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Methionine Oxidation Enhances κ-Casein Amyloid Fibril Formation

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Supporting Information

ABSTRACT: The effects of protein oxidation, for example of methionine residues, are linked to many diseases, including those of protein misfolding, such as Alzheimer's disease. Protein misfolding diseases are characterized by the accumulation of insoluble proteinaceous aggregates comprised mainly of amyloid fibrils. Amyloid-containing bodies known as corpora amylacea (CA) are also found in mammary secretory tissue, where their presence slows milk flow. The major milk protein κ-casein readily forms amyloid fibrils under physiological conditions. Milk exists in an extracellular oxidizing environment. Accordingly, the two methionine residues in κ-casein (Met_{95} and Met_{106}) were selectively oxidized and the effects on the fibril-forming propensity, cellular toxicity, chaperone ability, and structure of κ-casein were determined. Oxidation resulted in an increase in the rate of fibril formation and a greater level of cellular toxicity. β-Casein, which inhibits κ-casein fibril formation in vitro, was less effective at suppressing fibril formation of oxidized κ-casein. The ability of κ-casein to prevent the amorphous aggregation of target proteins was slightly enhanced upon methionine oxidation, which may arise from the protein's greater exposed surface hydrophobicity. No significant changes to κ-casein's intrinsically disordered structure occurred upon oxidation. The enhanced rate of fibril formation of oxidized κ-casein, coupled with the reduced chaperone ability of β-casein to prevent this aggregation, may affect casein−casein interaction within the casein micelle and thereby promote κ-casein aggregation and contribute to the formation of CA.

KEYWORDS: *casein proteins, amyloid fibril, methionine, oxidation*

INTRODUCTION

Casein is arguably the best characterized milk protein and constitutes over 70−80% of total bovine milk protein.[1] In milk, casein exists as large micelle-like particles that comprise four unrelated proteins (α_{s1}-, α_{s2}-, β-, and κ-casein) and calcium phosphate with a mass of approximately 10^8 Da and an average diameter of around 200 nm.[2] Owing to their high content of phosphate groups, which occur in clusters, α_{s1}-, α_{s2}-, and β-caseins have a strong tendency to bind metal ions, in particular calcium in milk. These calcium-binding caseins represent approximately 85% of total casein and are insoluble at calcium concentrations greater than ∼6 mM. Since bovine milk contains a calcium concentration of approximately 30 mM, it would be expected that the caseins would precipitate under the conditions prevailing in milk.[2] However, κ-casein, which contains only one to three phosphate groups and binds calcium very weakly, when mixed with the calcium-sensitive caseins, stabilizes them by forming large colloidal casein micelles. Like other caseins, κ-casein has little defined secondary or tertiary structure and is classified as an intrinsically disordered or natively unfolded protein.[3] κ-Casein is comprised of 169 amino acids which are arranged in an amphiphatic manner with a very hydrophobic N-terminal domain encompassing roughly residues Glu_1−Phe_{105} (para-κ-casein) and a polar C-terminal domain (Met_{106}−Val_{169}).[1] The flexible hydrophilic C-terminal region of κ-casein is thought to reside on the surface of the casein micelle and stabilizes the micelle through electrostatic and steric interactions. Separation of the hydrophobic and polar domains by chymosin-catalyzed hydrolysis of the Phe_{105}−Met_{106} peptide bond increases the surface hydrophobicity of casein micelles, allowing them to associate and coagulate, as occurs during cheese manufacture.[2]

Amyloid fibrils are ordered aggregates of peptides or proteins that are characterized by a highly structured array of cross β-sheet arranged into fibrils.[4] The assembly of partially folded proteins into amyloid fibrils is associated with a variety of devastating human diseases, including Alzheimer's (AD) and Parkinson's diseases.[5,6] Amyloid-like deposits have also been identified in bovine, rat, and canine mammary glands within calcified stones known as corpora amylacea (CA).[7,8] The majority of CA is located within the alveolar lumen, where milk is stored, and subsequently secreted into the ducts through ductules.[8] In addition, amyloid-like fibrils have been identified within the cytoplasm of mammary epithelial cells, which are involved in the synthesis and secretion of caseins and other milk constituents.[7,8] It has been postulated that CA can cause complications during late lactation by engorging luminal spaces and clogging small ducts, leading to milk stasis and involution.[8] Although the identity of the protein(s) involved in CA formation has not been determined conclusively, analysis of CA-derived peptides has suggested that several milk proteins, in particular caseins, are present.[9] It has been proposed that κ-casein plays a significant role in the formation of CA, as κ-casein readily forms amyloid fibrils in vitro under reducing and nonreducing physiological conditions.[10,11] Importantly, large scale fibril formation of κ-casein in vivo is prevented by

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the chaperone action of the other casein proteins due to their close association within the casein micelle.[11]

The formation of reactive oxygen species (ROS) is a byproduct of metabolism in cells. Under normal conditions, cells eliminate ROS by antioxidants, metal chelators, or enzymatic reduction.[12] However, when the level of ROS exceeds its defense mechanisms, the cell may suffer from oxidative stress. Proteins that are modified due to oxidation often accumulate during normal aging and are implicated in the pathogenesis of a wide range of disorders, including AD, Parkinson's disease, Huntington's disease, and cataract.[5,6] Post-mortem analysis of AD brains has shown high levels of protein oxidation, lipid peroxidation, and oxidative damage to mitochondria, indicating that oxidative stress is a characteristic of AD.[6] The oxidation state of the Aβ peptide, the putative causative agent in AD, is proposed to be critical to its neurotoxic properties in vivo whereby Met_{35} is involved in the generation of ROS.[13]

Protein oxidation in milk can be initiated enzymatically, e.g. by the lactoperoxidase system, by photo-oxidation, or by transition metal ions, which induce protein oxidation in the presence of ascorbic acid by a Fenton reaction.[14] Protein oxidation leads to various effects including covalent cross-linkages, fragmentation of covalent bonds, and modification of amino acids, e.g., methionine, histidine, tryptophan, and tyrosine.[14] Oxidation of methionine adds an oxygen atom to the sulfur atom of the side chain to form methionine sulfoxide.

Because of the relationship between oxidative stress and disease, along with methionine being one of the most readily oxidized amino acids,[15] we examined the effect of methionine oxidation on κ-casein's structure and function. Bovine κ-casein has two methionine residues at positions 95 and 106 which were selectively oxidized with hydrogen peroxide under mild conditions. Interestingly, while methionine oxidation in other fibril-forming proteins (e.g., α-synuclein, amyloid β, prion protein, transthyretin, and apolipoprotein C-II) inhibits fibril formation,[12,16−18] the oxidation of methionine residues in κ-casein increased fibril formation and enhanced cell toxicity. However, incubation of β-casein with oxidized κ-casein resulted in inhibition of κ-casein fibril formation, underlining the importance of the other caseins in preventing large-scale aggregation of κ-casein and hence the need for the incorporation of the casein proteins into the casein micelle.

MATERIALS AND METHODS

Materials. Bovine β-casein (chromatographically purified, minimum 90%), κ-casein (chromatographically purified, minimum 80%), and catalase (from bovine liver, 2000−5000 units/mg protein) were purchased from Sigma Aldrich. Alcohol dehydrogenase (ADH) was purchased from MP Bioscience. The fluorescent dyes thioflavin T (ThT) and 8-anilino-1-napthalene sulfonate (ANS) and the reducing agent 1,4-dithiotheritiol (DTT) were purchased from Sigma Aldrich. All other chemicals were of reagent grade, and all solutions were prepared with Milli-Q water.

Oxidation of Native κ-Casein. Native κ-casein (50 mg) was dissolved in 5 mL of 50 mM phosphate buffer (pH 7.4). To this solution was added 0.1% (v/v) hydrogen peroxide, and the reaction mixture was stirred overnight at 4 °C and then desalted using a HiPrep 26/10 desalting column (GE Healthcare) with 50 mM ammonium bicarbonate (pH 7.8) to remove the hydrogen peroxide. The sample was then lyophilized and stored at −20 °C. Prior to lyophilization, a 10 μL aliquot was taken for SDS−PAGE analysis and mixed with an equal volume of reducing sample buffer before being heated (95 °C, 5 min) and analyzed using 15% (w/v) acrylamide gels and standard techniques.

