{S2}-casein, possesses cysteine residues, two per molecule, which primarily participate in intermolecular disulfide bonding to form a homodimer under nonreducing conditions (*34*). α_S-Casein showed a minor sigmoidal increase in ThT fluorescence but only in the presence of a reducing agent (Figure 1). β-Casein, a protein devoid of disulfide linkages, showed no indication of fibril formation at 37 °C either in the presence or absence of a reducing agent (Figure 1).

Analysis by TEM confirmed the formation of fibrils by κ-casein. At room temperature, in the absence of reducing agents, κ-casein aggregates were essentially spherical with an average diameter of 20 nm (Figure 2A), comparable to that found in previous TEM studies (*35*). However, when incubated at 37 °C in the presence of DTT for 12 h, κ-casein formed threadlike structures, approximately 12−16 nm in diameter and 50−300 nm in length (Figure 2B). When DTT was substituted for another reducing agent, β-mercaptoethanol, fibrils of similar appearance were formed (not shown). Nonreduced κ-casein at 37 °C also formed fibrillar aggregates, suggesting that reduction is not essential to initiate the formation of κ-casein fibrils. These fibrillar species were similar in width to those formed by the reduced protein but were noticeably shorter (less than 200 nm; Figure 2C), consistent with the observed differences in ThT binding between reduced and nonreduced κ-casein (Figure 1). Although a small rise in ThT binding was observed, TEM indicated that reduced α_S-casein did not form amyloid fibrils. After 40 h at 37 °C, spherical particles rather than the fibrillar species typical of amyloid structures were observed (Figure 2D).

Analysis of κ-Casein Fibrils by X-ray Fiber Diffraction. X-ray fiber diffraction was employed to examine the substructure of fibrils formed by κ-casein incubated at 37 °C in the presence of DTT. Figure 3 shows the X-ray fiber diffraction pattern obtained for κ-casein fibrils and the values of the positions (in angstroms) of the reflection maxima. The meridional reflection at 4.7 Å corresponds to the spacing between adjacent hydrogen-bonded β-strands that lie perpendicular to the fibril axis. The broad equatorial reflection centered at 9.1−10.8 Å arises from the separation between the β-sheets that run parallel to the fibril axis and are stacked face-to-face to form the core structure of protofilaments. Reflections at approximately 4.7 Å on the meridian and approximately 10 Å on the equator are seen in all amyloid fiber diffraction patterns and are characteristic of a cross-β structure (*26*, *36*).

