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Heath Ecroyd
University of Wollongong, heathe@uow.edu.au

David Thorn
University of Adelaide

Yanqin Liu
University of Adelaide

John Carver
University of Adelaide

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The dissociated form of kappa-casein is the precursor to its amyloid fibril formation

Abstract

Bovine milk kappa-casein forms a self-associating oligomeric micelle-like species, in equilibrium with dissociated forms. In its native form, intra- and inter-molecular disulfide bonds lead to the formation of multimeric species ranging from monomers to decamers. When incubated under conditions of physiological pH and temperature, both reduced and non-reduced kappa-casein form highly structured beta-sheet amyloid fibrils. We investigated whether the precursor to kappa-casein fibril formation is a dissociated state of the protein or its oligomeric micelle-like form. We show that reduced kappa-casein is capable of forming fibrils well below its critical micelle concentration, i.e. at concentrations where only dissociated forms of the protein are present. Moreover, by regulating the degree of disulfide linkages, we were able to investigate how oligomerization of kappa-casein influences its propensity for fibril formation under conditions of physiological pH and temperature. Thus, using fractions containing different proportions of multimeric species, we demonstrate that the propensity of the disulfide-linked multimers to form fibrils is inversely related to their size, with monomeric kappa-casein being the most aggregation prone. We conclude that dissociated forms of kappa-casein are the amyloidogenic precursors to fibril formation rather than oligomeric micelle-like species. The results highlight the role of oligomerization and natural binding, partners in preventing amyloid fibril formation by disease-related proteins in vivo.

Keywords

amyloid, precursor, form, fibril, dissociated, formation, its, casein, kappa

Disciplines

Life Sciences | Physical Sciences and Mathematics | Social and Behavioral Sciences

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The dissociated form of κ-casein is the precursor to its amyloid fibril formation

Heath ECROYD*†[1,2], David C. THORN†[1], Yanqin LIU† and John A. CARVER†

*School of Biological Sciences, University of Wollongong, Wollongong, NSW 2522 Australia, and †School of Chemistry and Physics, The University of Adelaide, Adelaide, SA 5005 Australia

Bovine milk κ-casein forms a self-associating oligomeric micelle-like species, in equilibrium with dissociated forms. In its native form, intra- and inter-molecular disulfide bonds lead to the formation of multimeric species ranging from monomers to decamers. When incubated under conditions of physiological pH and temperature, both reduced and non-reduced κ-casein form highly structured β-sheet amyloid fibrils. We investigated whether the precursor to κ-casein fibril formation is a dissociated state of the protein or its oligomeric micelle-like form. We show that reduced κ-casein is capable of forming fibrils well below its critical micelle concentration, i.e. at concentrations where only dissociated forms of the protein are present. Moreover, by regulating the degree of disulfide linkages, we were able to investigate how oligomerization of κ-casein influences its propensity for fibril formation under conditions of physiological pH and temperature. Thus, using fractions containing different proportions of multimeric species, we demonstrate that the propensity of the disulfide-linked multimers to form fibrils is inversely related to their size, with monomeric κ-casein being the most aggregation prone. We conclude that dissociated forms of κ-casein are the amyloidogenic precursors to fibril formation rather than oligomeric micelle-like species. The results highlight the role of oligomerization and natural binding partners in preventing amyloid fibril formation by disease-related proteins *in vivo*.

Key words: amyloid fibril, casein, dissociating agent, disulfide bond, micelle, protein aggregation.

INTRODUCTION

Recently, there has been an increase in the number of diseases classified as amyloidoses, i.e. diseases with pathological hallmarks that include intra- or extra-cellular deposits of amyloid fibrils and/or amyloid plaques in tissues [1,2]. Amyloid fibril-related diseases include Alzheimer's disease, Parkinson's disease, Type 2 diabetes and Huntington's disease, and their incidence will increase substantially in the future as the population ages. Although there is still some debate over whether amyloid fibril formation is causative of disease, it is certainly intimately linked to disease progression, and the formation of fibrils, from both disease-related and non-disease-related proteins, is toxic to cells [3]. Thus investigations into the mechanism by which fibrils are formed are critical in attempts to understand, and therefore treat, such diseases.

Amyloid fibrils are an aggregated state of proteins that form when partially folded intermediates mutually associate and enter the off-folding pathway [1,2,4]. Their association is mediated by β-strand motifs, and stacking of these β-strands is the basis of fibril nucleation and elongation [5]. Since the β-strand is a physiochemical property of the polypeptide backbone, rather than of the type of amino acids, it has been proposed that the amyloid fibril state is a generic conformation accessible to all polypeptide chains, given the appropriate conditions [1,6]. Typically, fibril formation is described by a nucleation-dependent mechanism in which the rate-limiting step is the formation of the stable nucleus (which corresponds to the lag phase) [5]. However, recently, we proposed that fibril formation by RCMκ-CN (reduced and carboxymethylated bovine κ-casein), a major protein in milk, occurs through an alternative mechanism in which the rate-limiting step is the dissociation of an amyloidogenic species from an oligomeric state [7]. This mechanism was based on the finding that the lag phase of RCMκ-CN's fibril formation is independent of protein concentration and the rate of fibril formation does not increase upon addition of seeds (pre-formed fibrils) [7]. Such a mechanism may apply more broadly, e.g. to disease-related fibril-forming proteins, where the rate-limiting step to fibril formation is the dissociation of a monomeric precursor from a binding partner or multisubunit complex (e.g. transthyretin in systemic amyloidosis) [8–10], or its formation through proteolytic cleavage (e.g. amyloid β-peptides in Alzheimer's disease) [5].

κ-CN (κ-casein) is a 169-amino-acid 19 kDa glycoprotein that is one of the major casein proteins in milk micelles: the others are α_{s1}-, α_{s2}- and β-casein. The caseins exhibit a strong tendency to associate, both with themselves and each other, through hydrophobic and electrostatic interactions, leading to the formation of casein micelles [11]. κ-CN plays a critical role in the stability and structure of these micelles, which, because of the incorporation of Ca^{2+} and other ions, have a vital nutritional role for the newborn. All of the caseins belong to the group of 'intrinsically disordered' or 'natively unfolded' proteins as they possess relatively little ordered structure under physiological conditions [12,13]. As a consequence, no casein proteins have been crystallized; however, three-dimensional energy-minimized models are available [14–17]. With regard to κ-CN, spectroscopic and molecular modelling studies indicate that the monomeric form of the protein adopts a 'horse and rider' conformation with persistent secondary-structural elements/motifs, the most significant being two sets of antiparallel β-sheets which are rich

Abbreviations used: ACN, acetonitrile; CMC, critical micelle concentration; DTT, 1,4-dithiothreitol; FTIR, Fourier transform infrared; HMW, high-molecular-mass; κ-CN, κ-casein; LMW, low-molecular-mass; MALLS, multi-angle laser light scattering; MMW, moderate-molecular-mass; RCMκ-CN, reduced and carboxymethylated κ-CN; SEC, size-exclusion chromatography; TEM, transmission electron microscopy; ThT, thioflavin T.

[1] These authors contributed equally to this work.

[2] To whom correspondence should be addressed (email heath_ecroyd@uow.edu.au).

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in hydrophobic side chains [15]. Our work has shown that this putative β-sheet region is incorporated into the core of fibrils formed by κ-CN and probably provides the basis for its high propensity for fibril formation [7].

We have also shown that native (non-reduced) bovine κ-CN forms fibrils under conditions of physiological pH and temperature [18] and have proposed that this process may account for the proteinaceous deposits found within corpora amylacea of mammary tissue which are amyloid in nature [19–21]. κ-CN contains two cysteine residues and, in the native form of the protein, intermolecular disulfide bonds lead to the formation of multimers (ranging from dimers to decamers) [22], which associate further, via electrostatic, hydrophobic and hydrogen-bonding interactions, to form micelle-like oligomers of $\sim$18 nm in diameter [23]. When completely reduced, κ-CN forms a self-associating oligomeric species whose quaternary structure is maintained by these electrostatic, hydrophobic and hydrogen-bonding interactions. This oligomeric species has been likened to a micelle in that the hydrophobic regions of κ-CN are largely buried within the core of a spherical oligomer and are shielded from solution by hydrophilic regions of the protein [24]. We therefore define any oligomeric form of κ-CN resulting from non-covalent interactions as micelle-like in order to distinguish these species from smaller oligomeric forms (e.g. dimers or trimers) that are maintained by disulfide bonds. These latter forms we refer to as dissociated forms.