Mass Spectrometry. To confirm the oxidation of κ-casein methionine residues, a tryptic in-gel digest was performed on hydrogen peroxide-treated κ-casein according to the method of Shevchenko et al.[19] with slight modifications. The Coomassie blue-stained bands from an SDS−PAGE gel of native and oxidized κ-casein were cut into to 1 mm^2 blocks, rinsed in 100 mM NH_4HCO_3, and destained using 50 mM NH_4HCO_3 with 30% (v/v) acetonitrile and then incubated overnight with 100 ng of trypsin in 5 mM NH_4HCO_3 at 37 °C. The tryptic fragments were extracted from the gel by sonicating the samples for 15 min with 50% (v/v) acetonitrile in 0.1% (v/v) trifluoroacetic acid and then in 100% acetonitrile. All samples were prepared for mass spectrometry (MS) by spotting 1 μL of analyte solution into 1 μL of 2,5-dihydroxybenzoic (5 g/L) in 50% (v/v) acetonitrile, 0.1% (v/v) trifluoroacetic acid on a microtiter plate (Anchor Chip Target 600/384, Bruker Daltonics) and left to dry. Mass spectra were acquired on an Ultraflex III MALDI-TOF/TOF mass spectrometer (Bruker Daltonics) in reflector mode using Flex Control (Bruker Daltonics). The identity of peptides was confirmed by matching their observed masses to the expected masses of the in silico tryptic digest of the known κ-casein primary sequence using Biotools 3.0 software (Bruker Daltonics). All spectra were processed using FlexAnalysis software (Bruker Daltonics) with the following parameters: a maximum of 200 peaks was selected, with a signal-to-noise threshold of 5, and a quality factor of 75. A Gauss smoothing algorithm (0.02 m/z, 2 cycles) and a TopHat baseline subtraction were also performed. Electrospray MS experiments were also performed on native and oxidized κ-casein with a Q-Tof II spectrometer (Micromass UK) in positive ion mode. Protein solutions were made up to 1 mg/mL in acetonitrile and 0.1% formic acid (1:1 ratio) and diluted to a desired concentration using an acetonitrile and 0.1% formic acid solution (1:1 ratio). Spectra were smoothed (two channels, one cycle), baseline subtracted (cubic, 5% peak area), and deconvoluted using MassLynx 4.1 software (Waters).

Thioflavin T Assay To Monitor Amyloid Fibril Formation. The effect of oxidation on the fibril formation of native and reduced κ-casein was monitored using an in situ thioflavin T (ThT) binding assay. Native and oxidized κ-casein were incubated at 37 °C in 50 mM phosphate buffer pH 7.4, with and without DTT, to a final concentration of 20 mM. Samples were prepared in duplicate and incubated with 10 μM ThT in black μClear 96-microwell plates (Greiner Bio-One). The plates were sealed to prevent evaporation, and the fluorescence levels were measured with a FLUOstar Optima microplate reader (BMG Labtechnologies) with a 440/490 nm excitation/emission filter set. To monitor the effect of β-casein on κ-casein fibril formation, native and oxidized κ-casein (3.45 mg/mL) in 50 mM phosphate buffer, pH 7.4, was incubated with 20 mM DTT and increasing concentrations of β-casein up to a 1:1 molar ratio.

Chaperone Activity. The ability of native and oxidized κ-casein to inhibit the amorphous aggregation of various target proteins was monitored using a light scattering (turbidity) assay. Light scattering was monitored using a 96-well Falcon 3072 plate in a FLUOstar Optima microplate reader at a wavelength of 340 nm, which is indicative of protein aggregation. Alcohol dehydrogenase (ADH, 1 mg/mL) was incubated at 42 °C in 50 mM phosphate buffer (pH 7.4) with 2 mM EDTA for 3 h, while catalase (1 mg/mL) was incubated at 55 °C in 50 mM phosphate buffer (pH 7.4) for 2 h. The alteration with time in light scattering at 340 nm for each sample was measured. The change in turbidity with time in the absence of target protein was negligible in each assay. The percentage protection histograms were calculated from the light scattering assays and ThT binding assays using the following equation:

$$\%\text{protection} = \left(\frac{k_{\text{target}} - k_{\text{chaperone}}}{k_{\text{target}}}\right) \times 100$$

where k_{target} is the rate of aggregation of the target protein and $k_{chaperone}$ is the rate of aggregation of the target protein with the chaperone (κ-casein) present. The rates were determined from the maximum initial slope of the plots as described previously.[20]

Transmission Electron Microscopy. Samples for transmission electron microscopy (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 uranyl acetate (2% w/v, Agar Scientific). The grids were dried with Whatman 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 (Philips). Particle size measurements were performed using SIS Image Analysis software.

Cell Culture Toxicity Assays. The effect of methionine oxidation on κ-casein toxicity was investigated by treating pheochromocytoma-12 (PC-12) cells with native and oxidized κ-casein (200 μM), in 50 mM phosphate buffer (pH 7.4), preincubated at 37 °C for 20 h under reducing conditions (2 mM DTT). PC12 cells were grown in RPMI 1640 medium supplemented with 10% v/v horse serum, 5% v/v fetal bovine serum, 10 U/mL of penicillin, and 10 μg/mL of streptomycin. Cells were cultured in uncoated 75 cm^2 plastic flasks in an incubator with 95% air and 5% CO_2 at 37 °C. The medium was refreshed every 2−3 days. For treatment, cells from culture were seeded in 96-well plates at a density of 2×10^4 cells per well in 100 μL full-serum medium and incubated for 24 h. The plated cells were then treated (six replicates per treatment) with 10 μL of either native or oxidized κ-casein fibrils, which were preincubated for 20 h with 2 mM DTT, to give a final κ-casein concentration of 0.05, 0.5, or 2 μM. Control wells were treated with 10 μL of 2 mM DTT (in 50 mM phosphate buffer, pH 7.4) to ensure that the buffer was nontoxic to cells. RCM κ-casein induces cell death in a concentration-dependent manner.[21,22] Similar concentrations to those used by Dehle et al.[22] and Hudson et al.[21] were used. After incubation of the treated cells for 48 h, the treatment mixture was removed and 100 μL of serum-free medium containing MTT (0.6 mM) was added to each well. The cells were then incubated for an additional 2 h, and the MTT-containing medium was replaced with 100 μL of dimethyl sulfoxide. Formazan absorption was measured at 560 nm using a BMG Polarstar microplate reader (BMG Labtechnologies). Mean absorption readings from six wells were taken, and the percentage cell viability was calculated using the following equation:

$$\%\text{cell viability} = \left(\frac{A_{\text{treated}}}{A_{\text{untreated}}}\right) \times 100$$

where A_{treated} is the mean formazan absorption reading for each treatment and $A_{\text{untreated}}$ is the mean formazan absorption reading for cells treated with phosphate buffer only.

Extrinsic Fluorescence Spectroscopy. For extrinsic fluorescence measurements, 2−4 μL aliquots of a 10 mM stock solution of ANS, a hydrophobic probe, in 50 mM phosphate buffer (pH 7.4) were added to 25 μM native and oxidized κ-casein. The ANS fluorescence of each sample was measured using a Cary Eclipse fluorescence spectrophotometer (Varian), with the excitation wavelength set at 387 nm, and emission spectra were measured between 400 and 550 nm. The excitation and emission bandwidths were both 5 nm. The fluorescence intensity was plotted versus wavelength for each sample after the sequential addition of 2−4 μL aliquots of 10 mM ANS until the maximum fluorescence intensity was observed. The fluorescence emission of ANS in buffer alone was deducted from that obtained in the presence of protein.

Circular Dichroism Spectroscopy. Native and oxidized κ-casein at 0.2 mg/mL were dissolved in 10 mM phosphate buffer at pH 7.4, and 200 μL samples were incubated for 30 min at room temperature before being placed in a 0.1 mm quartz cuvette. Far-UV CD spectra were acquired on a JASCO π-star180 CD spectrophotometer at 25 °C over a wavelength range between 190 and 250 nm. The spectra were a sum of four scans with the ellipticity of buffer alone being subtracted from that obtained in the presence of protein. Spectra were plotted as molar ellipticity versus wavelength.

