Amyloid Fibril Formation by Bovine Milk α_{s2}-Casein Occurs under Physiological Conditions Yet Is Prevented by Its Natural Counterpart, α_{s1}-Casein[†]

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ABSTRACT: The calcified proteinaceous deposits, or *corpora amylacea*, of bovine mammary tissue often comprise a network of amyloid fibrils, the origins of which have not been fully elucidated. Here, we demonstrate by transmission electron microscopy, dye binding assays, and X-ray fiber diffraction that bovine milk α_{s2}-casein, a protein synthesized and secreted by mammary epithelial cells, readily forms fibrils *in vitro*. As a component of whole α_s-casein, α_{s2}-casein was separated from α_{s1}-casein under nonreducing conditions via cation-exchange chromatography. Upon incubation at neutral pH and 37 °C, the spherical particles typical of α_{s2}-casein rapidly converted to twisted, ribbon-like fibrils ~12 nm in diameter, which occasionally formed loop structures. Despite their irregular morphology, these fibrils possessed a β-sheet core structure and the ability to bind amyloidophilic dyes such as thioflavin T. Fibril formation was optimal at pH 6.5–6.7 and was promoted by higher incubation temperatures. Interestingly, the protein appeared to be less prone to fibril formation upon disulfide bond reduction with dithiothreitol. Thus, α_{s2}-casein is particularly susceptible to fibril formation under physiological conditions. However, our findings indicate that α_{s2}-casein fibril formation is potently inhibited by its natural counterpart, α_{s1}-casein, while is only partially inhibited by β-casein. These findings highlight the inherent propensity of casein proteins to form amyloid fibrils and the importance of casein−casein interactions in preventing such fibril formation *in vivo*.

The deposition of amyloid fibrils in human tissue is implicated in the pathogenesis of some of modern society's most debilitating diseases, including Alzheimer's and Parkinson's diseases, and type II diabetes (see ref *1* for a review). Amyloid-like deposits, possessing many of the characteristics of those associated with human disease, have been identified in bovine, rat, and canine mammary tissue within calcified stones known as *corpora amylacea* (CA)[1] (*2–4*). It has been postulated that CA can cause complications in cows during late lactation by accumulating in luminal spaces and blocking small ducts, leading to a reduction in milk secretion and flow (*5*). The identity of the protein(s) involved in the formation of the observed fibrils has not been determined conclusively, but immunoblotting and sequence analysis of peptides obtained from mammary CA has suggested that several milk proteins are present (*6, 7*).

The major milk proteins, the caseins (α_{s1}, α_{s2}, β, and κ), belong to a large group of proteins termed natively unfolded since they have little secondary or tertiary structure under physiological conditions; rather, they have extensive regions of disordered structure (*8*). A feature common to some natively unfolded proteins, most notably α-synuclein, tau, and islet amyloid polypeptide, is their propensity to form amyloid fibrils associated with degenerative diseases (*9*). The function of the caseins is largely nutritional; caseins associate with themselves and each other to form large colloidal aggregates (casein micelles), which serve as calcium-transport vesicles, providing young mammals with a concentrated, yet soluble, form of calcium as well as essential amino acids (*10*). We have recently provided further insights into their functional properties, showing that each of the caseins exhibits molecular chaperone-like activity (*11*). Caseins act as chaperones in a manner very similar to that of intracellular small heat-shock proteins, and the extracellular protein, clusterin (*12*), whereby they protect proteins against aberrant aggregation (*11, 13–16*), including amyloid fibril formation (*17*), when placed under conditions of cellular stress, for example, acidity, elevated temperature, reduction, or UV radiation.

α_s-Casein (s = sensitive) is the most calcium-sensitive fraction of casein. Originally, α_s-casein was considered to be a homogeneous protein, but later, it was shown to comprise two distinct gene products, designated α_{s1}- and α_{s2}-casein, which are unrelated in sequence but have very similar physicochemical properties (*10*). They readily associate with each other and are often isolated together (i.e., copurified)

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[1] Abbreviations: CA, *corpora amylacea*; ThT, thioflavin T; DTT, 1,4-dithiothreitol; TEM, transmission electron microscopy; A.U., arbitrary units; PPII, poly-L-proline.

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from milk. Of the four caseins, only α_{s2}- and κ-casein possess disulfide linkages, each with two cysteine residues per molecule. In κ-casein, these disulfides are mostly arranged intermolecularly to form multimers (ranging from dimers to decamers) (*18*) that further associate via hydrophobic and other interactions to form spherical particles of relatively uniform size (~18 nm in diameter) (*19*). Upon disulfide bond reduction, however, κ-casein rapidly assembles into amyloid fibrils (up to 1 μm in length) over a wide range of pH and temperature (*17, 20*). While κ-casein undergoes multimerization, α_{s2}-casein, under nonreducing conditions, forms either a monomer or a dimer (because of intra and interchain disulfide linkages, respectively), which are present in milk in similar proportions (*21, 22*). The corollary of this is that α_{s2}-casein, like κ-casein, is susceptible to destabilization by disulfide bond reduction. This and several other features shared by κ-casein prompted us to test whether α_{s2}-casein would exhibit a propensity to form amyloid fibrils under the same conditions.

α_{s2}-Casein is the least abundant and perhaps the most difficult of the casein proteins to isolate in a pure form. Consequently, it has been the least studied. Accordingly, in this study we separate α_s-casein into its individual components (i.e., α_{s1}- and α_{s2}-casein) via cation-exchange chromatography and (i) examine the fibril-forming propensity of each component; (ii) determine the optimal conditions (i.e., pH and temperature) for α_{s2}-casein fibril formation; and (iii) demonstrate that α_{s2}-casein fibril formation is inhibited by casein−casein interactions, particularly those with α_{s1}-casein, its natural counterpart.

MATERIALS AND METHODS

Materials. α_s-Casein and β-casein from bovine milk were purchased from Sigma Chemical Co. (St. Louis, USA). Thioflavin T (ThT) and β-mercaptoethanol were also obtained from Sigma. 1,4-Dithiothreitol (DTT) was obtained from Astral Scientific (Sydney, Australia). All other reagents were of analytical grade, and all solutions were prepared with ultrapure MilliQ water. Proteins were incubated in 50 mM sodium phosphate buffer (pH 7.0, unless stated otherwise) containing 2 mM EDTA and 0.02% (w/v) sodium azide. Prior to use, protein solutions were passed through 0.20 μm Supor syringe filters (Pall Corporation, East Hills, USA) to remove pre-existing aggregates, and protein concentrations were determined using a Cary 5000 UV–vis-NIR spectrophotometer (Varian, Melbourne, Australia) and extinction coefficients (A_{280}) of 1.06, 1.05, 1.10, and 0.44 (mL·mg^{-1}·cm^{-1}) for α_s-, α_{s1}-, α_{s2}-, and β-casein, respectively (*23*).

Purification of α_{s1}- and α_{s2}-Casein. Whole α_s-casein obtained from Sigma was separated into two major components by cation-exchange chromatography using a Mono S 5/50 GL column (Amersham Biosciences, UK) according to a method described by Rasmussen et al. (*22*), with minor modifications. The column was equilibrated with 50 mM ammonium acetate−8 M urea (pH 7.0) and eluted with a two-step gradient (i.e., steps of 50% and then 100% eluent) of 1 M ammonium acetate−8 M urea (pH 7.0). The eluted proteins were dialyzed at 4 °C against 20 mM ammonium bicarbonate for 3 days, then against MilliQ water for 1 day, replacing the dialysis buffer twice daily. The dialysate was then lyophilized.