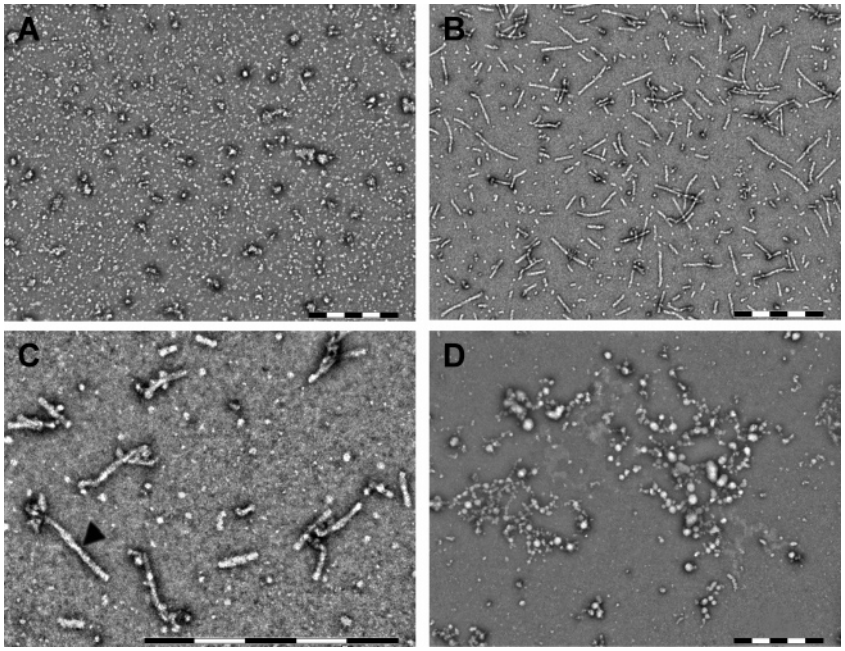


FIGURE 2: Electron micrographs of α_S- and κ-casein. (A) κ-Casein immediately after dissolution in 50 mM phosphate buffer (pH 7.0) without DTT. The following were incubated at 37 °C: (B) reduced κ-casein (2 mg/mL) at pH 7.2 with 20 mM DTT for 12 h, (C) nonreduced κ-casein (4 mg/mL) for 180 h, and (D) reduced α_S-casein (10 mg/mL) with 20 mM DTT for 40 h. Prior to analysis by TEM, all samples were diluted to a protein concentration of ~1 mg/mL, as stated in the Materials and Methods. In C, a short fibrillar aggregate of 180 nm in length is indicated by a tilted ▲. Scale bars represent 500 nm.

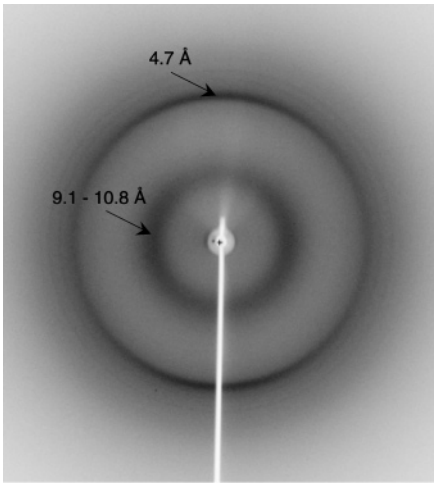


FIGURE 3: X-ray fiber diffraction pattern of κ-casein fibrils. κ-Casein (8 mg/mL) in 50 mM phosphate buffer (pH 7.0) with 20 mM DTT was incubated at 37 °C for 210 h. After dialysis against water for 7 days, fibril samples were prepared for X-ray fiber diffraction analysis as described in the Materials and Methods. The meridional and equatorial reflections are indicated at 4.7 and 9.1−10.8 Å, respectively.

Investigating the Amyloidogenicity of Reduced Sodium Caseinate. The fibril-forming propensity of κ-casein was compared to that of a preparation of sodium caseinate, the highly soluble sodium salt of whole casein [κ-casein accounts for ~15% (w/w) of whole casein (*6*)]. When incubated at 37 °C, a time-dependent rise in ThT fluorescence was observed for sodium caseinate but only under reducing conditions (Figure 4A); even after 215 h, the rate and magnitude of this fluorescence increase [~70 arbitrary units (AU)] was less than that observed for a solution having an equivalent concentration of κ-casein (~110 AU; Figure 4A). Despite the observed rise in ThT binding, electron microscopy of reduced sodium caseinate revealed that the particles resulting from incubation were essentially spherical, with average diameters of ~30 nm (Figure 4B; tilted ▲). For comparison, sodium caseinate in the absence of reducing agents also formed spherical particles (15−30 nm in diameter) and some more aggregated species (Figure 4C).

Effect of α_S- and β-Casein and BSA on κ-Casein Fibril Formation. Despite containing substantial amounts of κ-casein, sodium caseinate showed little indication of fibril formation under reducing conditions, implying that the ability of κ-casein to form amyloid fibrils is diminished by the presence of naturally occurring levels of other casein proteins. To investigate further the effects of α_S- and β-casein on κ-casein fibril formation, mixtures of κ-casein and α_S- or β-casein were incubated at 37 °C, with and without 20 mM DTT and fibril formation was monitored continually using an *in situ* ThT binding assay. For reduced κ-casein alone, incubation at 37 °C produced a steep rise in ThT fluorescence followed by a plateau and gradual recession (Figure 5; ●), as anticipated from previous experiments. In the presence of substoichiometric (~0.5 molar equivalent) α_S-casein (Figure 5A; ▼), the ThT fluorescence intensity still increased over time but to a significantly lesser extent, such that, by the end of the time course studied here, the fluorescence increase was 65% less than that of a sample of reduced κ-casein in the absence of α_S-casein. The presence of increasing amounts of α_S-casein progressively reduced the accumulation of material that binds ThT, with complete suppression of fibril formation resulting from a ~3.0-fold molar excess of α_S-casein (Figure 5A; ○). β-Casein was marginally more effective than α_S-casein at inhibiting fibril formation by reduced κ-casein, with near complete suppression achieved with a ~2.0-fold molar excess of β-casein (Figure 5B; ▲). Control assays of α_S- and β-casein incubated in the absence of κ-casein show no significant changes in ThT fluorescence over the time course of the experiment, as discussed