NMR Spectroscopy. Native and oxidized κ-casein were dissolved at 2 mg/mL in 50 mM deuterated phosphate buffer (pH 7.4 in D_2O). 1D ^1H and 2D $^1H-^1H$ total correlation spectroscopy (TOCSY) NMR spectra were acquired at 25 °C at 600 MHz on a Varian 600 Inova NMR spectrometer. Spectra were processed and analyzed using the Sparky 3.106 software package (Freeware). The spin lock mixing time in the TOCSY experiment was 60 ms. The residual HDO resonance was removed by presaturation during the delay between scans.

RESULTS

Oxidation of Native κ-Casein. The oxidation of native κ-casein was performed by incubating κ-casein with 0.1% (v/v) hydrogen peroxide overnight at 4 °C. Excess hydrogen peroxide was removed by desalting. Following purification, an in-gel tryptic digest of native (control) and oxidized κ-casein was performed. The subsequent solutions were analyzed by MALDI-TOF MS to verify that oxidation of κ-casein had occurred. A section of the raw mass spectra of native and oxidized κ-casein with tryptic peptide fragments of interest is presented in Figure 1. The two unmodified methionine residues in κ-casein, at positions 95 and 106, are indicated by peaks with a m/z of approximately 1193 and 1608 and correspond to the tryptic fragments $Ser_{87}-Arg_{97}$ and $His_{98}-Lys_{111}$, respectively. The identity of the more intense peak (m/z 1608.8) was also confirmed using MS/MS (data not shown). The oxidation of methionine is characterized by an addition of 16 Da. Thus, the two peaks observed in the mass spectra of oxidized κ-casein with a m/z of approximately 1209 and 1624 correspond to the oxidation of Met_{95} and Met_{106} within the tryptic fragments $Ser_{87}-Arg_{97}$ and $His_{98}-Lys_{111}$, respectively (Figure 1).

Native and oxidized κ-casein were also analyzed by electrospray ionization mass spectrometry (Supporting Information). Relatively poor MS spectra were obtained due to the heterogeneity of the protein. In κ-casein, the cysteine residues are randomly cross-linked by intermolecular disulfide bonds, resulting in the formation of polymers, from monomer to multimeric structures larger than decamers.[23] In addition to its phosphorylated residues, κ-casein is variously glycosylated on serine and threonine residues, resulting in significant heterogeneity such that a total of 19 variants make up this protein family.[24] Nevertheless, the deconvoluted MS spectrum of native κ-casein gave rise to peaks at 19 004, 19 036, 19 050, and 19 080 Da. The peak at 19 004 Da corresponds to monomeric κ-casein.[24] Deconvolution of the MS spectra of oxidized κ-casein resulted in various peaks at 19 034, 19 050, 19 068, 19 086, and 19 112 Da. The oxidation of two methionine residues is characterized by an addition of 32 Da. Therefore, the major peaks at 19 034, 19 068, and 19 112 Da in the MS spectrum of oxidized κ-casein correspond to the peaks at 19 004, 19 036, and 19 080 Da of native κ-casein that has had both of its methionine residues oxidized. Furthermore, MALDI and ES ionization MS showed that no other modifications were introduced by reaction of κ-casein with hydrogen peroxide.

Fibril-Forming Propensity of Native and Oxidized κ-Casein. Under reducing conditions, κ-casein forms long, rodlike aggregates at neutral pH and 37 °C that have the characteristics of amyloid fibrils.[10,11,20] Native κ-casein also has a propensity to form fibrils, although at a significantly reduced level compared to the reduced protein.[11] Various concentrations of native and oxidized κ-casein were incubated at neutral pH at 37 °C under reducing and nonreducing conditions to determine the effect of oxidation on fibril formation. Under both conditions, a concentration-dependent increase in ThT fluorescence was observed for both native and oxidized κ-casein (panels A−D of Figure 2) with oxidized κ-

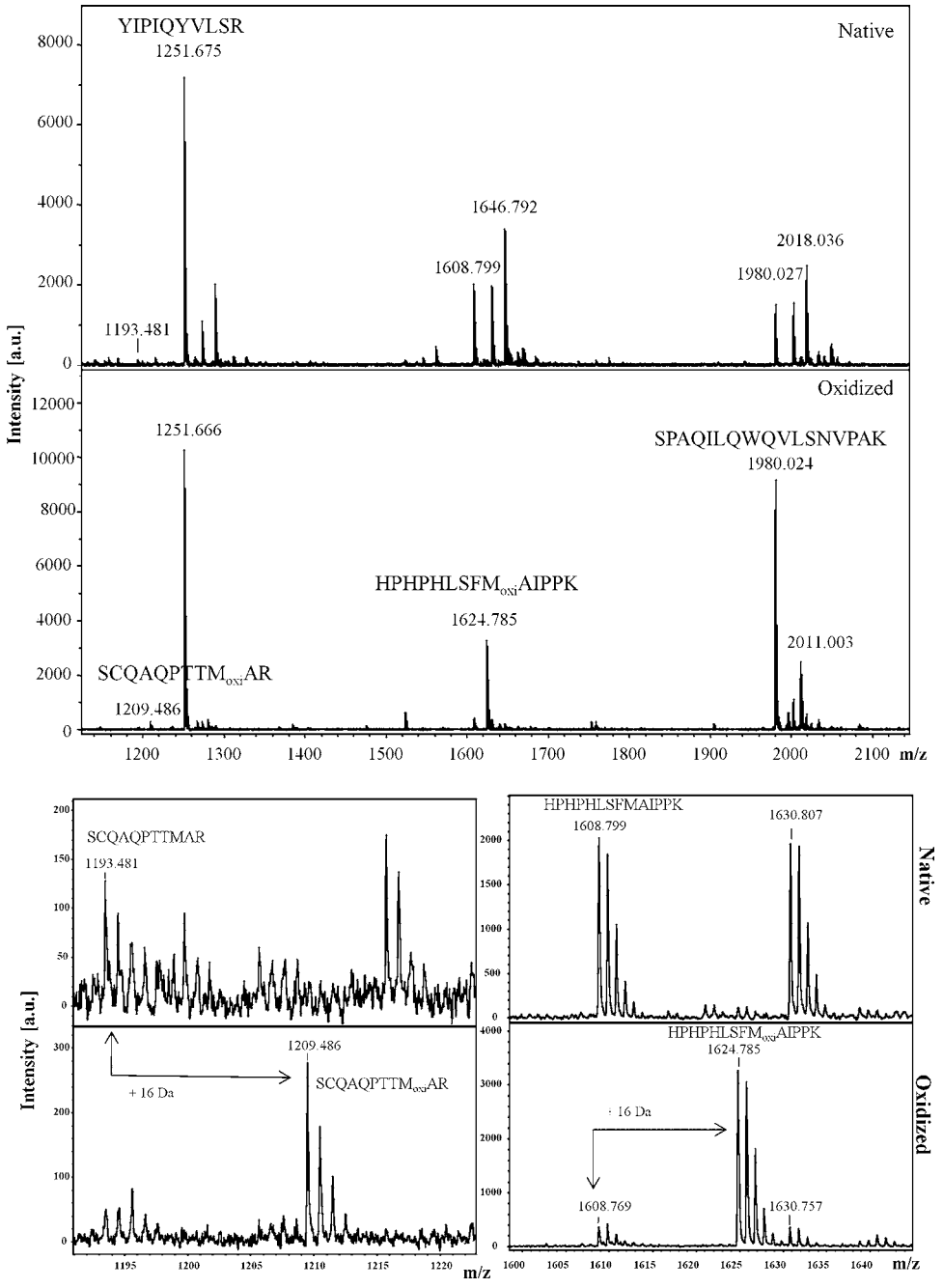

Figure 1. MALDI-TOF mass spectra of native and oxidized κ-casein following tryptic in-gel digest. The two spectral regions in the lower part of the figure are expansion of those above. Peak assignments corresponding to the tryptic κ-casein peptide fragments are indicated. An addition of mass 16 Da corresponds to the oxidation of a methionine residue (a.u., arbitrary units).