SDS−PAGE Analysis. Gel electrophoresis was performed on 12% (v/v) acrylamide gels according to Laemmli (*24*). Where reduction was required, samples were mixed with 2.5% (v/v) β-mercaptoethanol. All samples were heated (95 °C, 5 min) prior to loading onto the gel. Gels were stained with Coomassie Blue R-250.

Mass Spectrometry. Electronspray ionization spectrometery (ESI-MS) was performed on a Q-Tof2 quadruple orthogonal acceleration time-of-flight mass spectrometer (Micromass U.K., Manchester, U.K.). Samples were prepared by dissolving 5 μg/mL of either reduced α_{s1}- or α_{s2}-casein in 50% aqueous acetonitrile with 1% formic acid. The capillary voltage was 3.1 kV, and cone voltage was 50 V. The flow of nitrogen desolvation gas was optimized, the desolvation temperature set to 150 °C, and the source temperature set to 80 °C. Data was acquired in continuum mode with argon gas in the collision cell, and the samples were injected into the electrospray source at a rate of 3 μL/min. The spectra were processed with MassLynx software.

Thioflavin T binding. The time-dependent formation of amyloid fibrils was monitored via a ThT assay, as described previously (*17*). The low binding of ThT to α_{s2}-casein fibrils at neutral pH[2], that is, the optimal pH for fibril formation, precluded the use of *in situ* assays. As an alternative, 10 μL aliquots were periodically withdrawn from stock protein solutions that were incubated without shaking at either room temperature (25 °C), 37, or 50 °C and then snap frozen at −20 °C. Freezing had no noticeable affect on fibril morphology. At the completion of the time-course, each sample was thawed and then mixed with 1.6 mL of 10 μM ThT in 50 mM glycine buffer, raised to pH 9.0 with NaOH. The fluorescence was then measured using a glass cuvette (10 mm light path) and a Cary Eclipse spectrofluorimeter (Varian) with the excitation and emission wavelengths set at 442 and 490 nm, respectively, as described previously (*17*).

Transmission Electron Microscopy. Samples for TEM were prepared by adding 2 μL of undiluted protein solution to Formvar and carbon-coated nickel grids (SPI Supplies, Wester Chester, USA). The grids were then washed three times with 10 μL of water and negatively stained with 10 μL of uranyl acetate (2% (w/v); Agar Scientific, UK). The grids were dried with filter paper between each step. The samples were viewed under 25–64 K magnifications at 80 kV excitation voltages using a Philips CM100 transmission electron microscope (Philips, Eindhoven, The Netherlands). Particle size measurements were performed using SIS Image Analysis software.

CD Spectroscopy. Samples were prepared in 50 mM sodium phosphate buffer (pH 7.0), as detailed above, and incubated at either 37 or 50 °C. Where reduction was required, samples were mixed with 20 mM DTT 30 min prior to incubation and/or analysis. After one week's incubation, the resulting aggregates were purified according to Meehan et al. (*25*), that is, the protein solution was spun for 90 min at ~340,000*g* in a Beckman Coulter Optima TLX ultracentrifuge. The pellet was resuspended in 10 mM sodium phosphate buffer (pH 7.0) immediately prior to analysis. Samples were diluted to either 0.07 mg/mL α_{s1}-casein or 0.15–0.18 mg/mL α_{s2}-casein. Spectra (185 to 245 nm) were acquired at room temperature using a Jasco J-810 spectropo-

[2] Thorn, D. C., Ecroyd, H., and Carver, J. A., unpublished observations.

larimeter (Jasco, Victoria, Canada) and a 1 mm path length cell, recording the average of six measurements.

X-ray Fiber Diffraction. Samples for X-ray fiber diffraction were prepared via two methods. The native sample was prepared by dissolving the protein in water at a concentration of 10 mg/mL. Small aliquots were then dried down onto the surface of a quartz capillary and exposed to the X-ray beam. Alternatively, all other samples were prepared by incubating the protein in 50 mM phosphate buffer (pH 7.0) either with DTT at 37 °C, or without DTT at 50 °C. After one week's incubation, the resulting aggregates were purified via ultracentrifugation as described previously. The pellet was resuspended in a minimal volume of water. A fibril stalk was then prepared according to Serpell et al. (*26*). 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 alignment of amyloid fibrils in the fiber. A small stalk 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 K^{α} Rigaku rotating anode source (wavelength 1.5418 Å) and a mar345 image plate detector (MarResearch GmbH, Germany). Images were examined using marView (MarResearch), and reflections were measured.

RESULTS

Purification of α_{s1}- and α_{s2}-Casein. Whole α_s-casein was separated into its two major components, α_{s1} and α_{s2}, by cation-exchange chromatography using a simple two-step gradient, as shown in Figure 1A. A broad peak corresponding to the predominant α_{s1}-casein component eluted at 50 mM ammonium acetate−8 M urea, as observed previously (*22*). Once the tail of the α_{s1} peak trailed off, the step up to 50% eluent allowed the elution of α_{s2}-casein. Additionally, a minor peak containing peptide fragments and possibly other contaminants (e.g., β-casein) was observed after ~3 min (Figure 1A). SDS−PAGE analysis of the major eluted fractions showed that α_{s1}-casein was separated from α_{s2}-casein by the purification process (Figure 1B). On the basis of their electrophoretic mobility, α_{s1}-casein migrated as a single band of 27 kDa, and α_{s2}-casein migrated as two separate bands of 23 and 53 kDa, corresponding to the monomeric and dimeric forms, respectively. The dimer was largely converted to the monomer upon reduction at 95 °C. Meanwhile, reduction had no observable effect on the electrophoretic mobility of α_{s1}-casein. Analysis of the peak area of each fraction and taking into consideration the relative extinction coefficients of α_{s1} to α_{s2} at 280 nm provided a molar ratio of 4:1 α_{s1}/α_{s2}, consistent with the proportion in bovine milk reported by Davies and Law (*27*). The identity of the peaks obtained by cation-exchange chromatography was further verified by mass spectrometry. Each fraction exhibited substantial heterogeneity, attributable to the large number of post-translationally modified forms of each protein present in milk, for example, α_{s1}-casein carries between 7 to 10 phosphates, while α_{s2}-casein carries between 10 to 13 phosphates (*23*). Overall, the measured molecular mass corresponded to the expected range for each fraction, and the range between the two fractions differed by at least 1 kDa, which is expected for the two proteins (*23*). Furthermore, we obtained a molecular mass of 23,615.24 ± 0.34 Da for α_{s1}-casein, and 25,308.77 ± 1.81 Da for α_{s2}-casein. The former corresponds to the predominant α_{s1}-casein B-8P (theoretical mass, 23,614.7 Da), while the latter corresponds to α_{s2}-casein A-12P (theoretical mass, 25,308.3 Da), the major isoform of α_{s2}-casein present in milk.