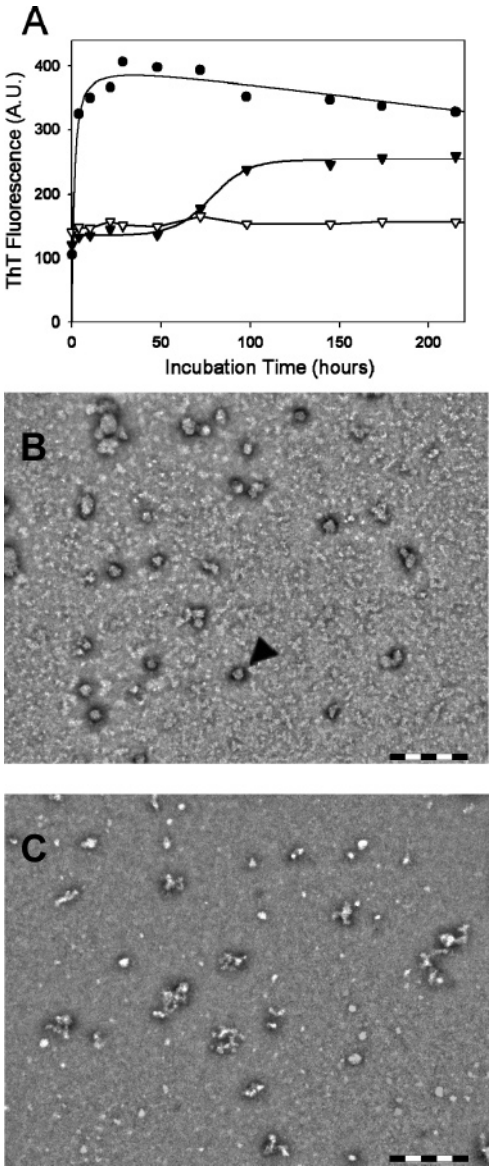


FIGURE 4: ThT fluorescence analysis and electron micrographs of reduced and nonreduced sodium caseinate. (A) Time course of ThT fluorescence for sodium caseinate (27 mg/mL; comprising ~4 mg/mL κ-casein) in 50 mM phosphate buffer (pH 7.0) with (▼) and without (▽) 20 mM DTT and κ-casein (4 mg/mL) with 20 mM DTT (●) incubated at 37 °C. Data shown are single measurements and are representative of two experiments. Electron micrographs show sodium caseinate (27 mg/mL) after 310 h of incubation at 37 °C in the (B) presence and (C) absence of 20 mM DTT. In B, a spherical aggregate of 30 nm in diameter is indicated by a tilted ▲. Scale bars represent 200 nm.

previously and shown in Figure 1. These results were confirmed by conventional ThT binding assays (data not shown), which also showed that α_S- and β-caseins inhibit fibril formation by reduced κ-casein. The α_S- and β-casein also act on native κ-casein to inhibit its fibril formation under nonreducing conditions (Figure 5). To determine the specificity of the inhibition of fibril formation, the ability of α_S- and β-casein to prevent κ-casein fibril formation was compared to that of an equivalent mass of BSA, an unrelated, ubiquitous protein that is also present in milk (*6*). At all concentrations tested, BSA exerted only a minor inhibitory effect on κ-casein fibril formation, under both reducing and nonreducing conditions (Figure 5C).

