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ARTICLE

Dephosphorylation of α_s- and β-Caseins and Its Effect on Chaperone Activity: A Structural and Functional Investigation

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Milk casein proteins can act as molecular chaperones: under conditions of stress, such as elevated temperature, molecular chaperones stabilize proteins from unfolding, aggregating, and precipitating. In this study, α_s- and β-caseins were dephosphorylated using alkaline phosphatase. A structural and functional investigation was undertaken to determine the effect of dephosphorylation on the chaperone activity of α_s- and β-caseins against two types of protein misfolding, i.e., amorphous aggregation and amyloid fibril assembly. The dephosphorylation of α_s- and β-caseins resulted in a decrease in the chaperone efficiency against both heat- and reduction-induced amorphously aggregating target proteins. In contrast, dephosphorylation had no effect on the chaperone activity of α_s- and β-caseins against the amyloid-forming target protein κ-casein. Circular dichroism and fluorescence spectroscopic data indicated that the loss of negative charge associated with dephosphorylation led to an increase in ordered structure of α_s- and β-caseins. It is concluded that the flexible, dynamic, and relatively unstructured and amphiphatic nature of α_s- and β-caseins is important in their chaperone action.

KEYWORDS: Casein proteins; molecular chaperone; protein aggregation; amyloid fibril

INTRODUCTION

The main biological function of milk casein proteins is nutritional: their open and flexible structure renders them susceptible to proteolysis, which facilitates their use as a source of amino acids (*1*). The caseins comprise four proteins (α_{s1}-, α_{s2}-, β-, and κ-caseins) that are unrelated in sequence. Casein proteins are all regarded as "natively unfolded" in conformation (*2*) with some elements of secondary structure but no defined tertiary structure (*3*). In milk, the casein proteins exist as a macromolecular complex that associates into large colloidal micellular aggregates (*2*). These micelles are $\sim 10^8$ Da in mass and $\sim$200 nm in diameter and comprise the four casein proteins and calcium phosphate (*1*). All of the caseins are phosphorylated to a certain extent (α_{s1}-casein = 8−9, α_{s2}-casein = 10−13, β-casein = 5, and κ-casein = 1 phosphate group), which contributes to their hydration, solubility, heat stability, and importantly their binding of calcium and other metals (*1*). The relative proportions of these proteins vary among species (*4*); in bovine milk, 65% of the casein fraction is comprised of α_{s1}- and α_{s2}-caseins, which are collectively known as α_s-casein, with α_{s1} the dominant component (*5*). α_{s1}- and α_{s2}-caseins associate very strongly via noncovalent interactions to form a complex and are often isolated together from milk (*2*). This close association is important because α_{s1} prevents α_{s2} from aggregating to form highly ordered linear species, known as amyloid fibrils (*6*).

While casein products have been used in the food industry for their nutritional value for many years (*7*), the observation that α_s-, β-, and κ-caseins act as molecular chaperones has opened a new avenue of research into casein proteins and their potential application. Under conditions of stress (e.g., elevated temperature), casein proteins interact with and stabilize other proteins (including other milk proteins) and prevent their aggregation and potential precipitation (*5*, *8*−*10*). In doing so, casein proteins exhibit a similar chaperone ability to mammalian small heat-shock proteins (sHsps) (*8*). The chaperone ability of casein proteins provides avenues for novel applications of casein proteins as well as further insight into the properties and stability of milk and milk products (*8*).

The aim of this study was to determine the effect of phosphate groups on the ability of caseins to act as molecular chaperones and give further insight in the mechanism of chaperone action. Previously, Matsudomi et al. (*9*) investigated the effects of dephosphorylation of α_s-casein on the heat-induced aggregation of ovotransferrin and observed that dephosphorylation reduced its ability to act as a molecular chaperone. We have extended these studies by preparing dephosphorylated α_s- and β-caseins and testing their chaperone action under thermal and reduction stresses against amorphously aggregating target proteins and against the fibril-forming target protein, κ-casein. In addition, the structural changes induced in α_s- and β-caseins after dephosphorylation were explored using a variety of spectroscopic techniques.