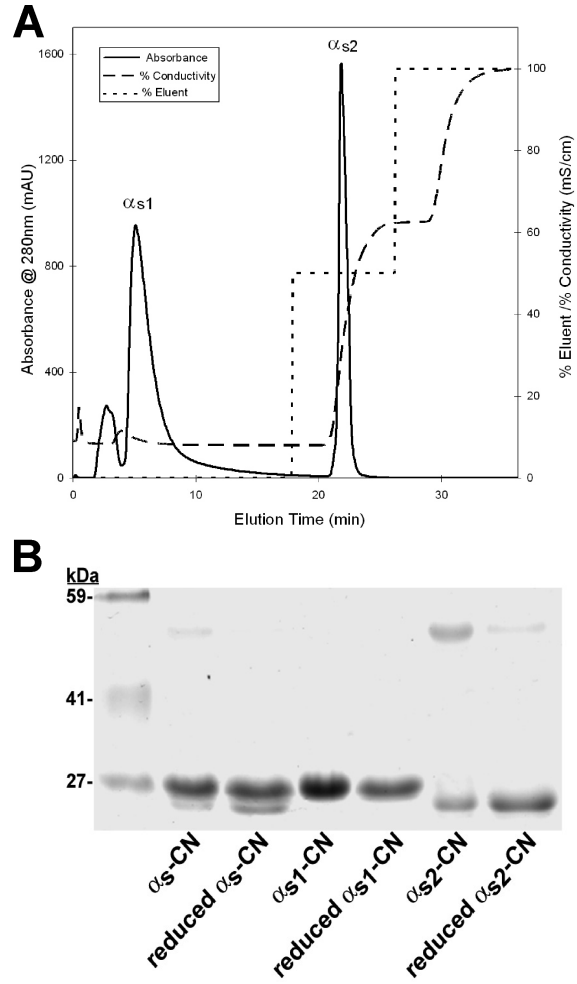

FIGURE 1: Purification of α_{s1}- and α_{s2}-casein. (A) Elution profile of α_s-casein separated by cation-exchange chromatography. Proteins were eluted with a two-step gradient of 1 M ammonium acetate−8 M urea (pH 7.0) on a Mono S column at a flow rate of 0.5 mL/min. (B) SDS−PAGE analysis of whole α_s-casein, and α_{s1}- and α_{s2}-casein fractions obtained from cation-exchange chromatography. Reduced samples were heated at 95 °C with β-mercaptoethanol for 5 min. Molecular weight calibrant standards are shown on the left.

Effect of Reduction on α_{s1}- and α_{s2}-Casein. κ-Casein fibril formation is induced by disulfide bond reduction at 37 °C (*17, 20*), a process that can be followed over time by monitoring the binding of ThT to the fibrils. Previously, we observed that reduced α_s-casein exhibits a time-dependent increase in ThT binding (*17*), suggesting that either one or both of its components also form fibrils under these conditions. Figure 2 shows the time course of ThT binding for reduced and nonreduced α_s-casein and its components, α_{s1}- and α_{s2}-casein, at 37 °C. As expected, ThT binding for α_s-casein increased sigmoidally over time but only in the presence of the reducing agent, DTT (Figure 2; ▽). Although a rise in ThT binding was observed, analysis by TEM indicated that α_s-casein did not form amyloid fibrils under

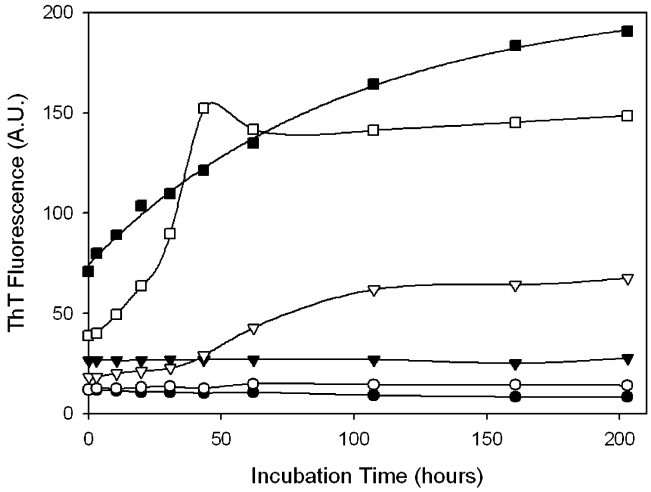

FIGURE 2: ThT fluorescence analysis of fibril formation by reduced and nonreduced α_s-casein proteins. α_{s1}- (●), α_{s2}- (■), and whole α_s- (▼) casein (120 μM) in 50 mM phosphate buffer (pH 7.0) were incubated at 37 °C. Under the same conditions and concentration, α_{s1}- (○) α_{s2}- (□), and whole α_s- (▽) casein were incubated in the presence of 20 mM DTT. Data shown are single measurements and are representative of at least three experiments.

these conditions, as after 150 h at 37 °C, spherical particles rather than fibrillar species were observed (*17*).

α_{s2}-Casein exhibited a much greater propensity to form fibrils than α_s-casein. Prior to incubation or reduction with DTT, the particles formed by α_{s2}-casein were essentially spherical with average diameters of ~10 nm, as shown by TEM (Figure 3A), along with some larger amorphous species. However, upon incubation at 37 °C, α_{s2}-casein formed twisted, ribbon-like fibrils, ~12 nm in diameter and up to 1 μm in length (Figure 3B) within a week of incubation. These fibrils had a tendency to curl to form irregular ring structures (up to 500 nm in diameter; Figure 3C), although the complete closure of these fibril loops was not common. TEM verified that α_{s2}-casein fibrils could be dissociated under nonreducing conditions by heating in 1% (w/v) SDS at 95 °C for 5 min (data not shown). Subsequent examination of the fibril solutions by SDS−PAGE revealed little evidence of proteolysis (data not shown), suggesting that the observed fibrils were formed from the intact protein. Surprisingly, fibril formation at 37 °C appeared to be prevented when α_{s2}-casein was incubated in the presence of DTT, leading instead to the formation of spherical aggregates (Figure 3D) similar to those of the native associated state (Figure 3A). Interestingly, reduced and nonreduced α_{s2}-casein each displays substantial capacities to bind ThT, both before and after incubation, yet the degree of initial binding and the kinetics of the increase in binding upon incubation vary significantly (Figure 2; ■ and □). For instance, in the absence of DTT (■), the fluorescence increase is immediate, with no discernible lag phase. ThT binding rises sharply during the initial stages of incubation, before plateauing over time (i.e., reminiscent of a saturation curve). However, upon addition of DTT (□), the initial fluorescence intensity for α_{s2}-casein immediately drops by ~50%. Moreover, incubation of this reduced form of α_{s2}-casein generates a sigmoidal increase in ThT binding (reminiscent of α_s-casein under reducing conditions), which is distinguished by a transient peak in fluorescence after 50 h, immediately preceding the plateau phase. Overall, with or without DTT, the rate and magnitude of the increases in fluorescence by α_{s2}-casein were significantly greater than those observed for solutions of α_s-casein.

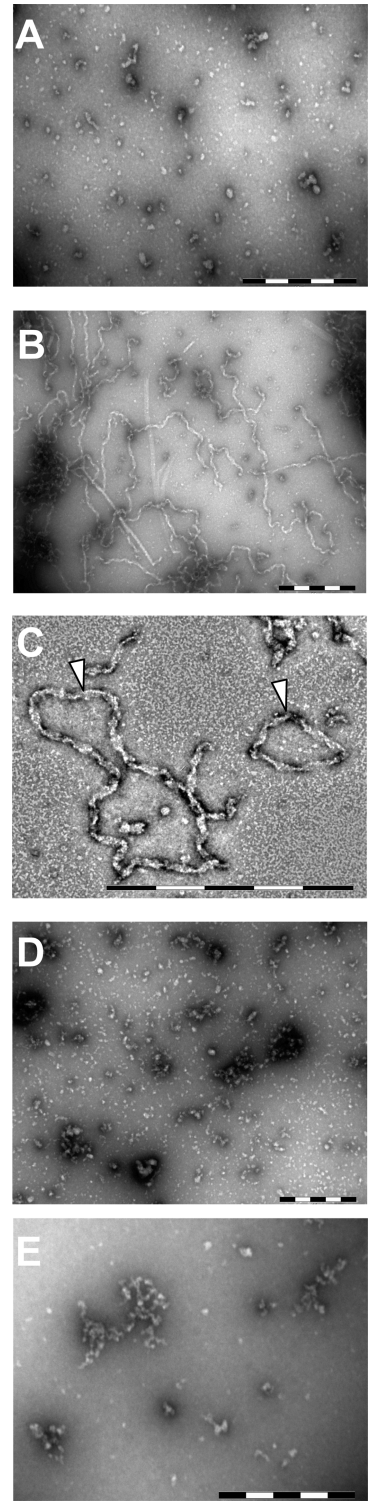

FIGURE 3: Electron micrographs of α_{s1}- and α_{s2}-casein. (A) α_{s2}-Casein immediately after dissolution in 50 mM phosphate buffer (pH 7.0) without DTT. The following proteins, at 120 μM, were incubated at 37 °C for one week: (B and C) α_{s2}-casein, nonreduced (D) α_{s2}-casein reduced with 20 mM DTT, and (E) α_{s1}-casein, nonreduced. In C, fibrillar loop species are indicated by (▽). The scale bars represent 500 nm.