Fibril formation by reduced κ-casein and its inhibition by α_S- and β-casein was verified by TEM. Electron micrographs of κ-casein incubated in the presence of either α_S- or β-casein at a 1:2 molar ratio (parts B and C of Figure 6) revealed a reduction in the apparent number and average length of fibrils compared to those formed when κ-casein was incubated in the absence of α_S- and β-casein under identical conditions and during the same time period (Figure 6A). While short fibrils were observed infrequently, larger aggregates observed in the presence of α_S- and β-casein revealed little or no evidence of ordered structure and appeared to be assembled from nonfibrillar components. In the case of α_S-casein, these amorphous aggregates appeared to be very large, occasionally with dimensions of 1 μm or more (Figure 6B; tilted ▲). In contrast, well-defined fibrillar structures were abundant on electron micrographs of samples containing κ-casein and BSA (Figure 6D).

Inhibition of κ-Casein Fibril Formation by ANS. ANS is an extrinsic fluorophore that binds noncovalently to clusters of hydrophobic aminoacyl residues in such a way that its fluorescence is strongly enhanced (*37*). ANS was tested for its ability to inhibit κ-casein fibril formation, prompted by the findings of Nielsen et al. (*29*), who observed that ANS affected the kinetics of insulin fibril formation. Accordingly, reduced κ-casein was incubated at 37 °C with increasing concentrations of ANS (0−8 mM). In the presence of 4 mM ANS, the time-dependent increases in ThT fluorescence otherwise observed for reduced κ-casein were completely suppressed (Figure 7A). Only at the lowest concentration tested (1 mM ANS) was a significant rise in ThT fluorescence observed. This increase, however, was short-lived because ThT fluorescence returned to preincubation levels (Figure 7A). TEM demonstrated that ANS, at a concentration of 8 mM, strongly inhibits the formation of fibrillar aggregates (Figure 7B). Interestingly, under these conditions, κ-casein formed instead large amorphous aggregates (Figure 7B) that are morphologically similar to those formed in the presence of α_S- and β-casein (Figure 6B; tilted ▲).

DISCUSSION

The natural function of caseins in milk is presumed to be primarily nutritional (*4*). Once phosphorylated, caseins bind relatively large amounts of insoluble calcium, supplying young mammals with a highly concentrated yet stable form of calcium phosphate in addition to essential amino acids (*4*). We have recently, however, provided further insights into the functional properties of caseins, by showing that bovine α_S-, β-, and κ-casein have molecular chaperone activities that are comparable to those of sHsps and clusterin (*9*). In the present study, we have extended this comparison by showing that α_S- and β-casein inhibit the formation of amyloid fibrils by κ-casein, their natural substrate.

In aqueous solution, κ-casein forms large spherical polymers of ~18 nm in diameter (*35*), with an average molecular mass of 1.18 MDa (38), which are assembled from smaller multimeric subunits (monomers to decamers) that result from intermolecular disulfide bonding (*33*). However, when disulfide bond formation is prevented by reduction and/or alkylation of sulfhydryl groups, such an arrangement is no longer favorable and κ-casein converts from predominantly spherical to fibrillar aggregates (*25*). Here, we show that these