MATERIALS AND METHODS

Materials. Bovine β-casein, α_s-casein, κ-casein, α-lactalbumin (calcium reduced > 85%), and ovotransferrin (OT, from chicken egg white,

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substantially iron free) were purchased from Sigma-Aldrich (St. Louis, MO). Alcohol dehydrogenase (ADH) and alkaline phosphatase (from calf intestine, activity > 1600 units/mg of protein) were purchased from MP Bioscience (Hilton, Derby). Snakeskin-pleated dialysis tubing with a 3.5 kDa cut off was obtained from Pierce Chemical Company (Rockford, IL). 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 unless otherwise stated, all solutions were prepared with Milli-Q water.

Dephosphorylation of α_s- and β-Caseins. Dephosphorylation of β-casein was carried out using alkaline phosphatase according to the method of Lorient and Linden (*11*). β-Casein (1%, w/v) was dissolved in 25 mM ammonium bicarbonate (pH 7.8) with 0.5 mM $MgCl_2$. A 0.5 mL enzyme solution of alkaline phosphatase (5 units/mL) was added to a 2 mL β-casein solution and placed in a narrow dialysis bag. The dialysis bag was added to 95 mL of external buffer (25 mM ammonium bicarbonate at pH 7.8 and 0.5 mM $MgCl_2$) at 37 °C with continuous stirring for 8 h. Freezing and lyophilizing the dephosphorylated β-casein quenched the reaction. The same protein was treated under identical conditions without the alkaline phosphatase for comparison. Dephosphorylation was carried out in a dialysis bag to allow for the inorganic phosphate to travel between the dialysis membrane and the external buffer and establish equilibrium. The method to determine the amount of inorganic phosphate in solution, adapted from Murphy and Riley (*12*), also detects organic phosphate in solution or phosphate bound to caseins. To ensure that only inorganic phosphate was being measured, dephosphorylation was performed in a dialysis bag and aliquots to determine dephosphorylation were taken from the external buffer. Dephosphorylation of α_s-casein was carried out under the same conditions as β-casein.

Determining the Extent of Dephosphorylation. The extent of dephosphorylation of α_s- and β-caseins was determined on 1 mL aliquots of buffer using the method of Murphy and Riley (*12*) at time points during the reaction. Aliquots (1 mL) were diluted to 5 mL with Milli-Q water, to which 0.4 mL of mixed reagent (see below) was added and left for 10 min at room temperature before being measured using a Cary Eclipse fluorescence spectrophotometer (Varian) at 890 nm. The absorbance of the samples was compared to a standard curve derived from stock solutions of potassium phosphate (0.05−0.5 mg/L in 25 mM ammonium bicarbonate at pH 7.8). A mixed reagent is formed by mixing the following solutions to make up a total volume of 100 mL: 50 mL of H_2SO_4 (5 N) with 5 mL of antimony potassium tartrate solution (0.0085 M), 15 mL of ammonium molybdate (0.032 M), and 30 mL of ascorbic acid (0.1 M).

Mass Spectrometry. To confirm dephosphorylation of β-casein, a tryptic in-gel digest was performed according to the method of Shevchenko et al. (*13*), with the following modifications. The Coomassie-Blue-stained bands from a 15% sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) (*14*) were cut into 1 mm^2 blocks, rinsed in 100 mM NH_4HCO_3, destained using 50 mM NH_4HCO_3 with 30% acetonitrile (v/v), 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% acetonitrile (v/v) in 0.1% trifluoroacetic acid (v/v) and then in 100% acetonitrile. The extracted peptide solution was dried down to a final volume of 5 μL using a vacuum centrifuge. The peptide solutions were then added to a phosphopeptide capturing kit, MB-IMAC Fe (Bruker Daltonics, Germany), with the following modification: the peptides were eluted off with 2 μL of 5 g/L 2,5-dihydroxybenzoic acid in 0.1% phosphoric acid and then analyzed with an Ultraflex III MALDI−TOF/TOF mass spectrometer (Bruker Daltonics) in reflector mode. The identity of peptides was confirmed by matching their observed masses to the expected masses of the *in silico* tryptic digest of the known β-casein primary sequence using Biotools 3.0 software (Bruker Daltonics). Electrospray mass spectrometry (MS) experiments were performed on a Q-Tof II spectrometer (Micromass UK, Manchester, U.K.) of native and dephosphorylated α_s-casein in positive 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).