Previous studies have suggested that, under reducing conditions, the micelle-like species is in equilibrium with a dissociated (monomeric) form [25]. As reduction of κ-CN enhances its ability to form fibrils, we [7,18] and others [26] have proposed that dissociated forms of the protein (i.e. monomers and disulfide-linked dimers or trimers) are the precursor to fibril formation. However, a recent study by Leonil et al. [27] using glycosylated and non-glycosylated forms of RCMκ-CN concluded that the micelle-like state of the protein is the amyloidogenic precursor. In the present paper, we describe experiments aimed at investigating this proposal further by examining the fibril-forming propensity of RCMκ-CN at concentrations below its CMC (critical micelle concentration), i.e. at concentrations at which only dissociated forms of the protein are present. In addition, we have conducted studies to isolate various oligomeric forms present in the native and partially reduced state of the protein in order to correlate their propensity for fibril formation with their oligomeric state. Together, these results show that oligomerization of κ-CN acts to suppress its fibril formation and highlight the role of oligomerization and of natural binding partners (i.e. α_S- and β-casein) in inhibiting this process *in vivo*.

MATERIALS AND METHODS

Materials

Bovine milk κ-CN (Swiss-Prot accession number P02668), without the N-terminal 21-amino-acid leader peptide, was purchased from Sigma Chemical Co. Protease inhibitors tablets (EDTA-free) were obtained from Roche, DTT (1,4-dithiothreitol) was obtained from Astral Scientific and uranyl acetate was purchased from Agar Scientific. All other reagents were obtained from Sigma and were of analytical grade, and all solutions were prepared with ultrapure milliQ water. The concentration of κ-CN was determined using a Cary 5000 UV–visible–near-IR spectrophotometer (Varian) and an absorption coefficient of 0.95 ml $\cdot$ mg^{-1} $\cdot$ cm^{-1} at 280 nm [28].

Determination of the CMC of κ-CN

The CMC of RCMκ-CN and κ-CN reduced with 20 mM DTT was determined using the Wilhelmy plate method, which is based on a change in the relationship between surface tension and concentration once the CMC has been reached. The surface tension of solutions of RCMκ-CN and κ-CN reduced with 20 mM DTT (in mN $\cdot$ m^{-1}) was recorded at room temperature (25 °C) and the results are representive of two independent experiments.

Preparation of RCMκ-CN and multimeric (partially reduced) RCMκ-CN

For some of the experiments, κ-CN was fully reduced and carboxymethylated as described previously [26,29]. Alternatively, κ-CN was partially reduced by initially isolating large oligomeric κ-CN species. Thus whole native κ-CN (100 mg) in 50 ml of 50 mM Tris/HCl and 8 M urea (pH 8.0) was concentrated to 5 ml over an ultrafiltration membrane with a 100 kDa molecular-mass cut-off membrane. To ensure thorough filtration, the sample was twice re-concentrated to 5 ml following the addition of two sequential 50 ml volumes of the same buffer. The final concentrate (i.e. κ-CN $>$100 kDa) was then subjected to the same reduction and carboxymethylation process as for native κ-CN, which was found to result in only partial reduction of the species present (see the Results section). We refer to this preparation as multimeric RCMκ-CN.

Fractionation of multimeric RCMκ-CN

Multimeric RCMκ-CN was separated into three major components by SEC (size-exclusion chromatography) using a Superdex-200 HR 10/30 column (GE Healthcare). Protein (1.5 mg) was injected in 100 μl volumes, and eluted with 50 mM ammonium acetate, 30 % (v/v) ACN (acetonitrile) (pH 7.2) at a flow rate of 0.5 ml/min. To minimize protein degradation, one tablet of EDTA-free protease inhibitor was added to 2 ml of sample before injection. Additionally, every precaution was taken to ensure the protein was kept at low temperature; the eluting buffer and sample loop were chilled in ice, and the eluted fractions were frozen with solid CO_2 immediately. The total eluate for each fraction ($\sim$30 ml) was later thawed and concentrated to 1–3 ml using an ultrafiltration membrane with molecular-mass cut-off of 30 kDa. After adding 30 ml of 50 mM ammonium bicarbonate, the samples were then concentrated to within 15 ml (to further remove the solvent and non-volatile salt from the samples) and then freeze-dried.

ThT (thioflavin T) binding

The formation of amyloid fibrils by multimeric RCMκ-CN and its components was monitored using a conventional ThT assay, as described previously [18,30]. Protein solutions were prepared at 3 mg/ml in 50 mM sodium phosphate buffer (pH 7.0) containing 2 mM EDTA. Fibril formation was induced by incubating protein solutions either with 40 mM DTT at 37 °C, or without DTT at 37 or 60 °C. Aliquots (3×10 μl) were periodically withdrawn from protein solutions during their incubation, 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

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and emission wavelengths set at 442 and 490 nm respectively, as described previously [18,30]. The results shown are averages of triplicate readings.

In situ ThT and light-scattering assays

The formation of amyloid fibrils by DTT-reduced κ-CN and RCMκ-CN was monitored using an *in situ* ThT binding or by a light-scattering assay as described previously [7,31]. Briefly, the protein was incubated at 37 °C in 50 mM sodium phosphate buffer (pH 7.2), with 20 mM DTT added to the solution in assays using native κ-CN. For the ThT assays, samples were incubated with 50 μM ThT at concentrations of 0.25–15 mg/ml. Owing to the decreased sensitivity of the light-scattering assay, data could only be collected for protein concentrations of 0.5–15 mg/ml. In each assay, the initial reaction rate was taken as the slope of the linear line-of-best-fit to either the ThT or light-scattering data over the first 180 min.

SDS/PAGE analysis

Gel electrophoresis was performed on 12 % (v/v) acrylamide gels using the method of Laemmli [32]. Samples were loaded without prior heating or reduction. Gels were stained with Coomassie Blue R-250. Densitometry was performed using ImageJ software (NIH) to estimate the proportion of each species in each sample.

Molecular mass measurements by SEC

The oligomer size distribution of κ-CN fractions under various conditions was assessed by SEC using a Superdex-200 HR 10/30 column. Proteins (0.15 mg) were injected in 100 μl volumes, and eluted at a flow rate of 0.5 ml/min. The column was calibrated with the following molecular-mass standards (Sigma): Blue Dextran (2000 kDa), thyroglobulin (669 kDa), apoferritin (443 kDa), β-amylase (206 kDa), ovotransferrin (76 kDa), ovalbumin (45 kDa), carbonic anhydrase (29 kDa), myoglobin (17 kDa) and dimeric insulin (12 kDa). Molecular-mass standards were prepared and eluted with 50 mM sodium phosphate buffer and 2 mM EDTA (pH 7.0).

MALLS (multi-angle laser light scattering)

The weighted-average molecular mass of oligomeric species eluting from the Superdex-200 HR 10/30 column was determined by MALLS. Multimeric RCMκ-CN at 10 mg/ml was separated by SEC at a flow rate of 0.25 ml/min, and the elution of the protein monitored by a miniDAWN™ TREOS triple-angle light scattering detector coupled with an Optilab rEx refractive index and UV light detector (Wyatt Technology). Data analyses were performed using Astra (Wyatt Technology).

TEM (transmission electron microscopy)

Samples for TEM were prepared by adding 2 μl of protein solution to formvar- and carbon-coated nickel grids (SPI Supplies). The grids were then washed three times with 10 μl of water and negatively stained with 10 μl of 2 % (w/v) uranyl acetate (Agar Scientific). 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 using a Philips CM100 transmission electron microscope.

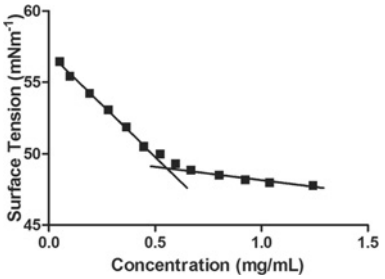


Figure 1 Determination of the CMC of RCMκ-CN

The CMC was determined as the intersection point of the line-of-best-fit for the initial portion of the curve where the surface tension shows a linear decline with increasing protein concentration and the baseline of minimal surface tension at the higher concentrations of protein, i.e. 0.54 ± 0.05 mg/ml.