casein having a greater initial rate of increase in ThT fluorescence compared to native κ-casein under both nonreducing (Figure 2E) and reducing conditions (Figure 2F) at the same concentration. Since the rate of increase in ThT fluorescence was dependent on the protein concentration (panels A–D of Figure 2), we determined the reaction order for the formation of fibrils by native and oxidized κ-casein under reducing and nonreducing conditions. The reaction order can be determined according to the equation $\ln k = A + n \ln c$, where k represents the initial reaction rate, c is the protein concentration, and n is the apparent reaction order.[20] The plot of $\ln k$ against $\ln c$ gave a slope $n = 0.82 \pm 0.14$ and 0.86 ± 0.07 for native and oxidized κ-casein under reducing conditions, respectively (Figure 2F). Thus, for both native and oxidized κ-casein under reducing conditions, the rate of increase in ThT fluorescence, in relation to the κ-casein protein concentration, approaches a first-order mechanism. The values are slightly lower than that for RCM κ-casein, in which a first-order mechanism was also found, with a reaction order of 1.13 ± 0.15.[20]

TEM was used to verify that the increase in ThT fluorescence of native and oxidized κ-casein under reducing and nonreducing conditions was the result of fibril formation. TEM is only a qualitative method for evaluating fibril

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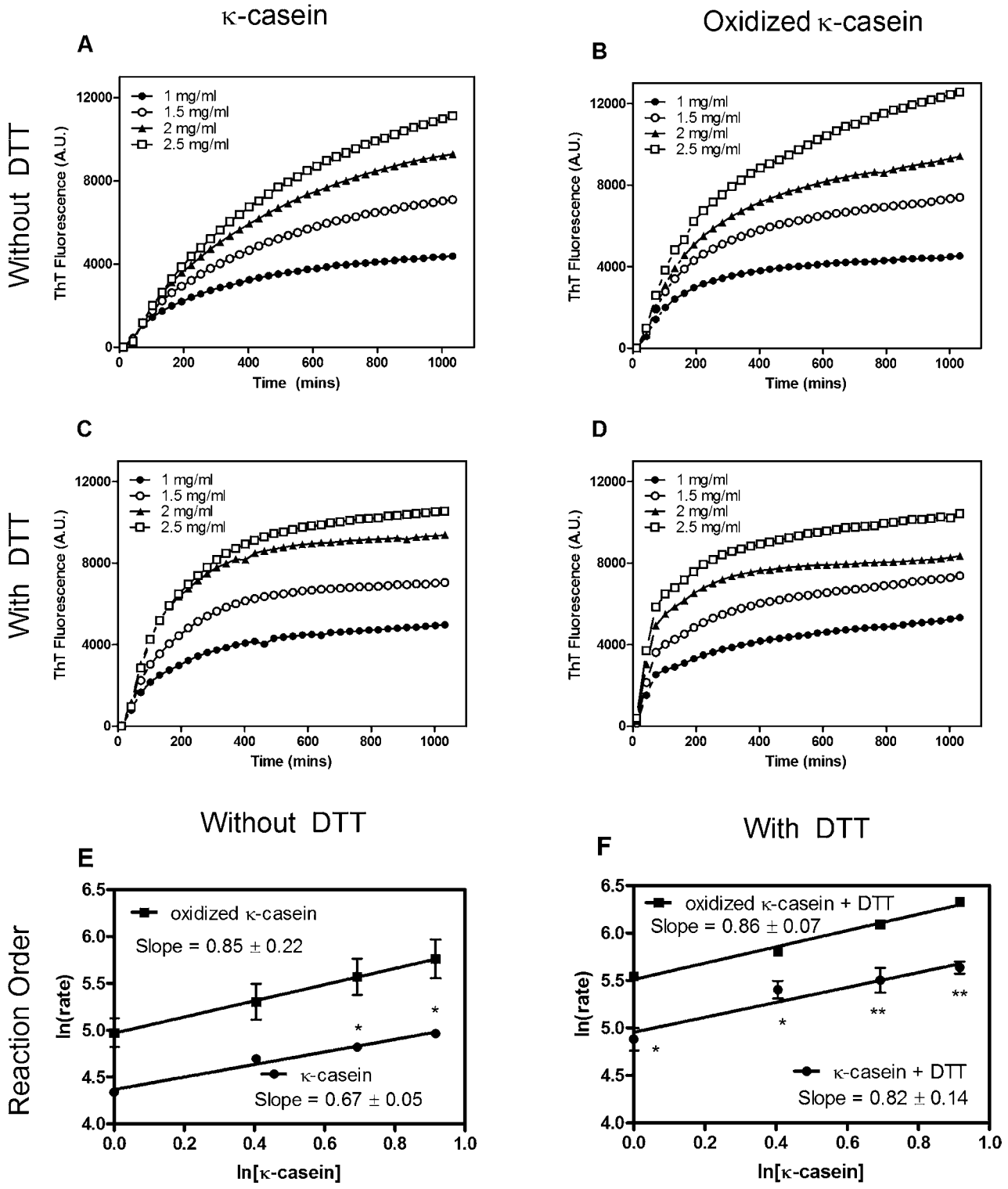

Figure 2. Fibril formation of native and oxidized κ-casein (1.0−2.5 mg/mL) monitored by ThT fluorescence over 18 h in the absence (A and B) or presence of 20 mM DTT (C and D). Samples were incubated in 50 mM phosphate buffer, pH 7.4, at 37 °C. The reaction order of native and oxidized κ-casein fibril formation was determined by plotting ln(rate) against ln[κ-casein], in the absence (E) or presence of DTT (F). The reaction rate was determined from the initial rate of increase in ThT fluorescence from the data shown in A−D (A.U., arbitrary units). Error bars represent standard error of at least three independent experiments; * and ** represent a *p*-value of <0.05 and <0.01, respectively (two-way ANOVA, Bonferroni post-test).

formation. Nevertheless, shorter fibrils were observed for native and oxidized κ-casein under nonreducing conditions (panels A and B of Figure 3), when compared to native and oxidized κ-casein in the presence of reducing agent (panels C and D of Figure 3), as was observed previously.[11] No significant differences in morphology were observed for native and oxidized κ-casein fibrils under the same conditions.

In the casein micelle, the fibrillar aggregation of κ-casein is prevented by the chaperone action of the other closely associated casein proteins (i.e., α_{s1}-, α_{s2}-, and β-casein).[11]

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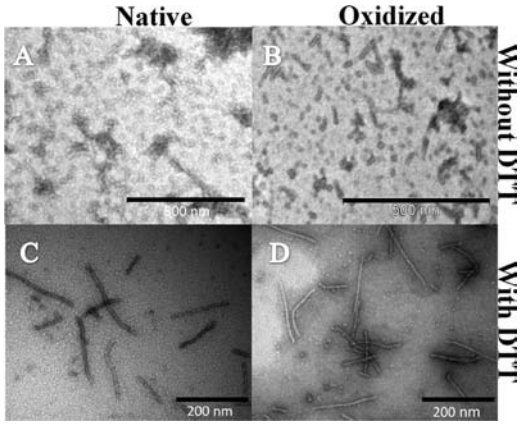

Figure 3. Transmission electron micrographs of native and oxidized κ-casein. Protein samples, at 1.5 mg/mL, were incubated in 50 mM phosphate buffer, pH 7.4 at 37 °C for 20 h. Native (A and C) and oxidized κ-casein (B and D) in the absence (A and B) or presence of DTT (C and D). Scale bars represent 500 and 200 nm for A and B, and C and D, respectively.

Accordingly, β-casein was incubated with native and oxidized κ-casein under reducing and nonreducing conditions to determine whether it could also prevent oxidized κ-casein from forming fibrils under these conditions (panels A–D of Figure 4). Under nonreducing conditions, β-casein suppressed the fibril formation of both native and oxidized as effectively (panels A and B of Figure 4). Thus, β-casein provided approximately 70% protection against fibril formation of both native and oxidized κ-casein at a 0.25:1.0 molar ratio of β-casein:κ-casein, while at a 1:1 molar ratio, around 90% protection was observed for both κ-casein species (panels A, B, and E of Figure 4). In contrast, under reducing conditions, β-casein was less able to suppress the fibril formation of oxidized κ-casein at lower concentrations (panels C and D of Figure 4). Thus, at a 0.25:1.0 molar ratio of β-casein:κ-casein, 35% protection was observed for κ-casein, whereas only 5% protection occurred for oxidized κ-casein (Figure 4F).