Chaperone Activity. The ability of native and dephosphorylated α_s- and β-caseins to inhibit the amorphous aggregation and subsequent precipitation of various target proteins was monitored using a light-scattering (turbidity) assay. Light scattering was monitored using a Fluostar Optima plate reader (BMG Labtechnologies) in a 96-well Falcon 3072 plate at a wavelength of 340 nm, which is indicative of protein aggregation. OT (0.5 mg/mL) was incubated at 60 °C for 4 h in 50 mM phosphate buffer (pH 7.4). ADH (1 mg/mL) was incubated at 42 °C in 50 mM phosphate buffer (pH 7.4) with 2 mM ethylenediaminetetraacetic acid (EDTA) for 2 h. α-Lactalbumin (2 mg/mL) was incubated at 37 °C in 50 mM phosphate buffer (pH 7.2) with 2 mM EDTA and 0.1 M NaCl. Aggregation of α-lactalbumin was initiated by the addition of DTT to a final concentration of 20 mM. The alteration with time in light scattering at 340 nm for each sample is presented in the plots. The change in turbidity with time in the absence of target protein was negligible in each assay.

The formation of amyloid fibrils by reduced and carboxymethylated (RCM) κ-casein (*15*, *16*) was monitored using an *in situ* ThT binding assay method adapted from Nielsen et al. (*17*). RCM κ-casein (1 mg/mL) was incubated at 37 °C for 20 h in 50 mM phosphate buffer (pH 7.4). Samples were prepared in duplicate and were incubated with 10 μM ThT in a 96-microwell plate. The plates were sealed to prevent evaporation, and the fluorescence was measured with a Fluostar Optima plate reader (BMG Labtechnologies) with a 440 nm excitation/490 nm emission filter set. The percentage protection histograms were calculated from the light-scattering 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 $k_{\text{chaperone}}$ is the rate of aggregation of the target protein with chaperone present. The rates were determined from the maximum slope of the plots.

Intrinsic and Extrinsic Fluorescence Spectroscopy. Native and dephosphorylated α_s- and β-caseins were dissolved in 50 mM phosphate buffer (pH 7.4) at 0.5 mg/mL (21 μM). A solution of 42 μM L-tryptophan was used to compare the fluorescence of free tryptophan to that of the protein-bound tryptophan. Intrinsic tryptophan fluorescence spectra were recorded at 37 °C using a Cary Eclipse fluorescence spectrophotometer (Varian) equipped with temperature control. The excitation wavelength was set at 295 nm, and emission was monitored between 300 and 450 nm. The excitation and emission slit widths were 5 nm. The fluorescence emission of buffer alone was subtracted from that obtained in the presence of protein.

Aliquots (2−4 μL) of a 10 mM stock solution of ANS (50 mM phosphate buffer at pH 7.4) were added to 21 μM native and dephosphorylated α_s- and β-caseins. 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 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. Between each addition and prior to measuring the next fluorescence reading, the solution was mixed for 1 min. The fluorescence emission of ANS in buffer alone was deducted from that obtained in the presence of protein.

Circular Dichroism (CD) Spectroscopy. Native and dephophorylated α_s- and β-caseins at 1 mg/mL were dissolved in 5 mM phosphate buffer at pH 7.4. Samples (1 mL) 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 195 and 250 nm. The spectra acquired were an average 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.

RESULTS AND DISCUSSION

Quantifying the Extent of Dephosphorylation of α_s- and β-Caseins. Dephosphorylation of α_s- and β-caseins was undertaken via the enzymatic action of alkaline phosphatase. The phosphate moieties are attached to the caseins as phosphomonoesters of serine, which upon dephosphorylation, produce serine and inorganic phosphate. The inorganic phosphate released was

determined using the method of Murphy and Riley (*12*). The phosphate mixes with the acidified ammonium molybdate to produce $PMo_{12}O_{40}^{3-}$, which has an α-keggin structure (*18*). This anion is then reduced by ascorbic acid to form the blue-colored β-keggin ion, $PMo_{12}O_{40}^{7-}$ (*18*). The amount of blue color produced is proportional to the concentration of phosphate ions present, with the absorption being measured at 890 nm with a colorimeter. For α_s- and β-caseins, this method enabled the extent of dephosphorylation over time to be determined (**Figure 1**), with approximately 90% dephosphorylation achieved for both proteins after 8 h of incubation with alkaline phosphatase.