RESULTS

Reduced κ-CN forms fibrils at concentrations below its CMC

The micelle-like behaviour of RCMκ-CN at high concentrations results in an equilibrium between oligomeric and dissociated forms of the protein [25]. To determine the effective CMC of RCMκ-CN, i.e. the concentration below which the protein does not form micelle-like species, we measured changes in surface tension with increasing concentrations of protein. We determined the CMC for RCMκ-CN (Figure 1) and DTT-reduced κ-CN (results not shown) to be 0.54 ± 0.05 and 0.46 ± 0.06 mg/ml respectively (i.e. 29 ± 2.6 and 24 ± 3.2 μM). This is in close agreement with the CMC reported previously for reduced κ-CN (0.53 mg/ml, i.e. 28 μM) [25]. Thus, at concentrations below $\sim$0.5 mg/ml (i.e. 26 μM), no micelle-like forms of RCMκ-CN or DTT-reduced κ-CN are present in solution.

We then investigated whether fibrils were formed at concentrations below the CMC. At concentrations of 0.1 and 0.2 mg/ml, we observed increases in ThT fluorescence indicative of fibril formation by RCMκ-CN and DTT-reduced κ-CN (see below). We examined these samples for the presence of fibrils by TEM. At 0.1 mg/ml, short unbranched fibrils were present, typical of those formed by RCMκ-CN that we have described previously [18,31] (Figure 2A). When RCMκ-CN was incubated at concentrations above its CMC (i.e. $>$0.5 mg/ml), the protein formed fibrils of the same overall morphology (Figure 2B). We found similar results when we examined samples of DTT-reduced κ-CN incubated at concentrations above and below its CMC, i.e. fibrils were formed with overall morphology similar to those described above.

The rate of κ-CN fibril formation is affected by its oligomeric state

We had reported previously that the initial rate of increase in ThT fluorescence associated with RCMκ-CN fibril formation was first-order with respect to protein concentration from 0.1 to 10 mg/ml (see Figure 1C of [7]). We performed further analysis of the kinetics of fibril formation by RCMκ-CN (Figure 3) and κ-CN reduced with DTT (results not shown) using higher protein concentrations (up to 15 mg/ml). The results show that, at concentrations below or close to the CMC (i.e. $<$0.5 mg/ml), the initial rate of increase (over the first 180 min) of ThT fluorescence (Figure 3A) and light scattering (Figure 3B) is linear with respect to RCMκ-CN concentration. However, as the protein concentration increases above the CMC, the initial rates approaches a maximum rate. These results suggest that, as the protein oligomerizes through non-covalent interactions to form

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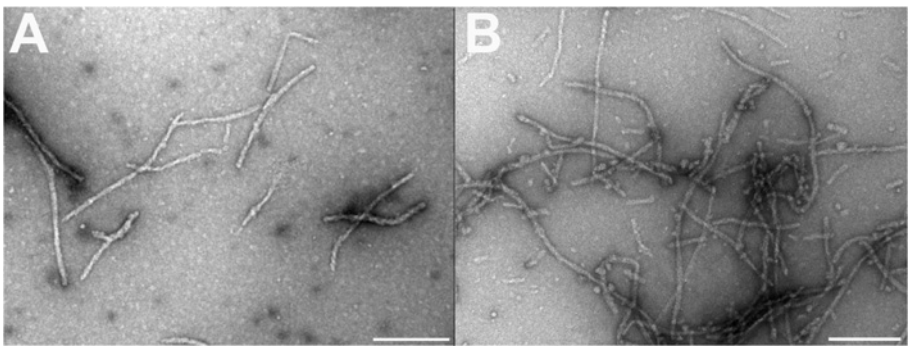


Figure 2 Transmission electron micrographs of fibrils formed by RCMκ-CN at concentrations below (A) and above (B) its CMC

RCMκ-CN was incubated at 0.1 mg/ml (**A**) and 1.0 mg/ml (**B**). Scale bars, 200 nm.

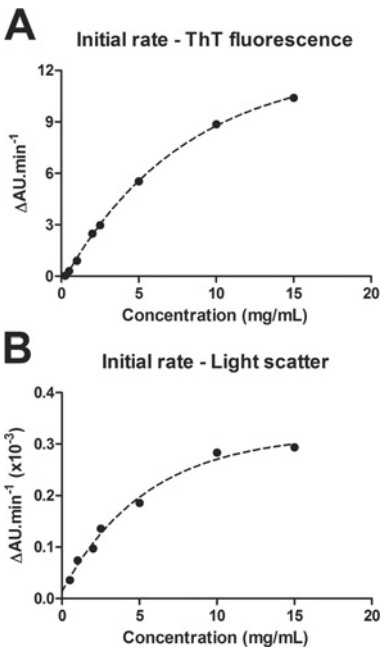


Figure 3 Rate of fibril formation of RCMκ-CN as monitored by ThT fluorescence (A) and light scattering at 340 nm (B)

For both (**A**) and (**B**), the initial reaction rate at each concentration was determined from the increase in the ThT fluorescence or light scattering data over the first 180 min of incubation. AU, absorbance units.

a micelle-like species, it is stabilized and its propensity to form fibrils decreases.

Isolating various multimeric fractions of partially reduced RCMκ-CN

A fraction of κ-CN that was more abundant in dissociated disulfide-linked species (e.g. dimer and trimer) was prepared by only partially reducing the protein, followed by carboxymethylation. First, peptide species that make up only a small proportion of the total sample were removed from native κ-CN via ultrafiltration. The resulting κ-CN sample (>100 kDa) was more extensively disulfide-linked than native κ-CN as judged by SDS/PAGE (results not shown), and subsequent reduction and carboxymethylation via standard methods yielded a mixture of disulfide-linked multimers with a unique size distribution, which we term 'multimeric' RCMκ-CN. Native κ-CN comprises a near-uniform distribution of multimers, with monomers to decamers each comprising approx. 10 % of the total protein [23]. Multimeric RCMκ-CN, however, comprised 23 % monomer, 23 % dimer and 13 % trimer, with the remainder encompassing a range of larger multimers (see below, Figures 5B and 5C).

From this multimeric RCMκ-CN sample, we endeavoured to isolate individual species and examine their propensity to form fibrils. A number of commonly used dissociating agents were investigated for their ability to disrupt the higher-order association inherent to native and multimeric RCMκ-CN (Figure 4). Native κ-CN eluted from the size-exclusion column as a broad peak centred at 7 ml, characteristic of the highly associated micelle-like state (Figure 4A). Addition of the dissociating agents SDS (2 %, w/v), urea (8 M) and ACN (33 %, v/v) all decreased the amount of micelle-like species and resulted in lower-molecular-mass species being produced. ACN was the most effective at disrupting the higher-order non-covalent oligomerization of native κ-CN, producing peaks with retention volumes of 10 and 15 ml. Multimeric RCMκ-CN eluted from the column as a broad peak centred at 8 ml and a smaller peak at 13 ml corresponding to its micelle-like and dissociated forms respectively (Figure 4B). Again, each of the dissociating agents disrupted the higher-order association of multimeric RCMκ-CN, with ACN (33 %, v/v) being the most effective. ACN caused the majority of the protein to elute in peaks with retention volumes between 10 and 15 ml and left very little of the protein remaining in the micelle-like state.