TEM was also performed on samples immediately following the ThT assay. Both native and oxidized κ-casein formed fibrils under reducing conditions in the absence of β-casein (panels A and E of Figure 5). Fibrils were also observed for both native and oxidized κ-casein at a 0.25:1.0 molar ratio of β-casein:κ-casein under reducing conditions (panels B and F of Figure 5). No fibrils were observed when κ-casein was incubated at a 0.5:1.0 molar ratio of β-casein:κ-casein under reducing conditions; instead, amorphous aggregates and small protofibrils were present (Figure 5C). In contrast, when oxidized κ-casein was incubated at a 0.5:1.0 molar ratio of β-casein:κ-casein, fibrils were still observed by TEM, although they were somewhat stunted (Figure 5G). Only amorphous aggregates were observed for both native and oxidized κ-casein under reducing conditions in the presence of a molar equivalent of β-casein (panels D and H of Figure 5). In summary, the TEM results are consistent with the ThT data, i.e., β-casein had a reduced ability to suppress the fibril formation of oxidized κ-casein compared to native κ-casein but is capable of doing so at higher β-casein concentrations.

Chaperone Activity of Native and Oxidized κ-Casein. Native κ-casein exhibits chaperone activity by inhibiting the amorphous aggregation of a variety of target proteins.[25] Native and oxidized κ-casein were incubated with various target proteins under heat stress to determine whether oxidation of methionine residues to the more polar methionine sulfoxide had an effect on κ-casein chaperone activity. When alcohol dehydrogenase (ADH) was incubated at 42 °C at neutral pH with EDTA to remove its constituent zinc ion, an increase in light scattering was observed at 30 min with aggregation reaching a plateau following 180 min of incubation (panels A and B of Figure 6). The presence of increasing amounts of native κ-casein led to a decrease in aggregation, such that, at a 2:1 molar ratio of κ-casein:ADH, the aggregation of ADH was almost completely suppressed (Figure 6A). Oxidized κ-casein was also similarly effective at suppressing the aggregation and subsequent precipitation of ADH (Figure 6B). Under more severe heating conditions, i.e. when catalase was incubated at 55 °C, an increase in light scattering was observed after 25 min with aggregation continuing over 120 min of incubation. The presence of an increasing concentration of native and oxidized κ-casein led to a decrease in the aggregation of catalase, such that, at a 2:1 molar ratio of κ-casein:catalase, the aggregation of catalase was completely suppressed (panels C and D of Figure 6). The rate of aggregation of ADH and catalase with and without chaperone present was quantified and expressed as a percentage of protection (panels E and F of Figure 6). Oxidized κ-casein offered slightly better protection against the aggregation of both catalase and ADH; however, its chaperone activity was not statistically different (p-values of 0.12 and 0.20 at 0.25:1.0 and 0.5:1.0 molar ratios, respectively).

Cellular Toxicity of Fibrillar Native and Oxidized κ-Casein. To assess the toxicity of oxidized κ-casein fibrils, pheochromocytoma-12 (PC12) cells were treated with native and oxidized κ-casein (4 mg/mL) preincubated at 37 °C for 20 h with 2 mM DTT in 50 mM phosphate buffer, pH 7.4. The samples were prepared in duplicate, one with and one without 10 μM ThT. The former sample was used to monitor the rate of fibril formation. The latter, without ThT, was utilized in the cell toxicity assays to ensure that the cells were not exposed to residual ThT, which has been shown to be toxic at certain concentrations.[21] The cells were incubated with a final κ-casein concentration of 0.05–2.0 μM in sextuplicate. Following incubation of the treated cells for 48 h, cellular survival was measured by methylthiazolyldiphenyltetrazolium bromide (MTT) reduction (Figure 7). Significantly greater amyloid-associated toxicity was observed for oxidized compared to native κ-casein fibrils, which correlated with a greater rate of fibril formation for oxidized κ-casein (Figure 2).

Structural Studies of Oxidized κ-Casein. Extrinsic fluorescence (ANS binding), intrinsic fluorescence, CD, and NMR spectroscopy were used to examine alterations in the structure of κ-casein upon oxidation. Aliquots of a stock solution of ANS were added to solutions of native and oxidized κ-casein (0.5 mg/mL) at neutral pH. The fluorescence intensity was plotted versus wavelength for each sample after sequential addition of ANS until the maximum fluorescence intensity was observed. The maximum κ-casein fluorescence intensity was 565 au, which increased to 650 au upon oxidation (Figure 8A), suggesting an increase in exposed and clustered hydrophobicity of κ-casein upon oxidation. Far-UV CD spectra spectra were used to examine the overall secondary structure of κ-casein upon oxidation. Figure 8B shows that the CD spectra of native and oxidized κ-casein are very similar. Both spectra exhibit a maximum negative mean residue ellipticity at 200 nm and a shoulder at 213 and 230 nm, which are indicative of a predominantly random coil structure and are very similar to