MALDI−TOF/TOF mass spectrometry was used to confirm dephosphorylation of β-casein, while electrospray mass spectrometry was used to confirm the successful dephosphorylation of α_s-casein. β-Casein has five phosphate groups attached to serine residues at positions 15, 17, 18, 19, and 35 (*2*) and produces two phosphopeptides upon incubation with trypsin: *m*/*z* 2061.790, which corresponds to the singularly phosphorylated tryptic peptide F33−K48, and the quadruply phosphorylated peptide of *m*/*z* 3122.080, which corresponds to the peptide fragment R1−R25. Both phosphopeptides are observed in the mass spectrum of native β-casein (**Figure 2A**) but are missing in the mass spectrum of dephosphorlated β-casein (**Figure 2B**). Instead, the mass spectrum of dephosphorylated β-casein shows large peaks at *m*/*z* 1981.913 and 2802.356, which correspond to the dephosphorylated tryptic peptides F33−K48 and R1−R25, respectively (i.e., a loss of 80 Da/phosphate group in mass). Both peptides are highly acidic, with five and seven aspartic and glutamic acid residues, respectively. The MB IMAC-Fe beads, which are routinely used to isolate phosphopeptides from mixtures, bind to negatively charged regions of peptides, in particular, phosphate groups, but will also bind to acidic peptides (*19*). Hence, both peptides are observed in the mass spectrum even though they are not phosphorylated.

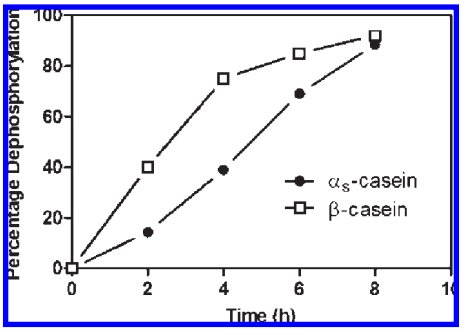

Figure 1. Dephosphorylation of α_s- and β-caseins over time using alkaline phosphatase in 25 mM ammonium bicarbonate (pH 7.8, 0.5 mM $MgCl_2$) at 37 °C.

The two proteins, α_{s1}- and α_{s2}-caseins, comprise α_s-casein and are 23.6 and 25.2 kDa in mass, respectively (*5*). Genetic variants contain a characteristic number of phosphorylated residues: 8 or 9 (α_{s1}) and 10−13 (α_{s2}). The main peak observed in the electrospray mass spectrum of native α_s-casein is at *m*/*z* 23 614, which corresponds to α_{s1} with eight phosphate groups attached, and α_{s1} with nine phosphate moieties attached has a peak at *m*/*z* 23 694; the other peaks are sodium adducts (addition of *m*/*z* 22) (**Figure 3A**). Dephosphorylated α_s-casein (**Figure 3B**) gives rise to a mass spectrum with the main peak at *m*/*z* 22 974 corresponding to the loss of eight phosphate groups from α_{s1}-casein and a peak at *m*/*z* 23 054, which corresponds to α_{s1}-casein with one phosphate group still attached. No peaks from native and dephosphorylated α_{s2}-casein were observed in the mass spectra, which most likely arises from α_{s1}-casein suppressing the ionization of α_{s2}-casein (α_{s1}-casein is the major component of α_s-casein, with an α_{s1}/α_{s2} ratio of approximately 4:1). In summary, the spectrophometric assay of phosphate content along with mass spectrometry confirmed that incubation with alkaline phosphatase led to almost complete dephosphorylation of α_s- and β-caseins.