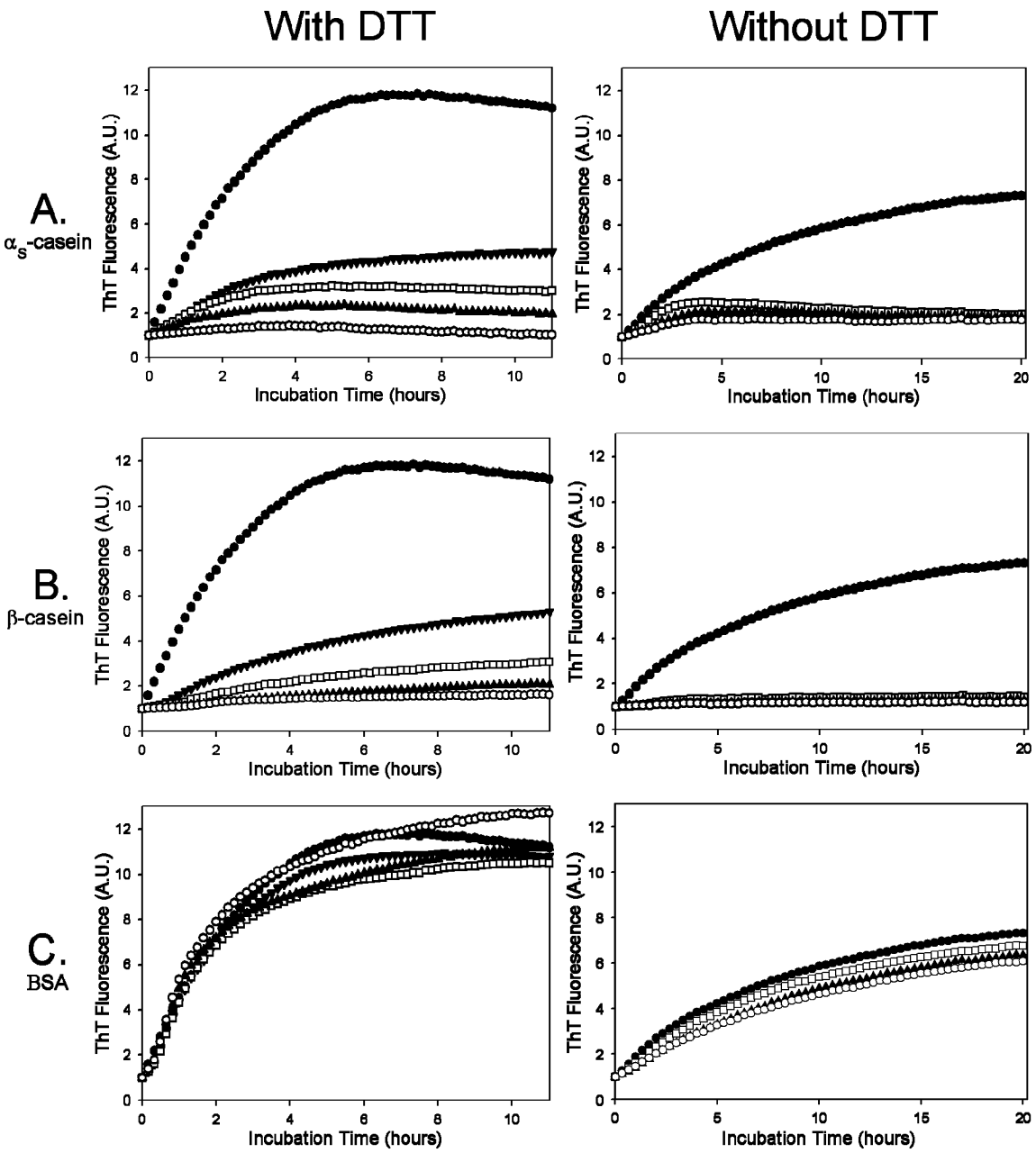


FIGURE 5: *In situ* ThT fluorescence analyses comparing the effect of casein proteins and BSA on κ-casein fibril formation under reducing and nonreducing conditions. κ-Casein (3 mg/mL) in 50 mM phosphate buffer (pH 7.0) with 10 μM ThT was incubated with and without 20 mM DTT at 37 °C in the absence (●) or presence of 1.88 mg/mL (▼), 3.75 mg/mL (□), 7.5 mg/mL (▲), and 11.25 mg/mL (○) of either (A) α_S-casein, (B) β-casein, or (C) BSA. Data shown are average values determined for duplicate samples and are representative of three independent experiments. To aid comparison between increases in ThT fluorescence, each curve was normalized by designating the initial fluorescence intensity as 1.0 AU.

fibrillar aggregates possess at least a core region with a cross-β structure, a defining feature of amyloid fibrils (*26*, *36*, *39*, *40*). Fibrillar aggregates are also formed from κ-casein in the absence of reducing agents at physiological temperature, and incubation of κ-casein at temperatures of 37 °C or higher induces a slow conformational rearrangement characterized by an increase in ThT binding (Figure 1). It is commonly accepted that native κ-casein polymerizes at 37 °C (*25*, *30*, *38*, *41*). For example, analytical ultracentrifugation shows that increasing the incubation temperature from 25 to 37 °C results in a 20% increase in the average molecular weight after 18 h (*38*). In the present work, TEM examination of nonreduced κ-casein incubated at 37 °C for 180 h clearly shows the presence of fibrils of up to 200 nm in length (Figure 2C). Without examination by X-ray fiber diffraction, it is not possible to conclude definitively whether these particular species represent amyloid fibrils, although our finding that native κ-casein undergoes a time-dependent transition into a conformation rich in β-sheet suggests that this is the case.