In contrast, α_{s1}-casein, a protein devoid of disulfide linkages, showed no capacity to bind ThT (Figure 2) or form fibrils (Figure 3E), irrespective of whether DTT was present

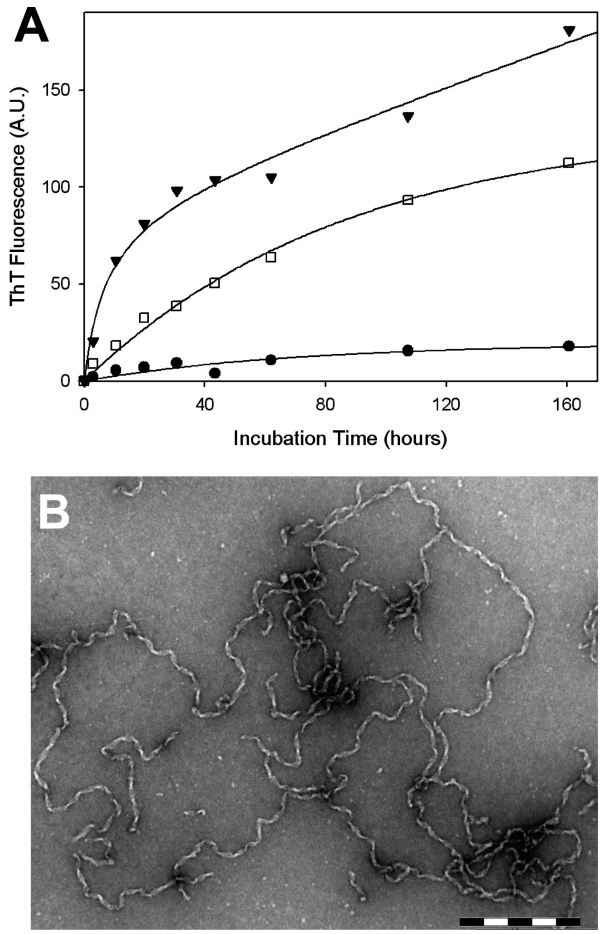

FIGURE 4: Temperature-dependence of α_{s2}-casein fibril formation. (A) Time-course of ThT fluorescence for α_{s2}-casein (120 μM) incubated at 25 °C (●), 37 °C (□), and 50 °C (▼). To aid the comparison between increases in ThT fluorescence, each curve was normalized by designating the initial fluorescence intensity as zero arbitrary units (A.U.). Data shown are single measurements and are representative of at least three experiments. (B) Electron micrograph showing α_{s2}-casein (120 μM) after one week of incubation at 50 °C. The scale bar represents 500 nm.

or absent. Hence, it is concluded that α_{s2}-casein is the fibril-forming component of whole α_s-casein.

Temperature Dependence of α_{s2}-Casein Fibril Formation. Owing to their inherent structural disorder, caseins are generally heat stable and resist structural deformations caused by high temperatures (*28*). Despite this, α_{s2}-casein formed fibrils at neutral pH under nonreducing conditions at 37 °C, implying that physiological pH and temperature are sufficient to induce fibril formation. To ascertain the effect of temperature on α_{s2}-casein fibril formation, the protein was incubated at 25, 37, and 50 °C in the absence of DTT. The initial rate and final magnitude of α_{s2}-casein fibril formation was strongly dependent on temperature, with fibril formation being promoted by higher temperatures (Figure 4A). At 25 °C, only a minor increase in ThT binding was observed over the course of the incubation (Figure 4A; ●). Examination by TEM verified the formation of fibrils at 37 °C (Figure 3B) and 50 °C (Figure 4B) but not at 25 °C (data not shown), demonstrating that α_{s2}-casein requires at least some heating in order to form fibrils. Fibrils formed at 50 °C appeared more rigid and longer (many over 1 μm in length) than those formed at 37 °C, but otherwise, raising the incubation

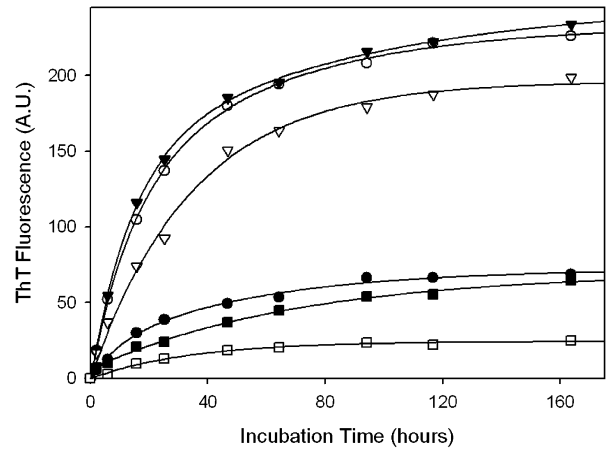

FIGURE 5: pH dependence of α_{s2}-casein fibril formation. (A) Time-course of ThT fluorescence for α_{s2}-casein (120 μM) incubated at 37 °C in 50 mM phosphate buffer at pH 6.0 (●), 6.5 (○), 6.7 (▼), 7.0 (▽), 7.5 (■), and 8.0 (□). All spectra were acquired at pH 9.0 by diluting protein samples 1:160 with 50 mM glycine (pH 9.0) prior to analysis, as described in Materials and Methods. To aid the comparison between increases in ThT fluorescence, each curve was normalized by designating the initial fluorescence intensity as zero arbitrary units (A.U.). Data shown are single measurements and are representative of at least three experiments.

temperature from 37° to 50 °C did not significantly alter fibril morphology.

pH Dependence of α_{s2}-Casein Fibril Formation. The effect of pH on α_{s2}-casein fibril formation was investigated by incubating α_{s2}-casein at 37 °C in phosphate buffers ranging from pH 6.0 to 8.0. While substantial increases in ThT binding were observed at pH values ranging from 6.5 to 7.0, the rate and magnitude of these increases were greatest at pH 6.5 and 6.7 (Figure 5; ○ and ▼). Thus, α_{s2}-casein fibril formation at 37 °C is optimal at slightly acidic pH, coinciding with the narrow pH range of fresh milk (pH 6.5–6.7) (*29*). As pH is increased beyond pH 6.7, there is a progressive loss of fibril-forming propensity, with almost a complete absence seen at pH 8.0 (Figure 5; □). Below pH 6.5, however, α_{s2}-casein was more prone to amorphous aggregation and precipitation. For example, incubation at pH 6.0 led to a visible increase in solution turbidity, and under these conditions, amorphous species of little or no ordered structure were visible by TEM (data not shown).