We therefore exploited the ability of ACN to disrupt the micelle-like nature of RCMκ-CN in order to separate the protein via SEC into various fractions of differing molecular mass (Figure 5A). Analysis of the peaks obtained from the column by non-reducing SDS/PAGE and quantitative densitometry of the resultant bands on the gel indicated that Fraction 1 (collected between 9.8 and 11.3 ml) consisted predominately of multimeric species with molecular masses of ~60 and ~80 kDa, as well as a large number of higher-molecular-mass species (i.e. >~200 kDa) (Figures 5B and 5C). On the basis of a mass of 19 kDa for the monomeric form of the protein, these species correspond to trimers, tetramers and larger multimers respectively. Fraction 2 (collected between 11.3 and 12.8 ml) consisted predominately of dimeric and trimeric species, whereas fraction 3 (collected between 12.8 and 14.3 ml) comprised mostly monomeric species. Fraction 3 therefore closely resembled the fully reduced form of the protein (Figures 5B and 5C). The minor band observed at 25 kDa in a number of κ-CN preparations most likely comprised

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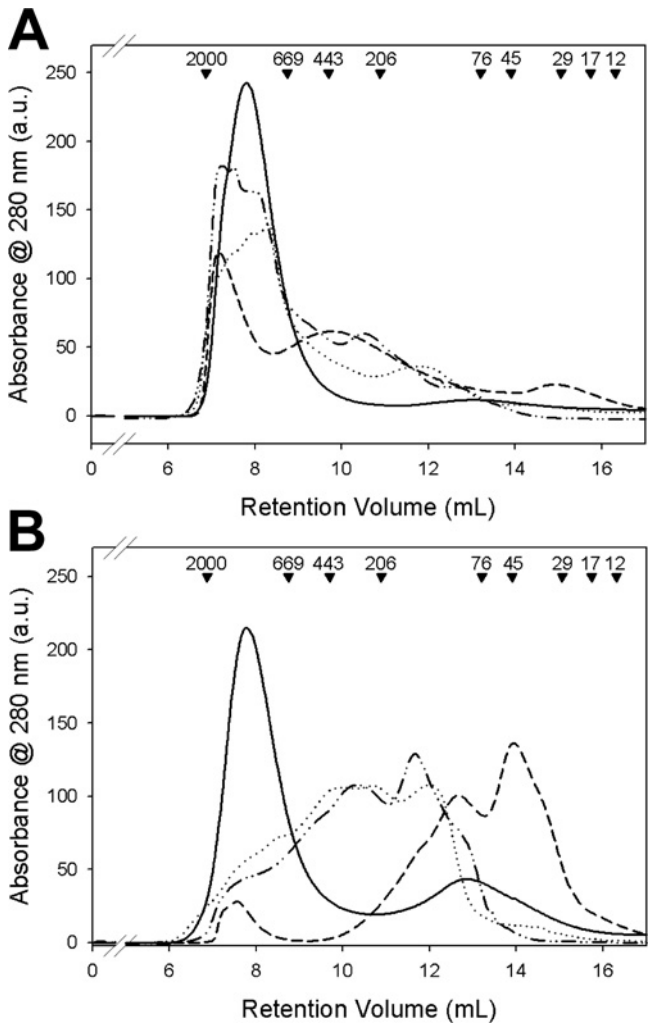


Figure 4 Dissociation of native κ-CN and multimeric RCMκ-CN

Elution profiles of native untreated κ-CN (**A**) and multimeric RCMκ-CN (**B**) separated by SEC. Proteins were eluted with either 50 mM sodium phosphate (pH 7.2) (——) or 50 mM ammonium acetate (pH 7.2) with one of three dissociating agents, i.e. 2 % (w/v) SDS (– · ·– · ·), 33 % (v/v) ACN (– –) or 8 M urea (· · · · ·), on a Superdex-200 HR 10/30 column at a flow rate of 0.5 ml/min. Molecular-mass calibrant standards (in kDa) are indicated by ▼. a.u., arbitrary units.

a mixture of the other caseins (i.e. α_{s1}-, α_{s2}- and β-casein) present in milk and are referred to as 'other'.

The oligomerization and fibril-forming propensity of multimeric RCMκ-CN fractions

Fractions 1–3, obtained under dissociating conditions (see above), were subsequently analysed by SEC under non-dissociating conditions in order to establish a relationship between their higher-order oligomerization (arising from non-covalent interactions) and their propensity for fibril formation (Figure 6). Each of the fractions eluted in various proportions in three discrete peaks centred at 7.0, 11.6 and 13.6 ml, and designated high- (HMW), moderate- (MMW) and low- (LMW) molecular-mass respectively. As expected, there was a strong correlation between the degree of intermolecular disulfide bonding and the overall degree of oligomerization. For instance, native κ-CN and fraction 1, which each comprised between 50 and 67 % higher-order multimers (i.e. >tetramer) (Figures 5B and 5C), assembled almost entirely into HMW oligomers (Figures 6A and 6B). Conversely, fully reduced RCMκ-CN and fraction 3, which each comprised approx. 60 % monomer (Figures 5B and 5C), remained essentially dissociated in the absence of ACN, with 15 % or less of the protein assembling into HMW species (Figures 6A and 6B). Interestingly,

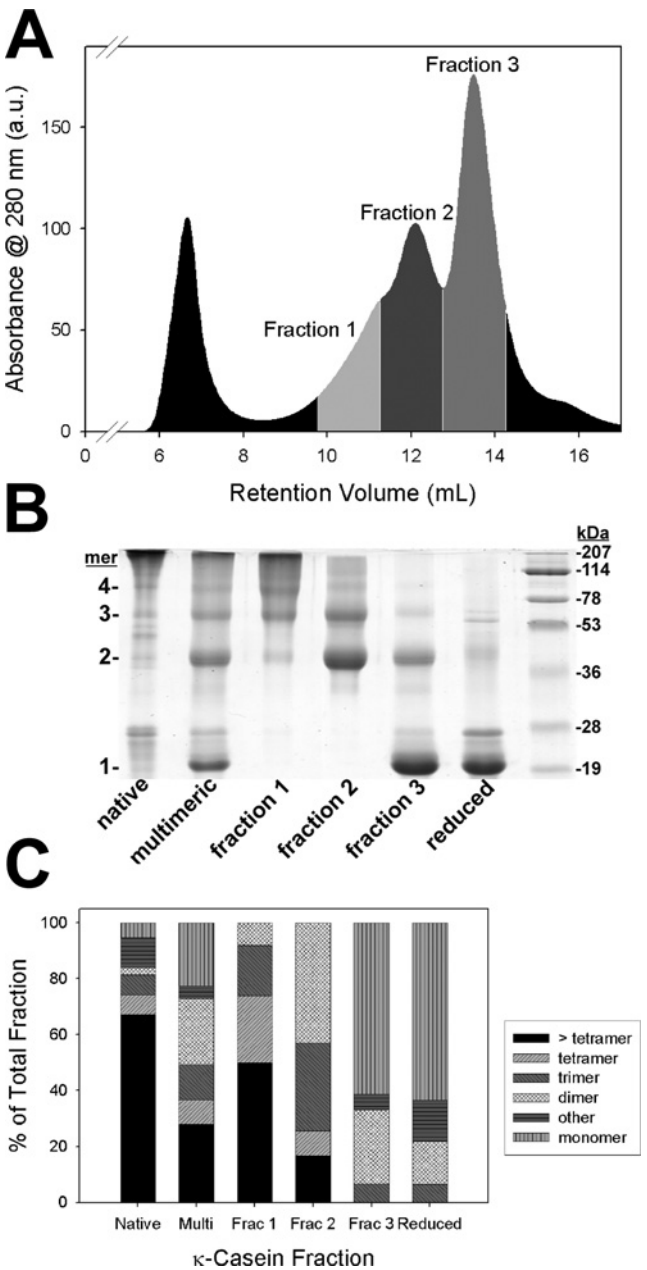


Figure 5 Fractionation of multimeric RCMκ-CN

(**A**) Elution profile of multimeric RCMκ-CN separated by SEC. Proteins were eluted with 50 mM ammonium acetate and 30 % (v/v) ACN (pH 7.2) on a Superdex-200 HR 10/30 column at a flow rate of 0.5 ml/min. (**B**) SDS/PAGE analysis under non-reducing conditions of, from left to right, native untreated κ-CN, multimeric RCMκ-CN, fractions 1, 2 and 3, obtained from separation of multimeric RCMκ-CN by SEC, and fully reduced RCMκ-CN. Molecular-mass markers are shown on the right-hand side of the gel and the position of bands in the gel assigned as monomer, dimer, trimer and tetramer are given on the left-hand side of the gel. (**C**) Percentage of disulfide-linked multimers in fractions of native and RCMκ-CN obtained by densitometric analysis of Coomassie Blue-stained protein separated by SDS/PAGE. 'Other' represents the proportion of protein that could not be ascribed to either monomer or dimer, corresponding to a molecular mass of ~25 kDa. a.u., arbitrary units.