It is reasonable to speculate that κ-casein reverts to a monomeric state before fibril formation ensues, as observed for a number of other proteins that exist as oligomers in their native state, e.g., transthyretin (*42*), insulin (*29*), and immunoglobulin light chain (*43*). Indeed, many, if not all, amyloidogenic proteins appear to adopt a partially unfolded, monomeric intermediate prior to self-assembly into fibrils (*44*). However, if intermolecular disulfide bridges hold native

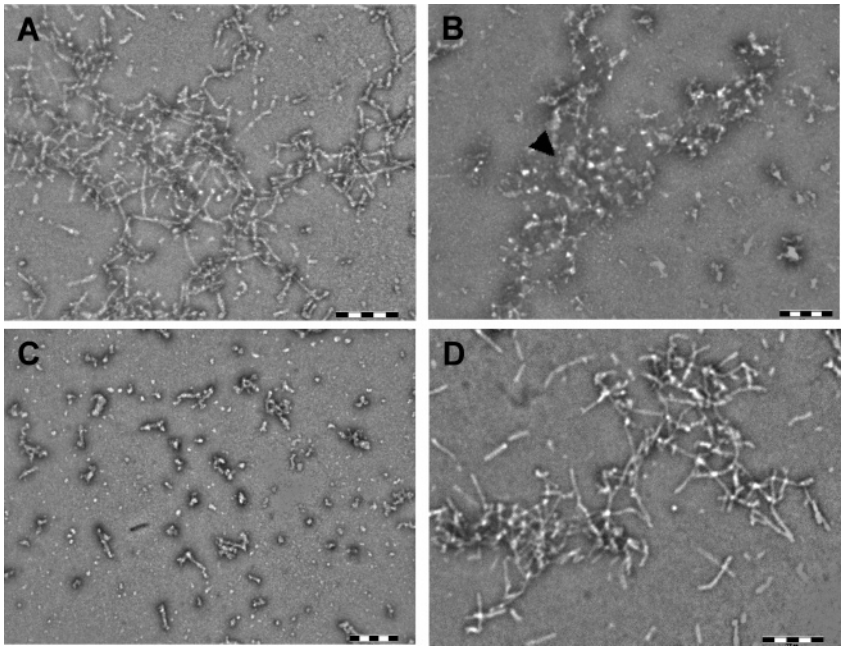


FIGURE 6: Electron micrographs of reduced κ-casein with and without casein proteins or BSA. κ-Casein (3 mg/mL) in phosphate buffer (pH 7.0) with 10 μM ThT and 20 mM DTT was incubated for 20 h at 37 °C in the absence (A) or presence of ~2.0 molar equivalents (7.5 mg/mL) of either α_S-casein (B) or β-casein (C) or an equivalent mass (7.5 mg/mL) of BSA (D). In B, a large conglomerate of irregularly structured aggregates is indicated by a tilted ▲. Scale bars represent 200 nm.