Analysis of α_{s1}- and α_{s2}-Casein by Far-UV CD. Secondary structural changes accompanying incubation and/or reduction of α_{s1}- and α_{s2}-casein were probed by far-UV CD. For α_{s2}-casein, the far-UV spectrum for the native protein (Figure 6A; —) exhibited a large negative ellipticity centered at 205 nm because of extensive regions of disordered structure (*8*). A smaller shoulder was observed at around 220 nm, representing the substantial proportion of α-helical (24 to 32%) and β-sheet (~30%) structures present in α_{s2}-casein, as predicted by Hoagland et al. (*30*). Upon reduction with DTT (Figure 6A; ---), there is an increase in ellipticity between 200 to 230 nm, that is, a general shift in the spectrum along the *y*-axis, while the overall shape of the curve is essentially unchanged. α_{s2}-Casein was then incubated in the absence of DTT for one week at 37 and 50 °C, and fibril formation was verified by TEM. Examination of fibrils pelleted by ultracentrifugation indicated that approximately 40% (37 °C) and 55% (50 °C), respectively, of the total amount of protein present had formed fibrils under these

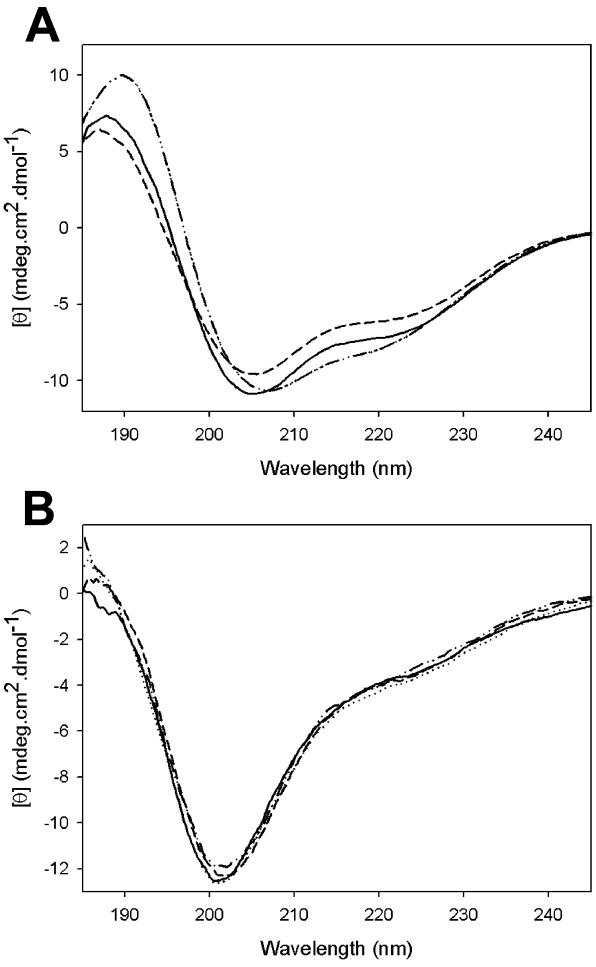

FIGURE 6: Analysis of α_{s1}- and α_{s2}-casein by far-UV CD. Spectra were acquired for (A) α_{s2}- and (B) α_{s1}-casein before and after one week's incubation at 37 °C under nonreducing (—, before; -•••-, after) and reducing (---, before; ••••, after) conditions. In A, the spectra for nonreduced (-••-••) and reduced (••••) α_{s2}-casein following incubation superimpose. These two spectra were each acquired on samples pelleted by ultracentrifugation and resuspended in buffer immediately prior to analysis, as described previously (*25*). Data shown are single measurements and are representative of two experiments.

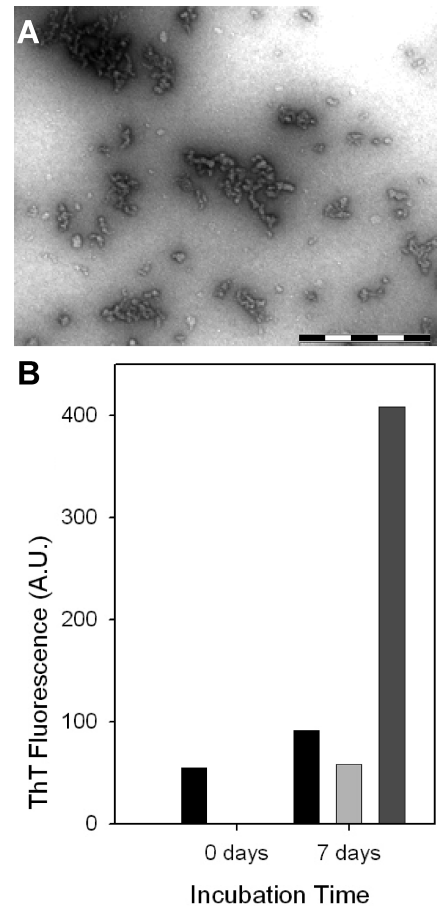

FIGURE 7: Analysis of reduced α_{s2}-casein following ultracentrifugation. (A) Electron micrograph shows disordered aggregates formed by α_{s2}-casein (120 μM) after one week of incubation at 37 °C in the presence of 20 mM DTT. Following incubation, the sample was pelleted via ultracentrifugation and resuspended in water, as described in Materials and Methods. The scale bar represents 5 μm. (B) Total ThT fluorescence for whole, unfractionated α_{s2}-casein (black bar) before and after one week of incubation at 37 °C in the presence of 20 mM DTT and for the resulting supernatant (light gray bar) and pellet (dark gray bar) obtained from incubation and subsequent ultracentrifugation. The pellet was diluted to the initial protein concentration of 120 μM prior to analysis.

conditions. Analysis of the pelleted fraction by far-UV CD revealed an increase in ellipticity between 190 to 200 nm (Figure 6A; -•••-). There was also a red shift of the minimum in ellipticity from 205 to 207 nm, and a decrease in ellipticity at ~218 nm. Taken together, these spectral changes indicate an increase in β-sheet structure, at the expense of disordered structure, in the fibrillar sample compared to the native protein and are consistent with the formation of amyloid fibrils.

A smaller pellet comprising 15% of the total protein was also obtained following incubation of α_{s2}-casein at 37 °C under reducing conditions. Examination of the pelleted fraction by TEM revealed the presence of relatively disordered aggregates of up to 5 μm in length (Figure 7A). Interestingly, the pelleted fraction had a dramatically higher propensity to bind ThT compared to the supernatant (Figure 7B), thus accounting for the time-dependent increase in ThT binding observed upon reduction and incubation of the protein (Figure 2; □). Despite their irregular morphology, the aggregated species present in the pellet (Figure 6A; •••) produced a far-UV CD spectrum identical to that of the fibrillar species (Figure 6A; -•••-).

No significant changes in far-UV CD spectra were observed upon reduction and/or incubation of α_{s1}-casein at 37 °C (Figure 6B), as expected. Moreover, no pellet was obtained following the incubation of α_{s1}-casein.