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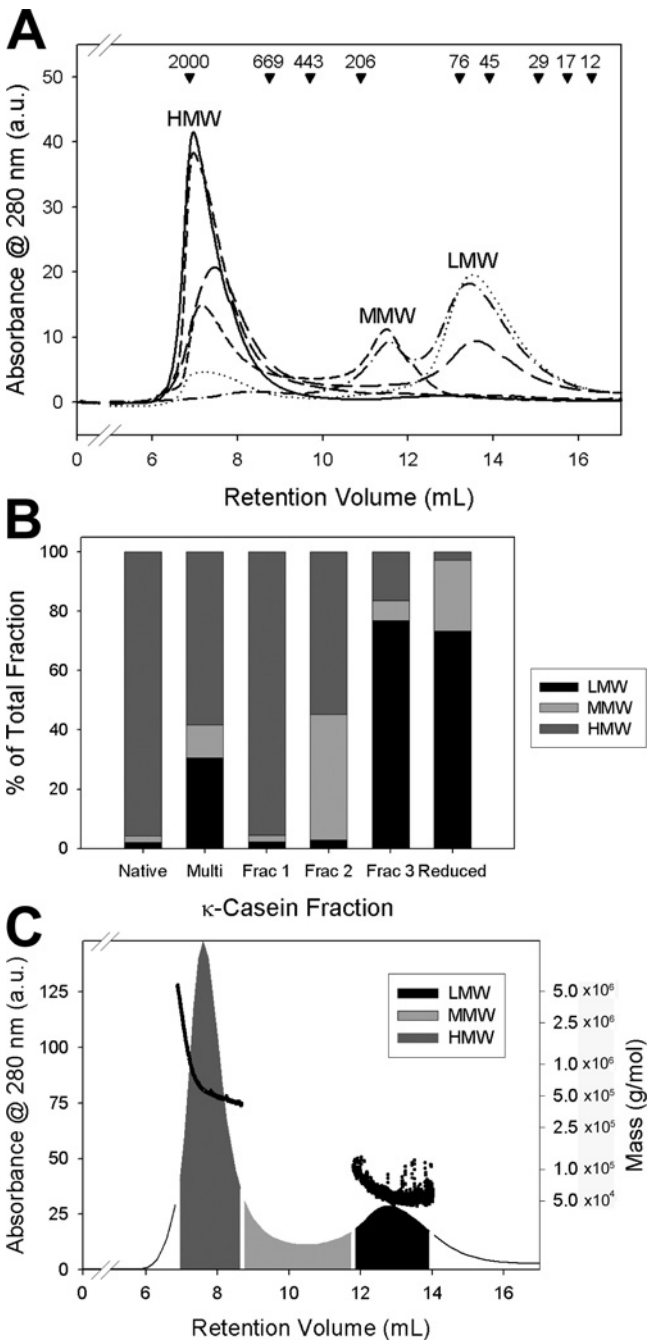


Figure 6 Higher-order oligomerization of RCMκ-CN fractions

(**A**) Elution profiles of native, untreated κ-CN (——), multimeric RCMκ-CN (— —), fractions 1 (– – –), 2 (- - - -), and 3 (· · · · · ·) of multimeric RCMκ-CN obtained by SEC, and fully reduced RCMκ-CN (– · – · –). Proteins (1.5 mg/ml) were eluted with 50 mM sodium phosphate (pH 7.2) on a Superdex-200 HR 10/30 column at a flow rate of 0.5 ml/min. Molecular-mass calibrant standards (in kDa) are indicated by ▼. (**B**) Percentage of LMW, MMW and HMW species in fractions of native κ-CN and RCMκ-CN based on SEC elution profiles in (**A**). (**C**) Weighted-average molecular mass of LMW and HMW species upon SEC elution of multimeric RCMκ-CN, as assessed by MALLS. a.u., arbitrary units.

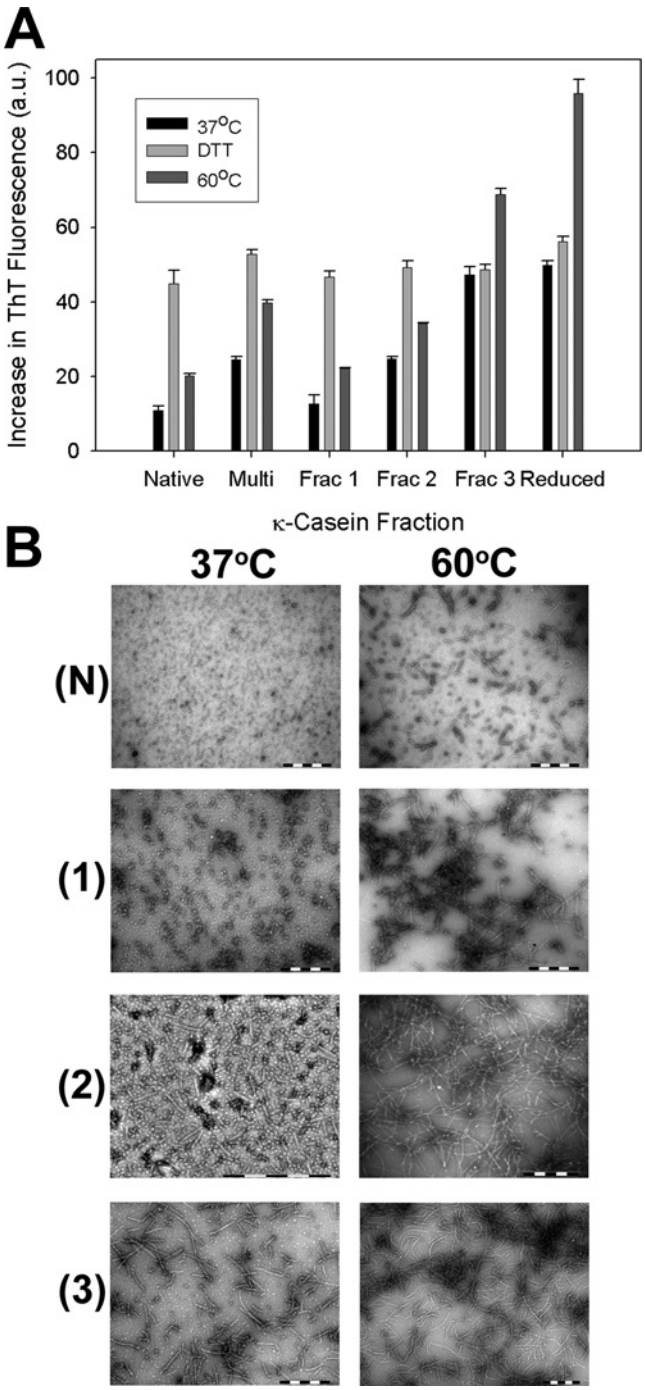


Figure 7 Fibril-forming propensity of native κ-CN and RCMκ-CN fractions

(**A**) ThT fluorescence analysis of native κ-CN, multimeric RCMκ-CN, fractions (1–3) of multimeric RCMκ-CN obtained from SEC and fully reduced RCMκ-CN, incubated for 2 days at 37 °C (black) and 60 °C (dark grey) under non-reducing conditions, and at 37 °C in the presence of 40 mM DTT (light grey). Results shown are means ± S.E.M. obtained from triplicate measurements and are representative of three experiments. a.u., arbitrary units. (**B**) Electron micrographs depict native (N) κ-CN, and fractions 1, 2 and 3 of multimeric RCMκ-CN obtained by SEC, following 2 days of incubation under non-reducing conditions at 37 °C and 60 °C. Scale bars, 500 nm.

fraction 2, characterized by an abundance of dimeric and trimeric disulfide-linked species (Figures 5B and 5C), formed instead a significant population (~40 %) of an intermediate (MMW) oligomeric species (Figures 6A and 6B). The remainder of fraction 2 formed mostly HMW species, with very little LMW species. MALLS measurements of multimeric RCMκ-CN showed that these HMW species varied greatly in terms of their molecular mass (Figure 6C), which, despite an average mass of 760 kDa (i.e. a 40-mer based on a monomeric mass of 19 kDa), ranged from 450 to 6000 kDa. The dissociated LMW species, however, were much less polydisperse with a molecular