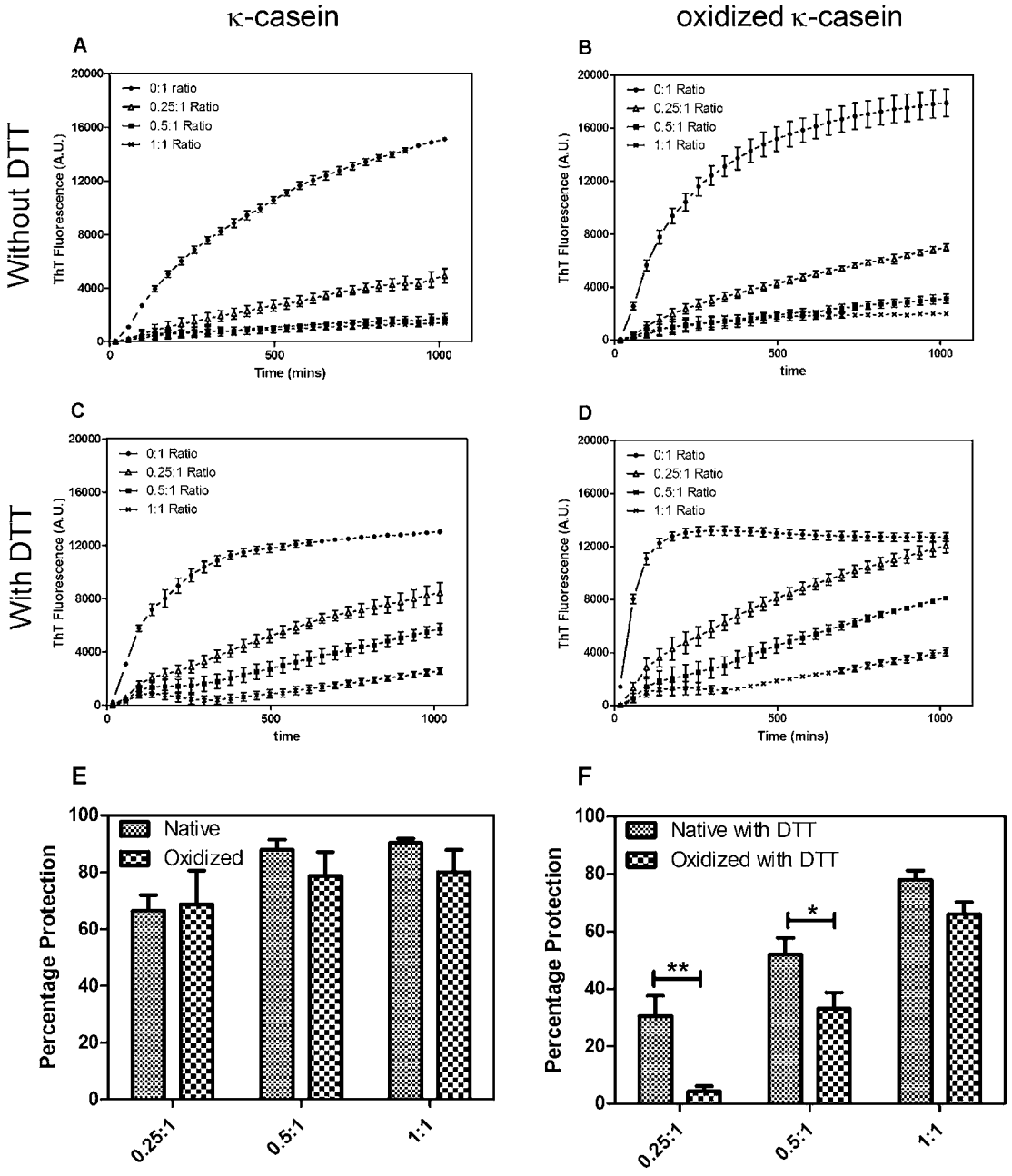

Figure 4. Amyloid fibril formation of native and oxidized κ-casein (3.4 mg/mL) incubated with increasing amounts of β-casein over 18 h in either the absence (A and B) or presence of 20 mM DTT (C and D). Ratios are indicated for β-casein:κ-casein. Samples were incubated in 50 mM phosphate buffer, pH 7.4 at 37 °C. The ability of β-casein to prevent fibril formation of native and oxidized κ-casein in the absence (E) or presence of 20 mM DTT (F) was quantified as a percentage protection. Percentage protection was determined by comparing the maximum change in ThT fluorescence with and without β-casein present. Molar ratios are indicated. Error bars represent the standard error of at least three independent experiments; * and ** represent a *p*-value of <0.05 and <0.01, respectively (two-way ANOVA, Bonferroni post-test).

previously published CD spectra of κ-casein[26] and RCM κ-casein.[20,22]

One- and two-dimensional ^{1}H NMR spectra were acquired on native and oxidized κ-casein at 25 °C in 50 mM deuterated phosphate buffer (pH 7.4). Native κ-casein in aqueous solution forms large spherical micelle-like polymers, with an average molecular mass of 1.18 MDa.[2] The micelles are assembled from smaller multimeric subunits (monomers to decamers) that result from intermolecular disulfide bonding.[23] An aggregate of this size would have a long correlation time, and thus, very broad, overlapping, and uninformative resonances would be expected to be observed, giving rise to a poorly resolved ^{1}H NMR spectrum. Instead, a series of comparatively sharp resonances are observed in the 1D ^{1}H NMR spectra of native and oxidized κ-casein (Figure 8C). The spectra are very similar to those of the κ-casein macropeptide (Met_{106}–Val_{169}),[27] the region of the polypeptide chain that extends into solution from the casein micelle. These relatively sharp resonances arise from protons in κ-casein that have independent flexibility (i.e., a much shorter correlation time) compared to the bulk of the protein. The 1D ^{1}H NMR spectra of native and oxidized κ-casein are very similar; however, an additional resonance at 2.75 ppm in the ^{1}H NMR spectra of oxidized κ-casein is observed (Figure 8C) which corresponds to the ε-methyl

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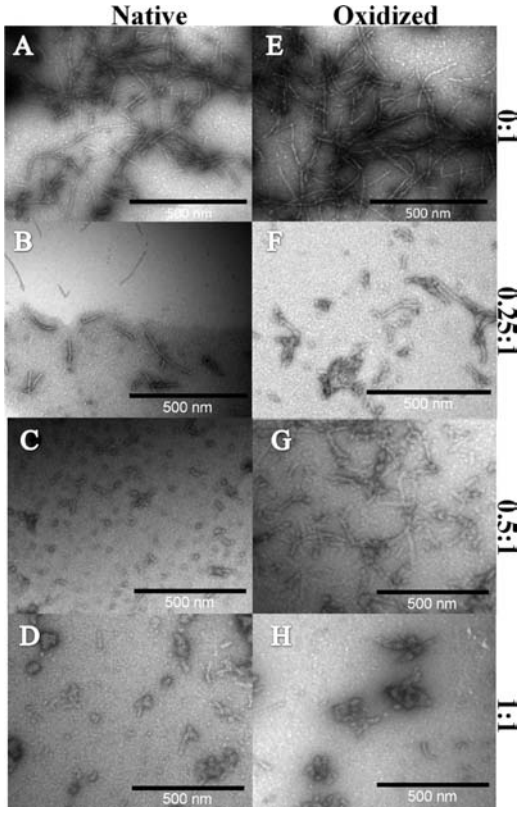

Figure 5. Transmission electron micrographs of native (A–D) and oxidized (E–H) κ-casein at the molar ratios of β-casein:κ-casein indicated. Protein samples, at 3.4 mg/mL, were dissolved in 50 mM phosphate buffer, pH 7.4, at 37 °C under reducing conditions with 20 mM DTT and incubated for 20 h with increasing concentrations of β-casein. Scale bars represent 500 nm.

protons of oxidized methionine and is approximately 0.6 ppm downfield compared to its shift in methionine,[28] due to the addition of the electronegative oxygen on the sulfur atom.

TOCSY experiments of native and oxidized κ-casein were also acquired (Figure 8D). No cross-peaks from methionine residues (Met_{95} and Met_{106}) were observed in the TOCSY spectrum of native κ-casein, presumably as they reside within or very near to the hydrophobic portion of para-κ-casein, encompassing residues from Glu_1 to Phe_{105}.[1] Therefore, the methionine residues are buried within with the κ-casein micelle and are not observed as a result of resonance broadening due to slow protein tumbling. In contrast, cross-peaks were observed for methionine sulfoxide residues in the TOCSY spectra of oxidized κ-casein, resulting from an increase in conformational flexibility of the methionine residue upon oxidation. The chemical shifts of the methionine sulfoxide resonances, i.e., H^{α} (4.47), $2H^{\beta}$ (2.10, 2.22), and $2H^{\gamma}$ (2.90, 2.91 ppm) (Figure 8D), were consistent with those in the literature.[29] The γ-methylene resonances are significantly more downfield than those of methionine[28] as a result of the electronegative oxygen on the sulfur atom.[29] Additional arginine cross-peaks were also observed in the TOCSY spectra of oxidized κ-casein (Figure 8D). The five arginine residues in κ-casein (at positions 10, 16, 34, 68, and 97) are located within the para κ-casein region and therefore would be buried within the κ-casein micelle and not observed in the TOCSY spectra due to resonance broadening. The presence of arginine cross-peaks in oxidized κ-casein, possibly arising from Arg_{97}, i.e. adjacent to Met_{96}, provides further evidence of localized unfolding of κ-casein upon oxidation. However, overall, no significant structural changes to κ-casein upon oxidation were observed by ^{1}H NMR spectroscopy, which is consistent with the CD and fluorescence data.

DISCUSSION

It is well-known that side chain modifications can have profound effects on protein stability and structure. The oxidation of methionine is regarded as a form of chemical "mutagenesis" in which the methionine side chain is substituted with methionine sulfoxide, resulting in a larger and more polar side chain.[30] It is therefore not surprising that oxidation of methionine can greatly perturb a protein's structure and affect its stability and hence activity. The oxidation of proteins has been implicated as a causative or contributing factor in many diseases.[5] In the case of protein misfolding diseases involving amyloid fibrils, the oxidation of methionine is a common modulator of fibril formation whereby it inhibits the aggregation of Aβ,[16] transthyretin,[17] α-synuclein,[12] and apolipoprotein C-II.[18] By contrast, oxidation of κ-casein had an opposite effect on its ability to form fibrils in the presence or absence of DTT; i.e., a greater rate of fibril formation occurred upon oxidation of κ-casein methionine residues. Furthermore, β-casein, which has been proposed to prevent large-scale fibril formation of κ-casein in vivo,[11] was slightly less efficient at suppressing amyloid fibril formation by oxidized κ-casein in the presence of DTT. The reduced chaperone efficiency of β-casein against oxidized κ-casein fibril formation may arise from the latter's enhanced rate of fibril formation. β-Casein, like small heat-shock proteins (sHsps), is a poor chaperone against rapidly aggregating target proteins.[31,32] However, at higher concentrations, β-casein is able to suppress the fibril formation of oxidized κ-casein and convert κ-casein from the fibril-forming to the amorphous aggregation pathway. The conversion of fibrillar κ-casein to amorphous aggregates at higher concentrations of β-casein is similar to the behavior observed for the interaction of fibril-forming α-synuclein, the putative causative agent in Parkinson's disease, with the sHsp αB-crystallin.[33]