Analysis of α_{s2}-Casein Fibrils by X-ray Fiber Diffraction. The substructure of the aggregates formed by the incubation of α_{s2}-casein under reducing and nonreducing conditions was further probed by X-ray fiber diffraction. For comparison, α_{s2}-casein was freshly dissolved in water, dried down, and exposed to the X-ray beam. The diffraction pattern obtained for the native state of the protein was dominated by a broad, diffuse halo, centered at ~4.3 Å (data not shown), consistent with the presence of extensive regions of disordered structure and the absence of ordered, repeating secondary structural elements (*31*). In contrast, the fibril pellet prepared by incubation of the protein at 50 °C under nonreducing conditions and subsequent ultracentrifugation produced a diffraction pattern indicative of cross-β structure (Figure 8A). The pattern showed

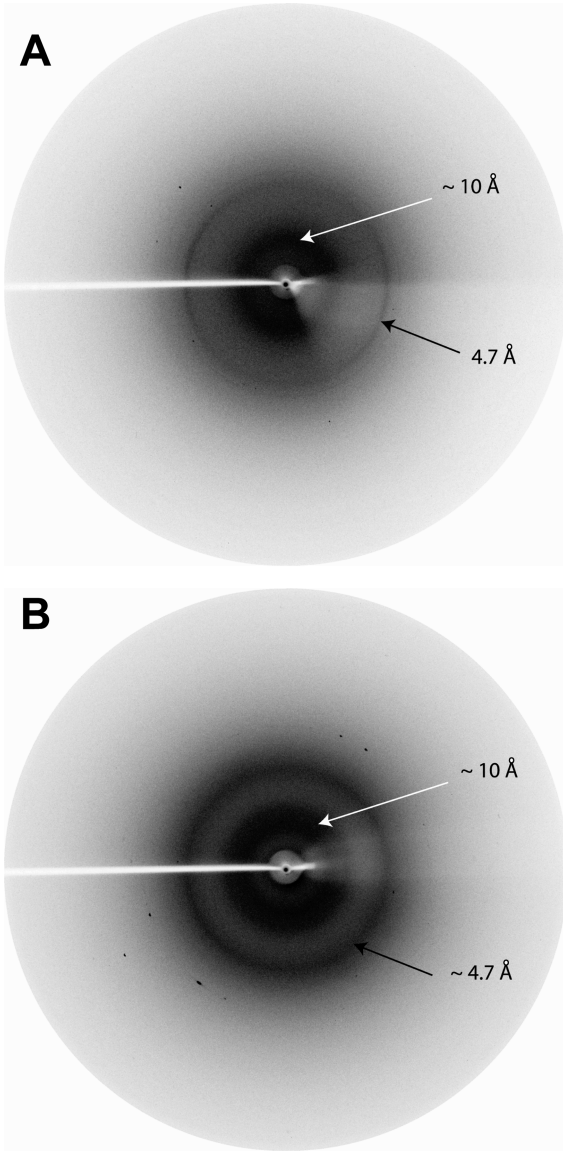

FIGURE 8: X-ray fiber diffraction pattern for α_{s2}-casein fibrils and disordered aggregates. (A) Fibrils were prepared by incubating α_{s2}-casein (240 μM) in 50 mM phosphate buffer (pH 7.0) at 50 °C. (B) Disordered aggregates were prepared by incubating α_{s2}-casein (120 μM) in 50 mM phosphate buffer (pH 7.0) at 37 °C in the presence of 20 mM DTT. Following one week of incubation, each sample was prepared for X-ray fiber diffraction analysis as described in Materials and Methods. The reflections at 4.7 Å and 10 Å are shown.

a sharp and intense meridional reflection at 4.7 Å, accompanied by a broad and diffuse reflection at approximately 10 Å. The sharpness and preferential alignment of the meridional reflection indicates that the spacing between adjacent hydrogen-bonded β-strands is ordered, repeating, and oriented in the direction of the long axis of the fibrils, as is a characteristic of amyloid fibrils (*32, 33*). The reflection at approximately 10 Å corresponds to 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. A similar diffraction pattern (Figure 8B) was obtained upon analysis of the disordered aggregates generated by incubation of the protein at 37 °C under reducing conditions and subsequent ultracentrifugation. This pattern displays two rings, one at ~4.7 Å and the other at ~10 Å. Here, however, in contrast to the pattern shown in Figure 8A, there is no preferential orientation of either of the reflections, but the spacings are consistent with the acquisition of β-structure, as suggested by ThT binding (Figure 2; □) and far-UV CD (Figure 6A). However, the underlying β-structure does not appear to be preferentially oriented or ordered over long distances, as is the case where fibrils have been produced by incubation of α_{s2}-casein under nonreducing conditions (Figures 3B and 4B).

Effect of α_{s1}- and β-Casein on α_{s2}-Casein Fibril Formation. Whole α_s-casein showed little propensity to form fibrils at 37 °C (Figure 2), despite comprising substantial amounts of α_{s2}-casein (~20%). This implies that fibril formation by α_{s2}-casein is inhibited by the other component of α_s-casein, namely, α_{s1}-casein. Previously, we showed that α_s-casein, along with β-casein, possessed the ability to prevent fibril formation by their micellar counterpart, κ-casein (*17*). In the present study, we investigated whether α_{s1}- (the major component of α_s-casein) and β-casein, could prevent fibril formation by α_{s2}-casein. Accordingly, α_{s2}-casein was incubated at 37 and 50 °C in the absence or presence of increasing amounts of either α_{s1}- or β-casein. ThT binding assays confirmed that α_{s1}- and β-casein do not form fibrils under these conditions (data not shown). For α_{s2}-casein alone, incubation under nonreducing conditions at 37 °C produced an immediate rise in ThT binding (Figure 9; ●), as seen previously (Figure 2; ■). In the presence of α_{s1}-casein (Figure 9A), the increases in ThT fluorescence associated with fibril formation by α_{s2}-casein was suppressed in a concentration-dependent manner, with near complete suppression achieved with a ~2.0-fold molar excess of α_{s1}-casein (Figure 9A; ○), a concentration that is substoichiometric compared to the 4:1 molar ratio of α_{s1}/α_{s2} found in milk (*27*). Inhibition of fibril formation was verified by TEM, which showed that amorphous aggregates, rather than fibrils, were formed when α_{s2}-casein was incubated in the presence of α_{s1}-casein (Figure 10A). At 50 °C, the rate of α_{s2}-casein fibril formation increased, as anticipated from previous experiments, reducing the required time for incubation. Elevating the incubation temperature, however, did not significantly affect the ability of α_{s1}-casein to suppress α_{s2}-casein fibril formation (Figure 9A).

β-Casein was clearly less effective than α_{s1}-casein, exhibiting only a marginal inhibitory effect (Figure 9B). Moreover, when α_{s2}-casein was incubated in the presence of β-casein, fibrils were clearly evident by TEM (Figure 10B). Nonetheless, the fibrillar species were noticeably shorter and much less prevalent than the amorphous species also observed in mixtures of α_{s1}- and α_{s2}-casein (Figure 10A).

DISCUSSION

Previously, we investigated the fibril-forming propensity of whole casein and its three major fractions, α_s, β, and κ (*17*). We observed that α_s-casein exhibits a time-dependent increase in binding to the amyloidophilic dye, ThT, but only under reducing conditions (Figure 2; ▽). The prerequisite of reduction suggested the involvement of its less abundant and disulfide-bonded subunit α_{s2}-casein. In the present study, we validated this supposition by demonstrating that α_{s2}-casein assembles into amyloid fibrils at 37 °C (Figure 3B) or higher temperatures (Figure 4B). Ironically, α_{s2}-casein, which comprises a mixture of monomers and disulfide-linked dimers (*21, 22*), formed fibrils most readily under nonreducing conditions. Indeed, the addition of DTT to solution