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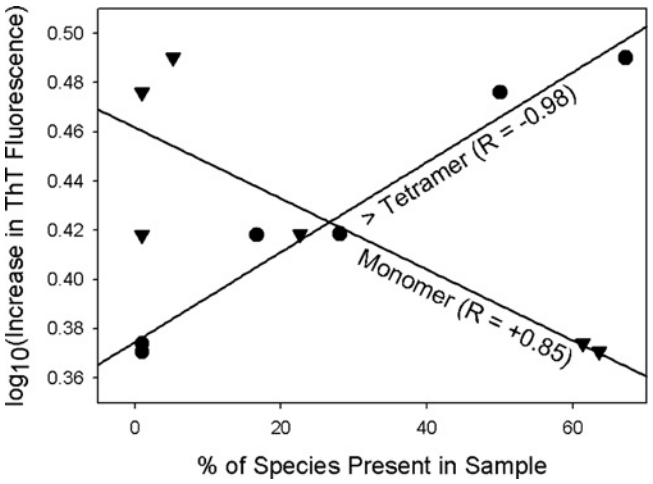


Figure 8 Correlation of the proportion of monomer and higher-order multimers present in a κ-CN sample with the sample's fibril-forming propensity

The percentage of monomeric species (▼) or species larger than tetramer (●), in each sample, was obtained by densitometric analysis of bands present in a SDS/PAGE gel (as shown in Figures 5B and 5C). This is plotted against the $\log_{10}$ of the increase in ThT fluorescence obtained from incubation of the sample at 37 °C without DTT (as shown in Figure 7A).

mass range of 45–130 kDa and an average mass of 62 kDa (i.e. a trimer).

The ability of these fractions to form fibrils was examined by ThT fluorescence under non-reducing conditions at 37 and 60 °C, and under reducing conditions at 37 °C (Figure 7A). As the extent of oligomerization increased (in terms of both the number of subunits in the disulfide-linked multimers and their subsequent propensity to form higher-order species via non-covalent interactions), the extent of fibril formation decreased. For example, fraction 1, an ensemble of predominately trimeric, tetrameric and larger disulfide-linked multimers that associated into HMW species (Figures 5 and 6), had the lowest propensity for fibril formation (Figure 7A). In contrast, fraction 3, which comprised a high degree of monomeric species that most closely resembled the fully reduced state of the protein, showed the biggest increase in ThT fluorescence indicative of fibril formation. Moreover, this increase in ThT fluorescence was not enhanced by reduction and mirrored that of the fully reduced protein. Upon addition of DTT, however, all of the fractions were found to have a similar increase in ThT fluorescence, indicating that the differences between the fractions are mediated by their disulfide-bonding patterns, which, in turn, governs their higher-order association.

These findings were verified by TEM (Figure 7B), which showed a progressive increase from fractions 1 to 3 in the length and apparent number of fibrils formed at 37 °C. Again, the differences between fractions were much less apparent upon addition of DTT (results not shown) or upon raising the incubation temperature to 60 °C. Thus each of the fractions, including native κ-CN, has the capacity to form fibrils, but their propensity to do so is restrained by their propensity for non-fibrillar oligomerization. To illustrate this further, the extent of fibril formation by each fraction at 37 °C in the absence of DTT, as indicated by their ThT fluorescence increase, was plotted against the percentage of each of their disulfide-linked components (monomer to higher-order multimer).

As shown in Figure 8 and Table 1, there is a strong relationship (R=0.85) between the percentage of monomer present in the

Table 1 Correlation between κ-CN species present in a sample with its fibril-forming propensity

Multimer indicates a species greater than a tetramer. The percentage of species was obtained by densitometric analysis of SDS/PAGE bands as shown in Figures 5(B) and 5(C). Correlation coefficients were produced by plotting the percentage of species in each fraction against the $\log_{10}$ of the increase in ThT fluorescence at 37 °C without DTT shown in Figure 7(A).

Species	*R* value	Mixture	*R* value
Multimer	−0.98		
Tetramer	−0.13	Tetramer and less	0.93
Trimer	−0.02	Trimer and less	0.97
Dimer	0.55	Dimer and less	0.98
Monomer	0.85		

sample (obtained from the values measured in Figures 5B and 5C) and its fibril-forming propensity (obtained from the increase in ThT fluorescence shown in Figure 7A). Moreover, there is also a very strong negative correlation (Figure 8, R=−0.98) between the proportion of higher-order multimers (i.e. species more than a tetramer) present in the sample (Figures 5B and 5C) and its propensity for fibril formation (Figure 7). The correlation between the percentage of dimer in the samples and fibril formation was weaker (R = 0.55). However, when the amount of monomer, dimer and trimer in the samples was correlated with the propensity of the sample to form fibrils, there was a very strong relationship (dimer and less, R=0.98; trimer and less, R=0.97) Thus the monomeric form of κ-CN appears to be the most amyloidogenic species, although the dimer and trimer, the predominant species in fraction 2 (Figure 5B), clearly have some propensity for fibril formation (Figures 7A and 7B).

DISCUSSION

Bovine milk κ-CN has an inherent propensity to form amyloid fibrils when incubated under conditions of physiological pH and temperature *in vitro*, the rate of which is enhanced by reduction of its disulfide bonds [18,26]. Reduction does not lead to any significant changes in the secondary or tertiary structure of κ-CN [26] and therefore the increased propensity of the reduced protein to form fibrils is largely mediated through changes to its quaternary structure. The ability of κ-CN to form oligomers, through covalent disulfide bonds and non-covalent interactions, leads to a heterogeneous population of species in solution, which is complicated further by the strong dependence of the association state of the protein on factors such as concentration, pH, temperature, method of purification and extent of reduction [26]. Thus, in the present study, we have differentiated between oligomeric micelle-like states (formed via non-covalent interactions) and dissociated states (consisting of monomers, dimers, trimers, etc., whereby the oligomers arise from disulfide bonds). We have shown that RCMκ-CN and DTT-reduced κ-CN are capable of forming fibrils at concentrations below their CMC, i.e. at concentrations at which only dissociated forms of the protein are present. At concentrations below or close to the CMC (i.e. <0.5 mg/ml) the initial reaction rate is linear and first-order with respect to protein concentration [7]. Moreover, we show that the propensity of κ-CN to forms fibrils is inversely correlated with its initial oligomerization state. Together, these data provide further support for our proposed model of fibril formation by κ-CN in which dissociated forms are the amyloidogenic precursor to fibril formation [7].

Whereas our current and previous data [7] indicate that dissociated forms of κ-CN are the precursor to fibril formation,

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it was proposed recently that a conformational change to the micelle-like state of RCMκ-CN is prerequisite to it forming fibrils and that the micelle-like state is therefore the amyloidogenic precursor [27]. This proposal was based on the following observations: (i) RCMκ-CN adopts a micelle-like form before fibril formation, as observed by TEM; (ii) the diameter of the fibrils is the same as the diameter of the oligomeric micelle-like species (i.e. 10–12 nm); (iii) there is a change in the intermolecular β-strands of the protein in the fibrillar state compared with the native micelle-like state as determined by FTIR (Fourier-transform IR) spectroscopy; and (iv) the rate constant of fibril formation reaches a plateau at high protein concentrations. Our results show clearly that RCMκ-CN and DTT-reduced κ-CN are both capable of forming fibrils at concentrations at which micelle-like forms of the protein are not present. Furthermore, we show that oligomerization stabilizes and prevents fibril formation by κ-CN. In its native state, above its CMC, reduced κ-CN exists in equilibrium between micelle-like species and dissociated forms [25,33]. Whereas the diameter of these oligomers is similar to that of the fibrils formed by the protein (i.e. 10–15 nm), it is well-established that the diameter of fibrils in general is in this range ($\sim$10 nm) [34], and, as such, this is likely a coincidence rather than evidence for the micelle-like state being the amyloidogenic precursor.

Our previous findings indicated that, upon fibril assembly, κ-CN undergoes structural rearrangement within the protein, particularly to the region that we have shown forms the protease-resistant β-sheet core of the fibrils (i.e. Tyr^{25}–Lys^{86}) [7]. It is unsurprising that fibril assembly involves alterations in the intermolecular β-sheet arrangement between protein monomers, since, in the native state, these intermolecular interactions facilitate micelle formation, whereas in the fibril, they are responsible for β-sheet stacking along the long axis of the fibril. Thus we propose that this β-sheet rearrangement accounts for the structural changes between RCMκ-CN before and after fibril formation that were observed previously by us [7] and by Leonil et al. [27]. Moreover, the FTIR data do not necessarily indicate that conformational rearrangement occurs within the micelle-like species itself to enable fibril formation, rather, that the intermolecular β-sheet arrangement in micellar native RCMκ-CN differs from that present in the fibrillar form of the protein.