Chaperone Activity of Dephosphorylated α_s- and β-Caseins. The effect of dephosphorylation on the chaperone activity of α_s- and β-caseins to prevent amorphous aggregation was examined by investigating the heat-induced aggregation of the target proteins, OT and ADH, and the reduction-induced aggregation of α-lactalbumin, a major whey milk protein. Turbidity was monitored by measuring the change in light scattering at 340 nm. The thermally induced aggregation at 60 °C of 0.5 mg/mL of OT commenced at around 60 min and reached a plateau after 240 min of incubation while exhibiting a sigmoidal aggregation profile (**Figure 4**). The presence of increasing amounts of β-casein led to a decrease in aggregation, such that, at a molar ratio of 3:1 native β-casein/OT, the aggregation of OT was almost completely inhibited (**Figure 4A**). Dephosphorylation of β-casein had a significant negative effect on its chaperone ability at preventing OT from aggregating (**Figure 4B**). Thus, a 3:1 molar ratio of native β-casein/OT led to 85% protection against OT aggregation, whereas the same ratio of dephosphorylated β-casein/OT

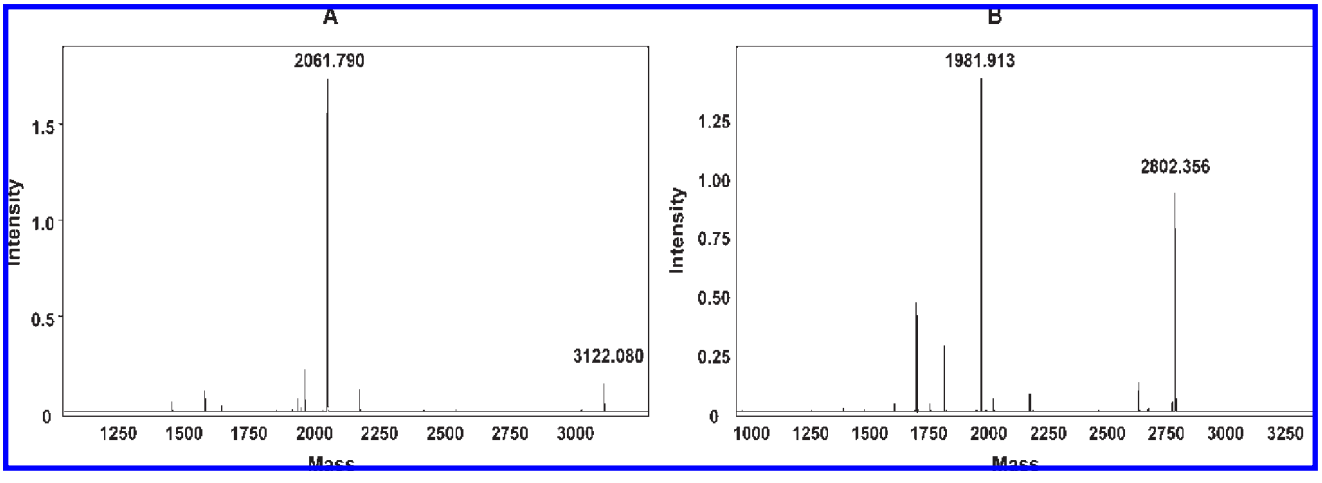

Figure 2. (A) MALDI−TOF/TOF mass spectrum of native β-casein after tryptic in-gel digest, MB IMAC-Fe separation, and elution with 5 g/L DHB in 50% ACN and 0.1% H_3PO_4. (B) Mass spectrum of dephosphorylated β-casein under the same conditions. Peak assignments corresponding to the tryptic phosphopeptides of β-casein are indicated.