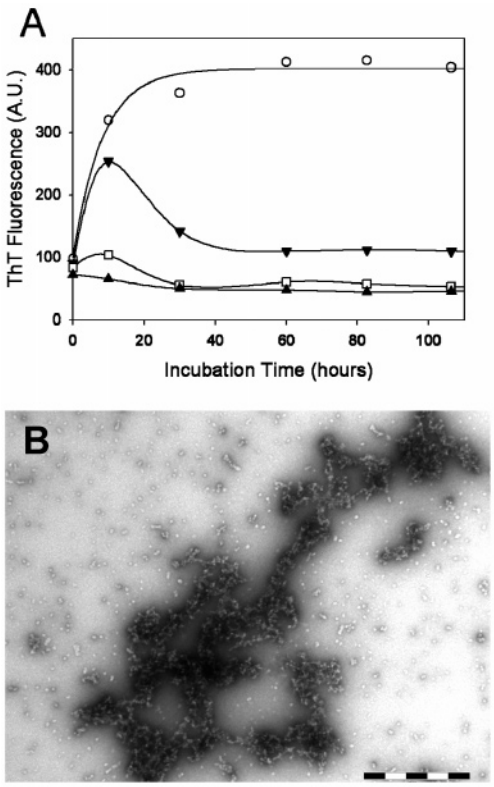
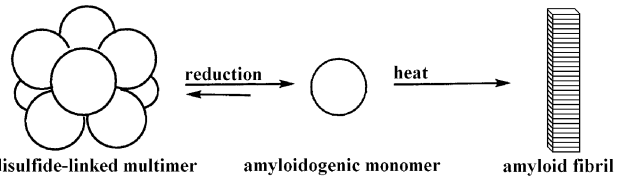


FIGURE 7: Inhibition of κ-casein fibril formation by ANS. (A) Time course of ThT fluorescence for κ-casein (3 mg/mL) in 50 mM phosphate buffer (pH 7.0) incubated at 37 °C with 20 mM DTT in the presence of either 0 mM (○), 1 mM (▼), 2 mM (□), or 4 mM (▲) ANS. Data shown are average values determined for duplicate samples. (B) Electron micrograph shows κ-casein (4 mg/mL) after 140 h of incubation at 37 °C with 20 mM DTT and 8 mM ANS. The scale bar represents 500 nm.

κ-casein in a stable multimeric state, why is fibril formation, albeit limited, observed under nonreducing conditions? With this question in mind, an important finding is that ~10% of

Scheme 1: Proposed Mechanism of κ-Casein Fibril Formation[a]



[a] Fibril formation is induced by quaternary structural destabilization caused by the reduction of intermolecular disulfide linkages. The dissociated, κ-casein monomer appears to be highly susceptible to temperature-dependent fibril formation.

κ-casein in milk migrates as a monomer on SDS−PAGE (*45*), since its sulfhydryl groups do not interact intermolecularly but instead form intrachain disulfide bridges (*46*). We therefore suggest that incubating κ-casein at 37 °C induces fibril formation via monomeric species that are present as minor components of the total protein. This suggestion is supported by the fact that the fully dissociated form of the protein, present in abundance under reducing conditions, appears to be sufficiently unstructured and flexible to allow ready assembly into amyloid fibrils (Scheme 1).

Despite our findings that κ-casein forms fibrils *in vitro* under near-physiological conditions (i.e., neutral pH, nonreducing, 37 °C), the occurrence of amyloid fibrils in milk, to the authors' knowledge, has not been reported. Farrell et al. (*25*) offered a possible explanation for this, observing that polymerization by reduced and carboxymethylated κ-casein was inhibited by α_{S1}-casein. The implication is that, in its natural environment, the fibril-forming tendency of κ-casein is kept at bay via its interaction with other caseins present in milk. To test this hypothesis, the amyloid fibril-forming propensity of purified κ-casein has been compared to that of a preparation of whole casein (sodium caseinate). Whole casein consists of several species, which, in the

absence of calcium at neutral pH, interact strongly with themselves and each other (*6*, *47*−*49*). We found that whole casein, while containing substantial amounts of κ-casein (~150 mg/g of whole casein), does not assemble into amyloid fibrils upon reduction (parts A and B of Figure 4). The other major components of whole casein, α_S- and β-casein, both show a concentration-dependent inhibition of κ-casein fibril formation, with a 3 times molar excess of either protein, resulting in near complete suppression of fibril formation (parts A and B of Figure 5). Under conditions where amyloid formation is strongly inhibited, the aggregates formed in the presence of α_S- and β-casein are less ordered, being somewhat amorphous in form. Such a phenomenon has also been reported with the intracellular chaperones, Hsp70 and Hsp40, and the sHsp, αB-crystallin, as a result of their interaction with huntington (*50*, *51*) and α-synuclein (*18*), respectively. It has been suggested that these chaperones shunt amyloidogenic proteins from the fibril-forming pathway to the amorphous aggregation pathway (*7*). Our observations imply that α_S- and β-casein do likewise with κ-casein.