Several amyloidogenic proteins whose fibril formation is inhibited by methionine oxidation have a key methionine residue located within their fibril core region. For example, Aβ has Met_{35}, positioned within the core fibril region, and its oxidation inhibits fibril formation.[16] Fibril formation of apolipoprotein C-II is particularly sensitive to oxidation or substitution of Met_{60},[18] which lies within one of its fibril-forming core regions. Likewise, in the peptide ccβ-Met, a number of site-specific hydrophobic interactions are responsible for the formation of the highly stable amyloid structure.[34] Perturbation of one of these residues by the insertion of a single oxygen moiety has drastic effect on the kinetics and dynamics of fibril assembly.[34] Therefore, in general, the particular environment and/or position of a methionine residue in a protein play an important role in regulating fibrillogenesis. In κ-casein, Met_{95} is situated in the hydrophobic N-terminal domain roughly encompassing resides Glu_1–Phe_{105} (para-κ-casein), while Met_{106} is the first residue at the start of the polar C-terminal domain.[1] We have shown that the fibril-forming core of κ-casein encompasses residues Tyr_{25}–Lys_{86},[20] which may explain why oxidation of Met_{95} and Met_{106} has no inhibitory effect on fibril formation. It may also explain why oxidation of native κ-casein makes it a slightly more effective

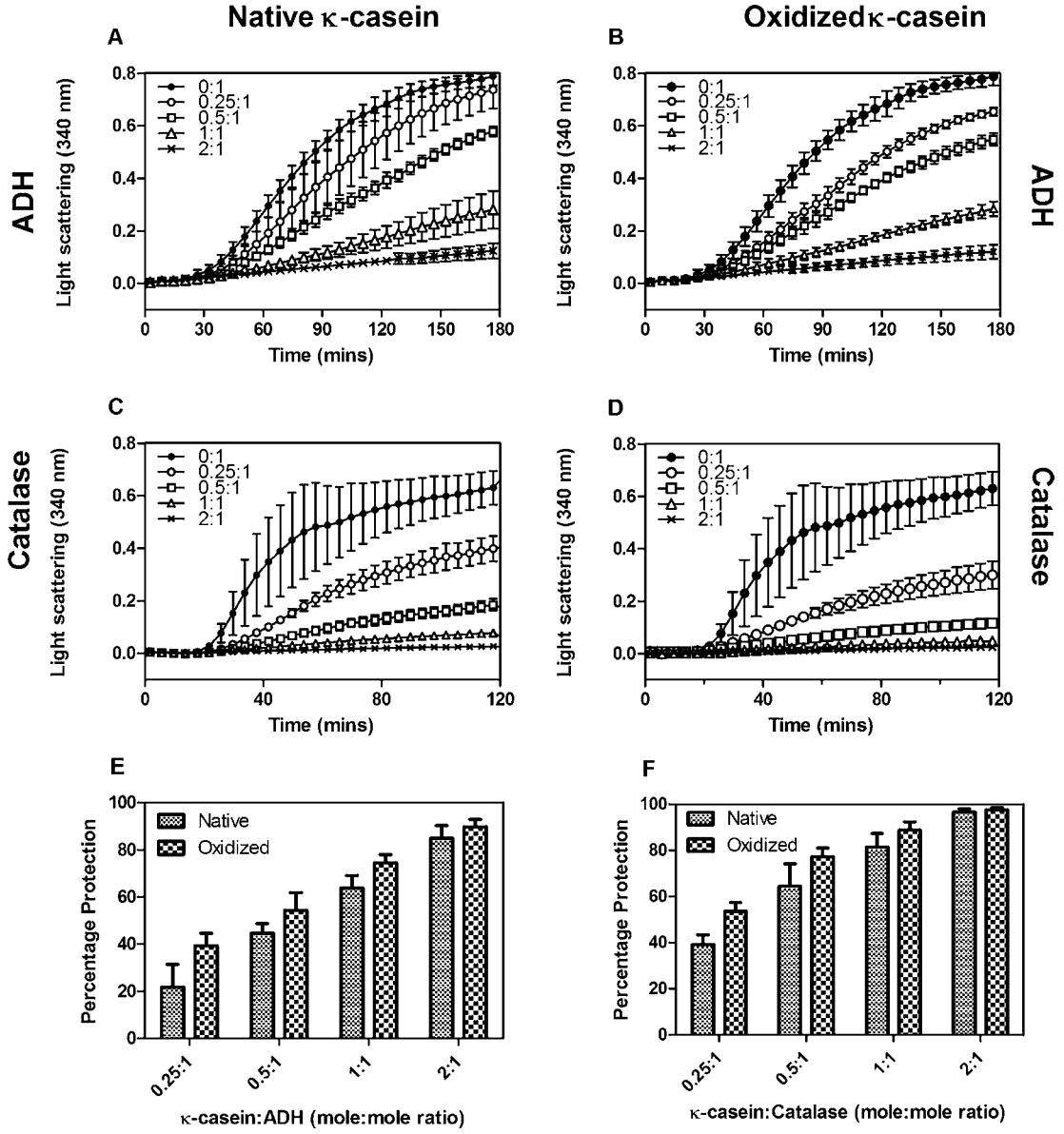

Figure 6. The thermal aggregation at 42 °C of ADH (1 mg/mL) in 50 mM phosphate buffer, pH 7.4, 2 mM EDTA, in the presence of an increasing concentration of native κ-casein (A) or oxidized κ-casein (B). The thermal aggregation at 55 °C of catalase (1 mg/mL) in 50 mM phosphate buffer, pH 7.4, in the presence of an increasing concentration of native κ-casein (C) or oxidized κ-casein (D). The chaperone activity of native and oxidized κ-casein was quantified by comparing the initial rate of aggregation with and without chaperone present (E and F). Molar ratios of κ-casein:target protein are indicated. Error bars represent the standard error of at least three independent experiments.

chaperone against the aggregating target proteins, catalase and ADH. The more polar methionine sulfoxide residues may help to solubilize κ-casein and the complex it forms with unfolding target proteins in an analogous manner to the phosphate groups which solubilize α_s- and β-casein complexes with partially folded target proteins during chaperone action.[35,36]

In contrast, α-synuclein has four methionines (Met_1, Met_5, Met_{116}, and Met_{127}) outside its fibril forming core, i.e., Glu_{22}–Glu_{105}, and its fibril formation is completely inhibited upon methionine oxidation.[12] Similarly, selective oxidation of Met_9 of apolipoprotein C-II, which is not in its fibril core, also inhibits fibril formation, but not to the same extent as oxidation of Met_{60}.[18] The difference in fibril-forming sensitivity to oxidation between κ-casein and α-synuclein may be attributed in their different modes of fibril aggregation. Fibril-formation of α-synuclein occurs via a standard nucleation-dependent polymerization mechanism, with a critical structural transformation from the unfolded conformation of α-synuclein to a partially folded species.[37] Fibril formation of RCM-κ-casein occurs by a novel mechanism whereby the rate of fibril formation is limited by the dissociation of the amyloidogenic monomer from an oligomeric state.[20,38] Structure prediction of the dissociated κ-casein monomer implies that, although predominately unstructured, its central region within the fibril-forming core can populate a structural conformation containing a large hydrophobic and aromatic-rich sheet–turn–sheet motif,[39] which is an ideal fibril precursor that can be readily incorporated into nuclei or growing fibrils, without the need for further unfolding or folding of the κ-casein monomer.[20] Thus, because folding/unfolding is not required for κ-casein fibril formation (i.e., its structure is "primed" for such) and methionine residues are absent in its fibril-forming core region,

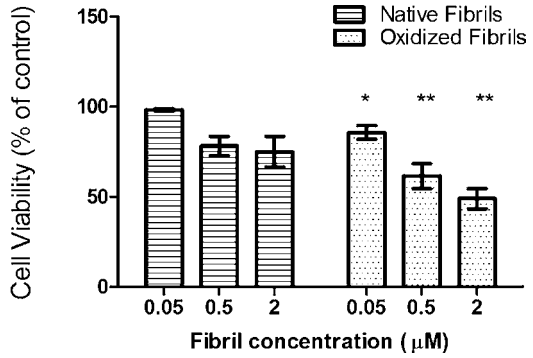

Figure 7. Viability of PC-12 cells treated with native and oxidized κ-casein preincubated at 37 °C for 20 h in the presence of 2 mM DTT. The final concentration of κ-casein is given. Error bars represent standard error of at least three independent experiments; * and ** represent a *p*-value of <0.05 and <0.01, respectively (two-way ANOVA, Bonferroni post-test).

it is conceivable that oxidation of methionine has no inhibitory effect on κ-casein fibril formation.