When the initial rate of ThT fluorescence (Figure 3A) and light scattering (Figure 3B) is plotted against RCMκ-CN concentration, our data show that it follows a relationship whereby, at concentrations below or close to the CMC, it is linear and first-order with respect to RCMκ-CN concentration [7,27], but at high concentrations, the rate reaches a plateau (i.e. the reaction is zero-order with respect to RCMκ-CN concentration). The first-order relationship at concentrations below or close to the CMC support our model in which dissociated forms of RCMκ-CN are responsible for fibril formation [7,27], as addition of protein would lead to a direct increase in the amount of amyloidogenic precursor available for fibril formation, thus increasing the reaction rate proportionally. The reaction rate reaching a plateau at concentrations well above the CMC can be rationalized by considering the effect of concentration on the association state of RCMκ-CN in solution. Above the CMC, the amount of dissociated species in solution increases proportionally until reaching a point at which the stability of the micelle-like state is maximal and the concentration of the dissociated species becomes independent of the total protein concentration [25,33]. Thus, under our proposed model [7,27], at high RCMκ-CN concentrations, the rate of fibril formation would become zero-order with respect to the total protein concentration, as was found to be the case in the present study. In contrast, if the amyloidogenic precursor were the micelle-like state of RCMκ-CN [27], then the reaction rate would be first-order with respect to protein concentration at all concentrations above the CMC.

Our previously proposed model predicts that factors which enhance the amount or rate of dissociation of κ-CN from its micelle-like state will increase the rate of fibril formation [7]. Reduction and heat are two such factors which most likely enhance the rate of fibril formation through such mechanisms and are consistent with ours and other studies [7,18,26]. Leonil et al. [27] also indicated that glycosylation decreases the rate of fibril formation by inhibiting dissociation. Therefore the increased rate of fibril formation by non-glycosylated RCMκ-CN (which has an increased rate of dissociation [27]) is accounted for in our model [7]. In the casein micelle, the amount of the dissociated amyloidogenic species is very low because of the interaction of κ-CN with the other casein proteins (i.e. α_S- and β-casein), both of which are effective inhibitors of κ-CN fibril formation in a chaperone-like manner [18,35].

The present study highlights the role of the intermolecular disulfide bonds to stabilize further the oligomeric micellar form of κ-CN and thus protect the protein against fibril formation. We have shown previously that native κ-CN, although extensively disulfide-linked, forms fibrils when subjected to temperatures of 37 °C or higher [18]. Our present findings indicate that the observed fibrils originate from dissociated monomeric, dimeric and trimeric species. This is significant in the light of our recent findings that α_{S2}-casein, a milk protein closely related to κ-CN, readily assembles into amyloid fibrils from a disulfide-linked dimer ([30] and D.C. Thorn, T. Kouldelka, H. Ecroyd, M. Sunde and J.A. Carver, unpublished work). Proteins that are natively associated are generally thought to revert to a monomeric intermediate state before fibril formation proceeds. This dissociation event is invariably induced by altering the solution or environmental conditions, and generally accompanies a variety of additional changes in the secondary and tertiary structure of the protein. For example, fibril formation by insulin or transthyretin is readily induced at 37 °C by acidic pH, causing denaturation and concomitant monomer formation [8,36]. Disulfide reduction of native oligomeric κ-CN, however, appears not to accompany significant changes in secondary or tertiary structure [26]. Thus, by regulating the chain of disulfide linkages in κ-CN, we have unambiguously shown how the amyloid-fibril-forming pathway may be progressively overcome by alternative aggregation pathways, e.g. native oligomerization.

We suggest that such a process of inhibition is far more significant than presently recognized, playing a broader role in the prevention of fibril formation by disease-related proteins. In particular, models of fibril formation in which the rate-limiting step is the dissociation of an amyloidogenic precursor [7], rather than formation of stable nuclei, may be more applicable to disease-related proteins such as transthyretin (dissociation from a multisubunit complex) or the amyloid β-peptides (formed by proteolytic cleavage). With regard to transthyretin, it is now well-established that the dissociation of monomer from its tetrameric state is the rate-limiting step in its fibril formation [8,37,38]. Accordingly, small molecules that stabilize the multimeric state of transthyretin (e.g. benzoxazoles) are effective inhibitors of fibril formation under both physiological and denaturing conditions [39], and are in current development as therapeutic agents for the treatment of familial amyloid polyneuropathy and other diseases associated with transthyretin fibril formation [40]. These small-molecule inhibitors bind to unoccupied thyroxine-binding sites and thus appear to mimic protein stabilization normally mediated by its natural ligand [41]. Similarly, κ-CN is stabilized and fibril formation is prevented by other casein proteins, its natural

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binding partners [18]. The crucial difference, however, between transthyretin and κ-CN is that transthyretin is a well-structured protein and fibril formation follows a pathway of partial unfolding and consequential dissociation, whereas κ-CN is unstructured and the amyloidogenic form of the protein is structurally analogous to its native state, albeit dissociated (i.e. fibril formation does not require partial unfolding). Whether small molecules could stabilize the micelle-like state of κ-CN and thereby limit fibril formation by this protein is therefore unclear.

In conclusion, our results indicate that dissociated forms of κ-CN are the amyloidogenic precursors to its fibril formation. The monomeric form has the highest propensity for fibril formation, although disulfide-linked dimeric and trimeric forms also retain some fibril-forming propensity. Moreover, higher-order oligomerization of κ-CN inhibits fibril formation. Thus interactions involving κ-CN, either with itself or with other casein proteins, are not only essential for the formation of the casein micelle (and therefore the nutritional qualities of milk), but are also important in preventing the accumulation of amyloid deposits in mammary tissue which would otherwise be favoured by the reducing environment of the epithelial cells that are responsible for casein synthesis and secretion. Although the vast majority of research to date has been aimed at developing strategies to reduce oligomerization/aggregation in general by disease-associated proteins, the present study also highlights the potential for the design of therapeutics that stabilize multimeric amyloidogenic proteins as a means of preventing fibril formation, as has been suggested for transthyretin [40,42].

AUTHOR CONTRIBUTION

Heath Ecroyd performed experiments, collated results and wrote the paper. David Thorn performed experiments, collated results and assisted with writing the paper. Yanqin Liu prepared the protein and performed experiments. John Carver assisted in the development of this work and data interpretation and edited the paper before submission.