Clusterin and the best characterized of the sHsp family, α-crystallin, both function as molecular chaperones by sequestering partially structured protein species that are prone to aggregation (*52*, *53*). Although lacking any sequence similarity, caseins, α-crystallin, and clusterin have many structural and behavioral features in common that make it likely that they prevent aggregation and amyloid formation via a similar mechanism (*7*, *9*). For instance, they are all dynamic and flexible proteins that have extensive regions of little ordered structure, high surface hydrophobicities, and a tendency to aggregate heterogeneously (*7*−*9*). A possible reason for some of their shared characteristics is that the polar and apolar residues in each of these proteins are not evenly distributed in their sequences but occur in clusters, yielding well-defined hydrophobic and hydrophilic regions (*4*, *7*, *11*). In sHsps and clusterin, these characteristics are likely to be vital to their chaperone functions; e.g., their exposed hydrophobic surfaces are believed to allow for selective interaction with partially unfolded target proteins, while flexible hydrophilic regions maintain the solubility of the chaperone-target protein complex (*7*, *11*). In a similar fashion, α_S- and β-casein may "chaperone" inherently unstable monomers of κ-casein by binding to and shielding their hydrophobic surfaces, thus prohibiting interactions with other κ-casein molecules that would otherwise facilitate self-assembly into fibrillar structures.

Reflecting the importance of hydrophobic interactions, κ-casein fibril formation is also inhibited by ANS. Fluorescence experiments show that κ-casein, in both its native and reduced state, has a high propensity to bind ANS,[2] as does α_S-casein (*9*) and β-casein (*10*, *54*). Such a property is commonly observed for partially structured proteins that expose clusters of hydrophobic residues (*37*). Comparable to the effect that α_S- and β-casein exert on κ-casein, ANS causes κ-casein to form amorphous aggregates under conditions that would otherwise give rise to fibrils. Nielsen et al. (*29*) observed that ANS has a very similar effect on insulin fibril formation. By disrupting specific hydrophobic interactions, ANS may inhibit the ordered assembly of fibrillar, β-sheet-rich aggregates, while still allowing residual hydrophobic interactions and hydrogen-bond formation that lead to amorphous aggregation.

The amyloid deposits found in bovine mammary CA consist of a tightly packed network of fibrils, 8−10 nm in diameter, which stain positively with amyloidophilic dyes such as ThT and Congo Red (*20*). Although the precise anatomical origin of their development is unclear, electron microscopy has identified numerous amyloid-like fibrils within the cytoplasm of mammary epithelial cells surrounding the CA (*20*, *23*). Such cells are actively involved in the synthesis and secretion of milk constituents, including caseins, into the alveolar lumina and their subtending ducts (*55*), within which 90% of CA are observed (*23*). A close correlation between CA prevalence and epithelial differentiation and an increased prevalence of CA as the lactation cycle progresses further suggest a dependence of CA development on synthetic and secretory processes (*23*) and highlight the possible involvement of casein proteins in the genesis of mammary CA amyloid. Certainly, the ease with which bovine κ-casein can be converted into fibrils strongly argues that CA-related amyloid fibrils are derived from this protein.

The ability of κ-casein to form amyloid fibrils at physiological pH and temperature and the ability of α_S- and β-casein to prevent such fibril formation make it probable that α_S- and β-casein perform such a role in mammary tissue to prevent large-scale fibril formation. It has been postulated that, prior to disulfide cross-linking, monomers of κ-casein first interact with α_{S1}-, α_{S2}-, and β-casein to form submicelles, followed by coalescence of the submicelles to form the complete micellar structure (*49*, *56*). Being in a reduced form, it is during these early stages preceding micelle formation that κ-casein would be particularly susceptible to fibril formation. Thus, the interactions between casein subunits, which are critical for the formation and structural stability of the casein micelle (*57*), may also play a vital role in protecting the mammary gland against localized amyloidosis.

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