In vitro, the rate of fibril formation upon κ-casein oxidation was greater than in the native state. To determine the cellular implications of this, the toxicity to PC12 cells of κ-casein upon oxidation was assessed. κ-Casein fibrils were formed by incubating κ-casein with 2 mM DTT for 20 h at physiological temperature. DTT protects against amyloid toxicity; e.g., β-amyloid$_{1-40}$ cytotoxicity to neurons in culture is prevented by 2 mM DTT.[40] However, in our experiments, the presence of residual DTT after dilution (20 μM) did not affect cell survival. Moreover, by first forming the amyloid fibrils in vitro, interference by protective extracellular molecular chaperones, such as clusterin and serum albumin, which are present in cell media, was avoided.[21] Oxidized κ-casein was significantly more toxic to cells compared to native κ-casein fibrils formed under the same conditions. Mature and protofibrillar RCM-κ-casein fibrils are more toxic than their prefibrillar counterparts.[21,22] Therefore, the increased rate of ThT fluorescence for oxidized κ-casein under reducing and nonreducing conditions may correlate to more mature and protofibrillar species being present after 20 h of incubation which increase cellular toxicity.

To determine the effect of methionine oxidation on κ-casein structure, CD, NMR, and extrinsic fluorescence spectroscopic studies were undertaken. Somewhat counterintuitively, the oxidation of methionine led to an increase in fluorescence of the hydrophobic probe ANS presumably through localized unfolding of the protein, leading to greater exposed, clustered regions of hydrophobicity at the protein surface. The enhanced surface hydrophobicity may contribute to the slightly better chaperone ability of oxidized κ-casein. The CD data showed no change in gross secondary structure upon oxidation of κ-casein.

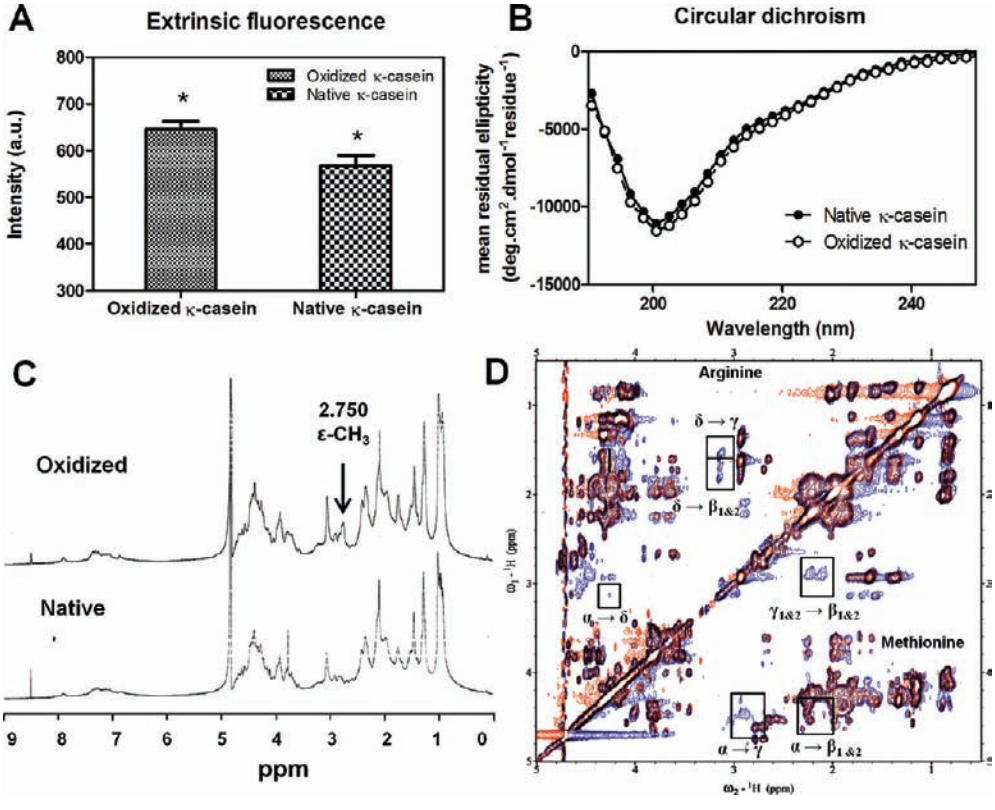

Figure 8. (A) Maximum extrinsic ANS fluorescence of native and oxidized κ-casein (0.5 mg/mL) in 50 mM phosphate buffer, pH 7.4. The bars represent the standard error of three independent experiments; * represents $p < 0.05$ (*t* test, two-tailed, unequal variance) (a.u., arbitrary units). (B) Far-UV CD spectra of native and oxidized κ-casein (0.2 mg/mL) in 10 mM phosphate buffer, pH 7.4, at 25 °C. (C) 1D ^{1}H NMR spectra of native and oxidized κ-casein (100 μM) in deuterated phosphate buffer (50 mM, pH 7.4) in D_2O recorded at 25 °C. The ε-methyl proton resonances of an oxidized methionine residue are indicated. (D) 2D ^{1}H−^{1}H TOCSY NMR spectra of native (red) and oxidized (blue) κ-casein in deuterated phosphate buffer (50 mM, pH 7.4) in D_2O at 25 °C. Cross-peaks for an oxidized methionine spin system are indicated: H^{α} (4.47 ppm), $2H^{\beta}$ (2.22, 2.10 ppm), $2H^{\gamma}$ (2.89, 2.91 ppm). Cross-peaks for an arginine spin system are also observed after oxidation: H^{α} (4.26 ppm), $2H^{\beta}$ (1.82, 1.71 ppm), $2H^{\gamma}$ (1.57 ppm), $2H^{\delta}$ (3.14 ppm). Resolved cross-peaks from arginine and methionine residues are indicated above and below the diagonal, respectively.

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Likewise, little alteration in the NMR spectra of κ-casein occurred upon oxidation apart from the additional presence of weak resonances corresponding to methionine and arginine, which suggest that these residues have greater conformational flexibility compared to the bulk of the protein upon κ-casein oxidation. The minor structural changes observed for oxidized κ-casein may be responsible for the poorer ability of β-casein to prevent oxidized κ-casein fibril formation under reducing conditions. Upon methionine oxidation, a similar situation is observed for other fibril-forming proteins upon oxidation; i.e., Aβ-(1−42) and α-synuclein do not exhibit significant alteration in their structure upon oxidation, with both forms of the proteins having little or no well-defined structure.[12,16]

While κ-casein readily forms fibrils at neutral pH,[10,11] oxidation of methionine residues leads to an increase in the rate of fibril formation. This may have implications in vivo as casein micelles are secreted and stored as milk in the alveolar lumen,[8] which is an extracellular oxidizing environment. Furthermore, β-casein, which has been proposed to prevent large-scale fibril formation of κ-casein in vivo,[11] is less efficient at suppressing amyloid fibril formation by oxidized κ-casein. Thus, under an oxidative regime, the casein micelle may not function as effectively as a regulator of κ-casein aggregation, leading to potential accumulation of κ-casein amyloid fibrils and its concomitant cellular toxicity. Potentially, such behavior could occur in the development of calcified deposits associated with CA.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Additional information as described in the text. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS USED

AD, Alzheimer's disease; ADH, alcohol dehydrogenase; ANS, 8-anilino-1-napthalene sulfonate; CD, circular dichroism; CA, corpora amylacea; DTT, 1,4-dithiothreitol; MTT, methylthiazolyldiphenyltetrazolium bromide; PC-12, pheochromocytoma-12; RCM, reduced and carboxymethylated; ROS, reactive oxygen species; sHsp, small heat-shock protein; TOCSY, total correlation spectroscopy; ThT, thioflavin T; TEM, transmission electron microscopy

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