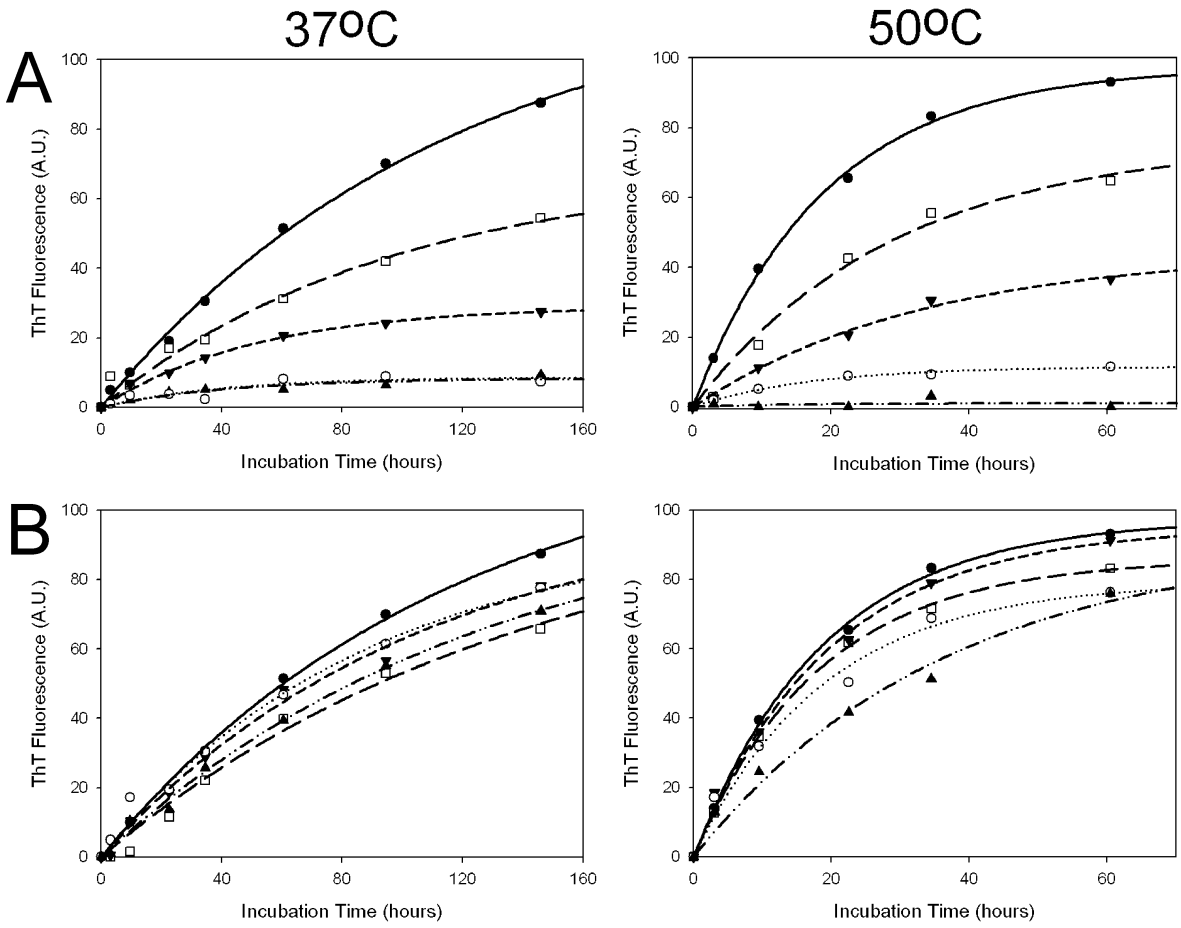

FIGURE 9: Effect of other caseins on α_{s2}-casein fibril formation at 37 and 50 °C. α_{s2}-Casein (120 μM) in 50 mM phosphate buffer (pH 7.0) was incubated at 37 and 50 °C in the absence (●; —) or presence of 60 μM (□; — —), 120 μM (▼; ---), 240 μM (○; •••••), and 360 μM (▲; –••–••) of either (A) α_{s1}-casein or (B) β-casein. To aid the comparison between increases in ThT fluorescence, each curve was normalized by designating the initial fluorescence intensity as zero arbitrary units (A.U.). Data shown are single measurements and are representative of at least three experiments.

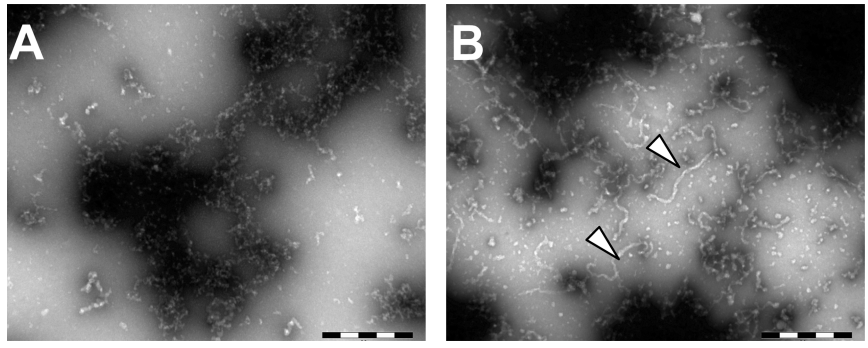

FIGURE 10: Electron micrographs of α_{s2}-casein with and without other caseins. α_{s2}-Casein (120 μM; ~3 mg/mL) in 50 mM phosphate buffer (pH 7.0) was incubated for one week at 37 °C in the presence of 2.0 mol equiv (240 μM; ~7.5 mg/mL) of either α_{s1}-casein (A) or β-casein (B). In B, short fibrillar species (600 nm in length) are indicated by (▽). The scale bars represent 500 nm.

appeared to inhibit fibril formation, leading instead to the formation of small spherical aggregates (Figure 3D). In contrast, κ-casein fibril formation is promoted following the reduction of its intermolecular disulfide linkages (*17*). Nonetheless, incubation of α_{s2}-casein under reducing conditions was accompanied by the formation of larger nonfibrillar aggregates having a substructure similar to that of amyloid fibrils, as suggested by far-UV CD and X-ray diffraction. Accordingly, the aggregated species readily bound ThT and possessed increased β-sheet structure. While their precise relationship to amyloid fibril structure is still unclear, it is possible that these disordered species represent intermediates in fibril formation that have subsequently diverted to a less ordered aggregation pathway.

Ribbon-Loop Morphology of α_{s2}-Casein Fibrils. Under nonreducing conditions, the aggregates formed from α_{s2}-casein possessed a β-sheet core structure characteristic of amyloid fibrils. Morphologically, however, these fibrils resembled those of human apolipoprotein C-II (apoC-II), which, in the absence of lipid, forms flat, twisted ribbons ~12 nm in diameter (*34*). Additionally, because of their inherent flexibility, apoC-II fibrils have a tendency to form loops (*35*), as do α_{s2}-casein fibrils (Figure 3C). The structural commonality between fibrillar α_{s2}-casein and apoC-II suggests

that the conformational states adopted by each protein prior to fibril assembly may have features in common. Indeed, prior to fibrillation, apoC-II and α_{s2}-casein are believed to possess extensive regions of disordered structure (*30, 36*).

pH Dependence of α_{s2}-Casein Fibril Formation. α_{s2}-Casein is the least soluble of the caseins, especially in the presence of Ca^{2+} and at 37 °C is highly prone to aggregation. Even at room temperature, α_{s2}-casein associates via electrostatic interactions to form spherical particles (Figure 3A), the size of which depends upon the ionic strength (*37*) and pH of the solution. One of the forces driving this association is the exceptionally high number of negatively charged phosphate residues (10–13 per molecule) and positively charged lysine residues (24 per molecule) in α_{s2}-casein (*23*). The uneven distribution of negative and positive charges (i.e., clusters) along its polypeptide endows the protein with a dipolar nature (*37*). It comes as no surprise, therefore, that α_{s2}-casein aggregation at 37 °C is highly sensitive to changes in pH and ionic strength and that fibril formation occurs readily only within a narrow pH range (pH 6.5–7.0). Importantly, this range corresponds to the pH of fresh milk, and thus fibril formation would be favored under physiological conditions. Outside this range, α_{s2}-casein was found to aggregate amorphously. At lower pH, aggregation is so extensive that precipitation occurs, presumably, because of the protonation of uncompensated negatively charged groups that normally combat hydrophobically driven association (e.g., as occurs during the isoelectric precipitation of casein). Conversely, at higher pH, α_{s2}-casein aggregation is partially abrogated, perhaps by electrostatic repulsion between the particles due to the overall net negative charge of the protein. A similar phenomenon was observed at low ionic strength, for example, in the absence of buffer (data not shown), where the rate of fibril formation is decreased and intermolecular electrostatic repulsion favors the dissociation of α_{s2}-casein (*37*).