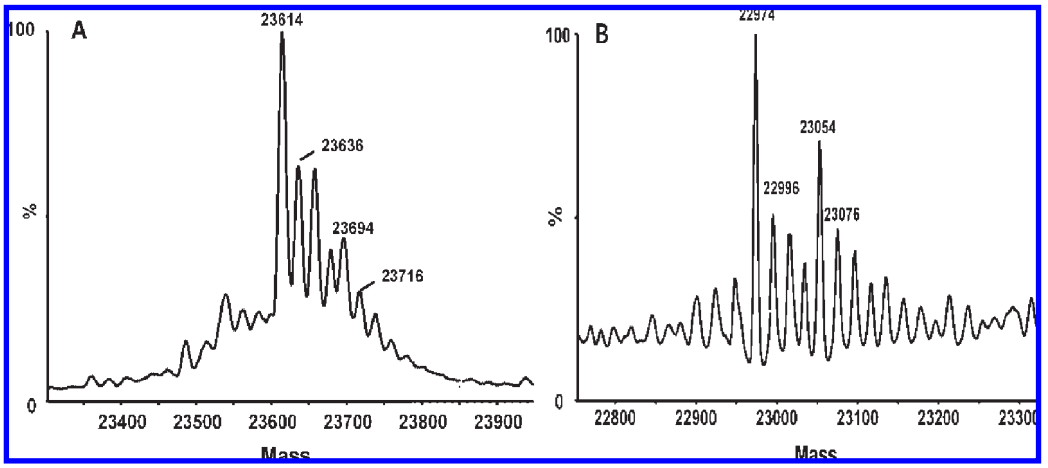

Figure 3. (A) Electrospray mass spectrum of native α_s-casein in acetonitrile/0.1% formic acid (1:1, v/v) and (B) dephosphorylated α_s-casein under the same conditions. Assignments for peaks arising from α_{s1}-casein are indicated.

only provided 40% protection (**Figure 4B**). A very similar effect was observed for native α_s-casein; a 3:1 mol ratio of α_s-casein/OT almost completely prevented aggregation of OT (**Figure 4C**), while dephosphorylated α_s-casein was less effective at inhibiting OT aggregation and subsequent precipitation (panels **D** and **E** of **Figure 4**). The chaperone action of native and dephosphorylated α_s- and β-caseins was quantified by comparing the initial rate of aggregation of target protein with and without the chaperone (**Figure 4E**). Dephosphorylation of α_s-casein and its impact on chaperone ability on the thermally induced aggregation of OT was also studied by Matsudomi et al. (*9*). However, these workers heated an OT solution at 80 °C at pH 7.0 for 20 min to promote aggregation. They came to a similar conclusion in that dephosphorylated α_s-casein had reduced ability to suppress the heat-induced aggregation of OT.

Under much milder heating conditions, when ADH was incubated at 42 °C with 2 mM EDTA to chelate the bound zinc ion, an increase in light scattering was observed at approximately 25 min with aggregation continuing over 120 min of incubation. The addition of native and dephosphorylated β-casein had an "antichaperone" effect, leading to an increase in the amount of light scattering with both ADH and β-casein co-precipitating (panels **A** and **B** of **Figure 5**) as assessed by 15% acrylamide gels (v/v) using standard techniques (*14*). The implication is that the complex formed between the two proteins is inherently unstable. A similar behavior is observed upon the interaction of reduced α-lactalbumin and a destabilizing mutant of αB-crystallin, R120G, which was attributed to their complex exposing excessive hydrophobicity to solution and precipitating out of solution (*20*). Analogously, a similar behavior may occur upon the interaction of β-casein with destabilized ADH. Consistent with this, β-casein is the most hydrophobic of the casein proteins (*1*) . In contrast, the presence of increasing amounts of α_s-casein led to a decrease in aggregation of ADH, such that, at a molar ratio of 3:1 native α_s-casein/ADH, the aggregation of ADH was completely inhibited (**Figure 5C**). Dephosphorylated α_s-casein was less efficient at inhibiting the aggregation of the unfolding ADH, such that, at a molar ratio of 3:1, dephosphorylated α_s-casein/ADH only provided 50% protection against ADH aggregation (**Figure 5D**). The rate of aggregation of ADH with and without chaperone present was quantified and expressed as a percentage of protection (**Figure 5E**).

The aggregation of α-lactalbumin was induced by the addition of DTT to reduce its four disulfide bonds (*21*), and the resultant light scattering was monitored over 360 min. A sigmoidal increase in light scattering was observed at approximately 25 min, which began to plateau after 6 h. Morgan et al. (*8*) investigated the effects of α_s-casein on the reduction-induced aggregation of α-lactalbumin under the same conditions and observed that α_s-casein inhibited aggregation in a concentration-dependent manner. In this study herein, both α_s- and β-caseins had very similar chaperone efficacies in preventing α-lactalbumin aggregation, consistent with previous studies (*8*). Both dephosphorylated α_s- and β-caseins had a markedly reduced propensity to inhibit aggregating α-lactalbumin compared to native α_s- and β-caseins. The rate of aggregation of α-lactalbumin with and without chaperone present was quantified and expressed as a percentage of protection (**Figure 6**).