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REFERENCES

1 Chiti, F. and Dobson, C. M. (2006) Protein misfolding, functional amyloid, and human disease. Annu. Rev. Biochem. **75**, 333–366
2 Dobson, C. M. (2004) Experimental investigation of protein folding and misfolding. Methods **34**, 4–14
3 Bucciantini, M., Giannoni, E., Chiti, F., Baroni, F., Formigli, L., Zurdo, J., Taddei, N., Ramponi, G., Dobson, C. M. and Stefani, M. (2002) Inherent toxicity of aggregates implies a common mechanism for protein misfolding diseases. Nature **416**, 507–511
4 Ecroyd, H. and Carver, J. A. (2008) Unraveling the mysteries of protein folding and misfolding. IUBMB Life **60**, 769–774
5 Harper, J. D. and Lansbury, Jr, P. T. (1997) Models of amyloid seeding in Alzheimer's disease and scrapie: mechanistic truths and physiological consequences of the time-dependent solubility of amyloid proteins. Annu. Rev. Biochem. **66**, 385–407
6 Dobson, C. M. (1999) Protein misfolding, evolution and disease. Trends Biochem. Sci. **24**, 329–332
7 Ecroyd, H., Koudelka, T., Thorn, D. C., Williams, D. M., Devlin, G., Hoffmann, P. and Carver, J. A. (2008) Dissociation from the oligomeric state is the rate-limiting step in amyloid fibril formation by κ-casein. J. Biol. Chem. **283**, 9012–9022
8 Lai, Z., Colon, W. and Kelly, J. W. (1996) The acid-mediated denaturation pathway of transthyretin yields a conformational intermediate that can self-assemble into amyloid. Biochemistry **35**, 6470–6482
9 Colon, W. and Kelly, J. W. (1992) Partial denaturation of transthyretin is sufficient for amyloid fibril formation *in vitro*. Biochemistry **31**, 8654–8660
10 Quintas, A., Vaz, D. C., Cardoso, I., Saraiva, M. J. and Brito, R. M. (2001) Tetramer dissociation and monomer partial unfolding precedes protofibril formation in amyloidogenic transthyretin variants. J. Biol. Chem. **276**, 27207–27213
11 Swaisgood, H. E. (1992) Chemistry of the caseins. In Advanced Dairy Chemistry. 1: Proteins (Fox, P. F., ed.), pp. 63–110, Elsevier, London
12 Uversky, V. N. (2002) What does it mean to be natively unfolded? Eur. J. Biochem. **269**, 2–12
13 Syme, C. D., Blanch, E. W., Holt, C., Jakes, R., Goedert, M., Hecht, L. and Barron, L. D. (2002) A Raman optical activity study of rheomorphism in caseins, synucleins and tau: new insight into the structure and behaviour of natively unfolded proteins. Eur. J. Biochem. **269**, 148–156
14 Kumosinski, T. F., Brown, E. M. and Farrell, Jr, H. M. (1991) Three-dimensional molecular modeling of bovine caseins: α_{s1}-casein. J. Dairy Sci. **74**, 2889–2895
15 Kumosinski, T. F., Brown, E. M. and Farrell, Jr, H. M. (1993) Three-dimensional molecular modeling of bovine caseins: a refined, energy-minimized κ-casein structure. J. Dairy Sci. **76**, 2507–2520
16 Kumosinski, T. F., Brown, E. M. and Farrell, Jr, H. M. (1993) Three-dimensional molecular modeling of bovine caseins: an energy-minimized β-casein structure. J. Dairy Sci. **76**, 931–945
17 Farrell, Jr, H. M., Malin, E. L., Brown, E. M. and Mora-Gutierrez, A. (2009) Review of the chemistry of α_{S2}-casein and the generation of a homologous molecular model to explain its properties. J. Dairy Sci. **92**, 1338–1353
18 Thorn, D. C., Meehan, S., Sunde, M., Rekas, A., Gras, S. L., MacPhee, C. E., Dobson, C. M., Wilson, M. R. and Carver, J. A. (2005) Amyloid fibril formation by bovine milk κ-casein and its inhibition by the molecular chaperones α_S- and β-casein. Biochemistry **44**, 17027–17036
19 Beems, R. B., Gruys, E. and Spit, B. J. (1978) Amyloid in the corpora amylacea of the rat mammary gland. Vet. Pathol. **15**, 347–352
20 Reid, I. M. (1972) Corpora amylacea of the bovine mammary gland: histochemical and electron microscopic evidence for their amyloid nature. J. Comp. Pathol. **82**, 409–413
21 Taniyama, H., Kitamura, A., Kagawa, Y., Hirayama, K., Yoshino, T. and Kamiya, S. (2000) Localized amyloidosis in canine mammary tumors. Vet. Pathol. **37**, 104–107
22 Rasmussen, L. K., Hojrup, P. and Petersen, T. E. (1992) The multimeric structure and disulfide-bonding pattern of bovine κ-casein. Eur. J. Biochem. **207**, 215–222
23 Farrell, Jr, H. M., Kumosinski, T. F., Cooke, P. H., King, G., Hoagland, P. D., Wickham, E. D., Dower, H. J. and Groves, M. L. (1996) Particle sizes of purified κ-casein: metal effect and correspondence with predicted three-dimensional molecular models. J. Protein Chem. **15**, 435–445
24 Hill, R. J. and Wake, R. G. (1969) Amphiphile nature of κ-casein as the basis for its micelle stabilizing property. Nature **221**, 635–639
25 Vreeman, H. J., Brinkhuis, J. A. and van der Spek, C. A. (1981) Some association properties of bovine SH-κ-casein. Biophys. Chem. **14**, 185–193
26 Farrell, Jr, H. M., Cooke, P. H., Wickham, E. D., Piotrowski, E. G. and Hoagland, P. D. (2003) Environmental influences on bovine κ-casein: reduction and conversion to fibrillar (amyloid) structures. J. Protein Chem. **22**, 259–273
27 Leonil, J., Henry, G., Jouanneau, D., Delage, M. M., Forge, V. and Putaux, J. L. (2008) Kinetics of fibril formation of bovine κ-casein indicate a conformational rearrangement as a critical step in the process. J. Mol. Biol. **381**, 1267–1280
28 Fox, P. F. and McSweeney, P. L. H. (1998) Dairy Chemistry and Biochemistry, Blackie Academic and Professional, London
29 Schechter, Y., Patchornik, A. and Burstein, Y. (1973) Selective reduction of cystine 1–8 in α-lactalbumin. Biochemistry **12**, 3407–3413
30 Thorn, D. C., Ecroyd, H., Sunde, M., Poon, S. and Carver, J. A. (2008) Amyloid fibril formation by bovine milk α_{s2}-casein occurs under physiological conditions yet is prevented by its natural counterpart, α_{s1}-casein. Biochemistry **47**, 3926–3936
31 Ecroyd, H., Meehan, S., Horwitz, J., Aquilina, J. A., Benesch, J. L., Robinson, C. V., MacPhee, C. E. and Carver, J. A. (2007) Mimicking phosphorylation of αB-crystallin affects its chaperone activity. Biochem. J. **401**, 129–141
32 Laemmli, U. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227**, 680–685
33 Vreeman, H. J., Both, P., Brinkhuis, J. A. and van der Spek, C. (1977) Purification and some physicochemical properties of bovine κ-casein. Biochim. Biophys. Acta **491**, 93–103
34 Sunde, M. and Blake, C. (1997) The structure of amyloid fibrils by electron microscopy and X-ray diffraction. Adv. Protein Chem. **50**, 123–159
35 Thorn, D. C., Ecroyd, H. and Carver, J. A. (2009) The two-faced nature of milk casein proteins: amyloid fibril formation and chaperone-like activity. Aust. J. Dairy Technol. **64**, 36–40
36 Nielsen, L., Khurana, R., Coats, A., Frokjaer, S., Brange, J., Vyas, S., Uversky, V. N. and Fink, A. L. (2001) Effect of environmental factors on the kinetics of insulin fibril formation: elucidation of the molecular mechanism. Biochemistry **40**, 6036–6046

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37 Jiang, X., Smith, C. S., Petrassi, H. M., Hammarstrom, P., White, J. T., Sacchettini, J. C. and Kelly, J. W. (2001) An engineered transthyretin monomer that is nonamyloidogenic, unless it is partially denatured. Biochemistry **40**, 11442–11452
38 Quintas, A., Saraiva, M. J. and Brito, R. M. (1999) The tetrameric protein transthyretin dissociates to a non-native monomer in solution: a novel model for amyloidogenesis. J. Biol. Chem. **274**, 32943–32949
39 Razavi, H., Palaninathan, S. K., Powers, E. T., Wiseman, R. L., Purkey, H. E., Mohamedmohaideen, N. N., Deechongkit, S., Chiang, K. P., Dendle, M. T., Sacchettini, J. C. and Kelly, J. W. (2003) Benzoxazoles as transthyretin amyloid fibril inhibitors: synthesis, evaluation, and mechanism of action. Angew. Chem. Int. Ed. Engl. **42**, 2758–2761
40 Connelly, S., Choi, S., Johnson, S. M., Kelly, J. W. and Wilson, I. A. (2010) Structure-based design of kinetic stabilizers that ameliorate the transthyretin amyloidoses. Curr. Opin. Struct. Biol. **20**, 54–62
41 Miroy, G. J., Lai, Z., Lashuel, H. A., Peterson, S. A., Strang, C. and Kelly, J. W. (1996) Inhibiting transthyretin amyloid fibril formation via protein stabilization. Proc. Natl. Acad. Sci. U.S.A. **93**, 15051–15056
42 Johnson, S. M., Wiseman, R. L., Sekijima, Y., Green, N. S., Adamski-Werner, S. L. and Kelly, J. W. (2005) Native state kinetic stabilization as a strategy to ameliorate protein misfolding diseases: a focus on the transthyretin amyloidoses. Acc. Chem. Res. **38**, 911–921

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