Role of α_{s2}-Casein Fibril Formation in Vivo. Networks of fibrils displaying many of the hallmarks of amyloid are, on occasion, observed extracellularly within the proteinaceous deposits or inclusions of bovine mammary *corpora amylacea* (CA). Additionally, electron microscopy has identified numerous fibril-like structures within the cytoplasm itself, in mammary epithelial cells surrounding the CA (*3, 5*). Although the identity of the proteins forming these fibrillar structures is not explicitly known, the relationship between CA prevalence and epithelial synthetic and secretory processes point to casein proteins as the likely candidates (*5*). Indeed, the ease with which κ-casein could be converted into fibrils previously led us to suggest that CA-related amyloid fibrils originate from this protein (*17*). For this same reason, it is likely that α_{s2}-casein is also involved in the genesis of mammary CA amyloid. Strongly supporting this, several peptide fragments derived from α_{s2}-casein have been isolated from inclusion bodies within mammary CA (*6*). Designated AαS2-C (according to the nomenclature for newly discovered amyloid proteins), the group of peptides appears to be a product of trypsinolysis. α_{s2}-Casein is particularly susceptible to digestion by trypsin and plasmin, and like many of the amyloidogenic proteins associated with disease (*1*), fibril formation may be triggered by proteolytic cleavage. Niewold et al. (*6*) postulated that specific enzymatic breakdown of protein in the alveolar lumen (within which 90% of CA are observed (*5*)) toward the end of the lactation period generates peptides of α_{s2}-casein (and potentially, κ-casein) that are highly amyloidogenic. While our *in vitro* studies suggest that the intact proteins are, themselves, capable of forming fibrils, it is likely that *in vivo* proteolysis plays a significant role in casein fibril formation.

Structural Basis for Casein Fibril Formation. As early as 2002, Syme et al. (*38*) speculated that casein proteins would exhibit a propensity to form fibrils because of their structural parallels with the amyloidogenic proteins, tau and α-synuclein, which are characterized by being essentially unfolded and rich in poly-L-proline (PPII) helical structure. The PPII helix is a more extended left-handed helix that is thought to bestow a flexible, open character to the structure of tau and α-synuclein, and the caseins, which, in itself, has been implicated in the formation of fibrils in disease (*9*).

While a partially unfolded conformation is clearly an important requisite for fibril formation, in caseins, other factors must also be considered since only α_{s2}- and κ-casein form fibrils under physiological (nondenaturing) conditions. Interestingly, α_{s2}- and κ-casein have a number of structural features in common. For instance, of the four caseins, only α_{s2}- (Figure 2; ■) and κ-casein (*17, 20, 39*) readily bind ThT in their prefibrillar state. This highlights the possible importance of native β-sheet structure for casein fibril assembly. Indeed, far-UV CD and FTIR spectroscopy both indicate that substantial regions (~30%) of the α_{s2}-casein polypeptide have a propensity to form β-sheet structure (*30*). Considering that there is a substantial proportion of β-sheet structure already in place within the native protein under fibril-forming conditions, it is, therefore, perhaps not so surprising that only a minor increase in β-sheet content is observed upon α_{s2}-casein fibril formation. According to structure prediction algorithms, the region encompassing residues Ile^{85} to Val^{112} is particularly prone to adopt β-sheet structure and likely accounts for much of the β-sheet present in the native protein (*30*). This region is exceptionally hydrophobic and possesses 43% sequence similarity to residues Ile^{28} to Phe^{55} in κ-casein (*40*), a region predicted to adopt two sets of antiparallel β-sheets (*41*). This sheet-turn-sheet motif has been postulated to initiate κ-casein fibril formation (*20*) and is incorporated into the β-sheet core structure of fibrils formed by κ-casein (*42*). A relatively exposed and hydrophobic β-sheet structure may serve a similar function in α_{s2}-casein fibril formation. Importantly, this region of α_{s2}-casein ($Ile^{85}-Val^{112}$) incorporates part of AαS2-C, that is, the peptide fragments, 32 to 45 residues in length and all starting from Ala^{81}, which have been isolated from amyloid deposits *in vivo* (*6*).

Inhibition of α_{s2}-Casein Fibril Formation by Other Caseins. The propensity of α_{s2}- and κ-casein to assemble into fibrils at physiological pH and temperature raises an interesting question: why are amyloid fibrils not more frequently observed in mammary tissue or for that matter in milk? With this in mind, it is important to note that all of the caseins exhibit a strong tendency to associate, either with themselves or with each other, forming a wide range of homo and heteropolymers, respectively (*43*). It is these interactions, for instance, that in the presence of calcium and other minerals present in milk lead ultimately to the formation of casein micelles. The implication is that in their natural environment, the fibril-forming tendency of α_{s2}- and κ-casein

is kept in check via their interactions with other caseins present in milk. Consistent with this hypothesis, we showed previously that α_s- and β-casein inhibit fibril formation by κ-casein (*17*). In the present study, we have substantiated this further by showing that α_{s1}-casein inhibits α_{s2}-casein fibril formation in a concentration-dependent manner, with a two times molar excess of α_{s1}-casein resulting in near complete suppression of fibril formation. The corollary of this is that α_s-casein's propensity to form fibrils is suppressed by the interaction between α_{s1}- and α_{s2}-casein, which are present in α_s-casein at a molar ratio of 4:1 (*27*). However, α_{s2}-casein fibril formation was only marginally inhibited by β-casein, yet we have shown previously that β-casein is equally as effective as α_s-casein at inhibiting fibril formation by κ-casein (*17*). The reasons for these differences are not entirely clear. However, it is interesting to note that β-casein is the most hydrophobic of the caseins, whereas α_{s2}-casein is the most hydrophilic (*23*). Thus, the reduced efficacy in β-casein's inhibition of α_{s2}-casein fibril formation may be largely accounted for by the relatively weak interaction between these two proteins. In any case, α_{s2}- and κ-casein fibril formation is likely to be initiated by partially exposed regions of the polypeptide that have a high propensity to adopt β-sheet structure, for example, via the sheet-turn-sheet predicted for κ-casein (*41*). Inhibition of fibril formation, therefore, presumably arises from the binding of other caseins, that is, α_{s1}- and β-casein, and shielding of the aforementioned surfaces, thereby prohibiting sheet−sheet interactions that would otherwise facilitate fibril assembly.

Cells and other biological compartments have adopted a number of strategies to combat the accumulation in tissue of amyloid fibrils. One such strategy employs the molecular machinery of chaperone proteins, which are believed to sequester other proteins that are prone to fibril formation. For instance, it has been suggested that the intracellular chaperones, Hsp70 and Hsp40, and the sHsp, αB-crystallin, shunt amyloidogenic proteins from the fibril forming pathway to a more favorable amorphous aggregation pathway (*44, 45*), allowing misfolded and aggregated protein to be more easily degraded and disposed of by quality control mechanisms. Our findings suggest that α_{s1}- and β-casein protect the mammary gland from localized amyloidosis in a similar fashion. It is evident that interactions between the caseins begin to take place early after synthesis in the endoplasmic reticulum (*46*). α_{s1}-Casein, in particular, appears to play a pivotal role during this period, overseeing the transport of other caseins from the endoplasmic reticulum to the Golgi apparatus and thereby preventing aberrant aggregation of susceptible caseins (*46*). Thus, casein−casein interactions, which are, indeed, vital for the formation and stability of the casein micelle, may also play a role in the prevention of mammary CA amyloidosis.

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