Previous *in vitro* studies have shown that RCM κ-casein spontaneously forms long, rod-like aggregates at neutral pH and 37 °C that have the characteristics of amyloid fibrils (*15*, *22*). *In vivo*, fibrillar aggregation of κ-casein is kept in check by the chaperone ability of the other casein proteins in milk (i.e., α_s- and β-caseins) (*23*). Native and dephosphorylated α_s- and β-caseins were incubated with RCM κ-casein to examine their ability to prevent the fibrillation of RCM κ-casein. Interestingly, native and dephosphorylated α_s- and β-caseins had very similar chaperone ability; i.e., dephosphorylation had no effect on their chaperone ability to prevent κ-casein fibril formation (**Figure 7**).

In this study, the chaperone ability of dephosphorylated α_s- and β-caseins has been investigated with target proteins undergoing amorphous and fibrillar aggregation, different stress conditions (heat and reduction), and with various physiologically and nonphysiologically relevant target proteins. Dephosphorylation of α_s- and β-caseins had a significant effect in decreasing their chaperone ability against amorphously aggregating target milk proteins. Similar to other molecular chaperones, α_s- and β-caseins prevent the irreversible amorphous aggregation of target proteins induced by thermal and nonthermal stress by providing hydrophobic surfaces to the unfolding target proteins (*5*). The caseins are flexible, relatively unstructured molecules; their lack of stable secondary structure is due to their high content of proline residues, e.g., 8.5 and 17% for α_{s1}- and β-caseins, respectively, and high surface hydrophobicity (*2*, *24*). Another important feature responsible for casein chaperone activity is their amphiphatic nature; for example, α_{s1}-casein at pH 6.6 has a large net negative charge of −20.6 between residues E41−T80, while the remainder of the molecule has very little charge (*2*). Likewise, β-casein has a highly charged N-terminal domain containing an anionic phosphoserine cluster with a very distinct hydrophobic C-terminal domain; i.e., the first 21

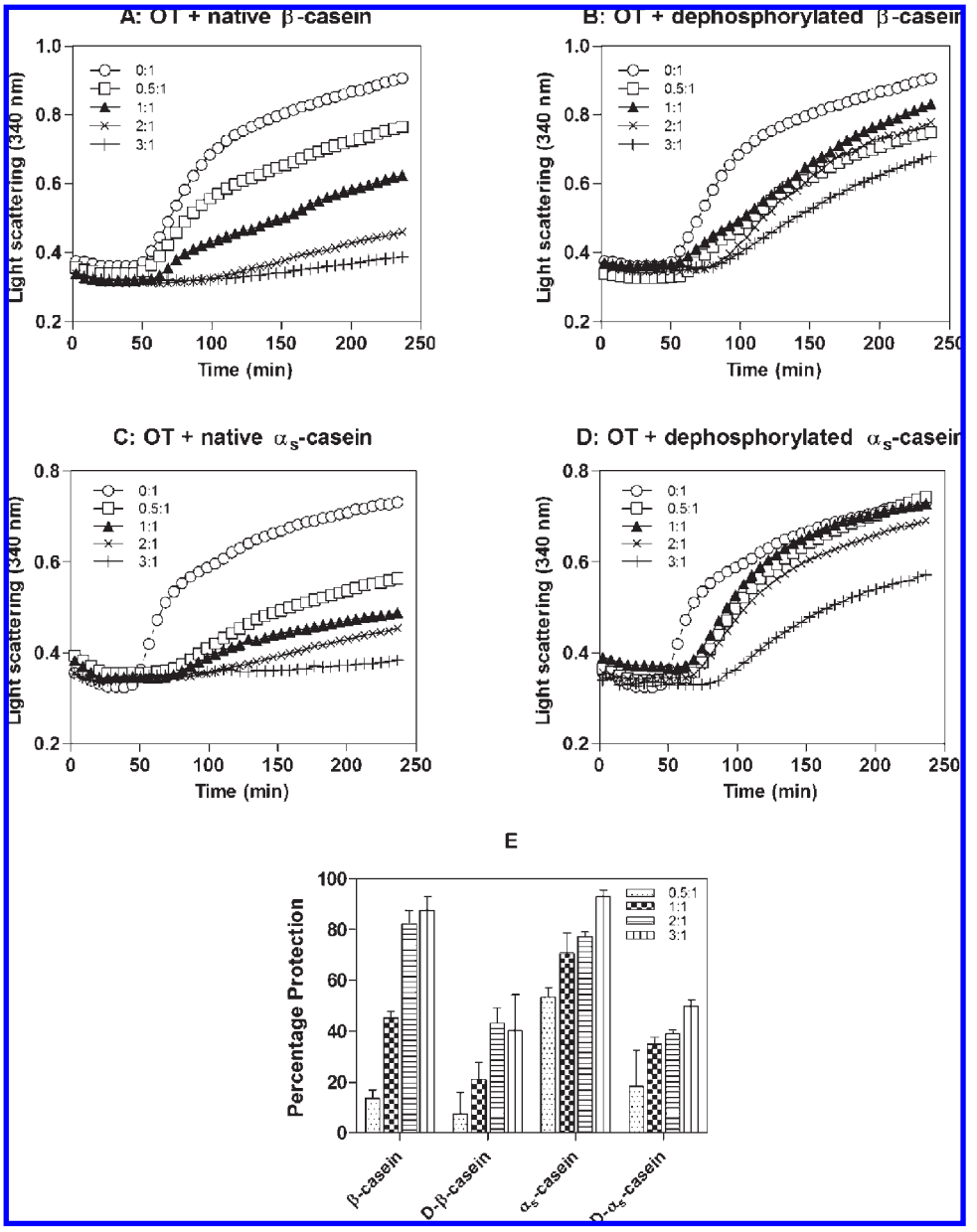

Figure 4. Thermal aggregation at 60 °C of OT (0.5 mg/mL) in 50 mM phosphate buffer at pH 7.4 in the presence of (A) increasing amounts of native β-casein, (B) dephosphorylated β-casein, (C) native α_s-casein, and (D) dephosphorylated α_s-casein. (E) Chaperone activity of native and dephosphorylated α_s- and β-caseins was quantified by comparing the initial rate of aggregation with and without chaperone present. Molar ratios are indicated. Error bars represent the standard error of at least three independent experiments.

residues have a net charge of −11.5 at pH 6.6, while the rest of the protein has essentially no charge, making it extremely amphiphatic (*1*). Thus, α_s- and β-caseins have exposed hydrophobic regions, which can bind to partially folded target proteins and solubilize the resultant complex via their highly flexible phosphoserine-rich polar regions. This structural arrangement is analogous to sHsps, where a flexible and polar C-terminal extension solubilizes the chaperone itself and the complex it forms with the target protein (*25*−*28*). Dephosphorylation of α_s- and β-caseins results in a decrease in hydrophilicity of the polar region of the protein, leading to a loss in overall amphiphatic nature and, potentially, chaperone ability. Similarly, it has been proposed that casein proteins interact with exposed hydrophobic regions of partially folded target proteins, and then the polyanion on the surface of the complex (i.e., the phosphate groups) prevents the coalescence of the complex through electrostatic repulsion, thus inhibiting large-scale aggregation and precipitation (*8*, *9*).

Interestingly, the loss of the amphiphatic nature of α_s- and β-caseins as a result of dephosphorylation had no effect on the chaperone ability of these proteins to prevent fibril formation of RCM κ-casein. κ-Casein, similar to the other caseins, is very amphiphatic; its N-terminal domain (Q1−F105) constitutes a hydrophobic core with a high content of aromatic residues, including the amyloid fibril core region (*15*), while its C-terminal domain (M106−V169) is highly hydrophilic and bears up to six O-glycosylation sites, which solubilizes the protein and has a predominant role in stabilizing the casein micelle (*29*). The N-terminal domain is proposed to contain two adjacent antiparallel β strands (*30*), which would provide a template for the stacking of κ-casein molecules to form the core of the amyloid fibril (*15*). RCM κ-casein has a unique mechanism of fibril formation, in which the rate-determining step is the dissociation from the aggregated state, leading to a monomeric species, which is highly amyloidogenic (*15*, *23*). The rapid rate of RCM κ-casein

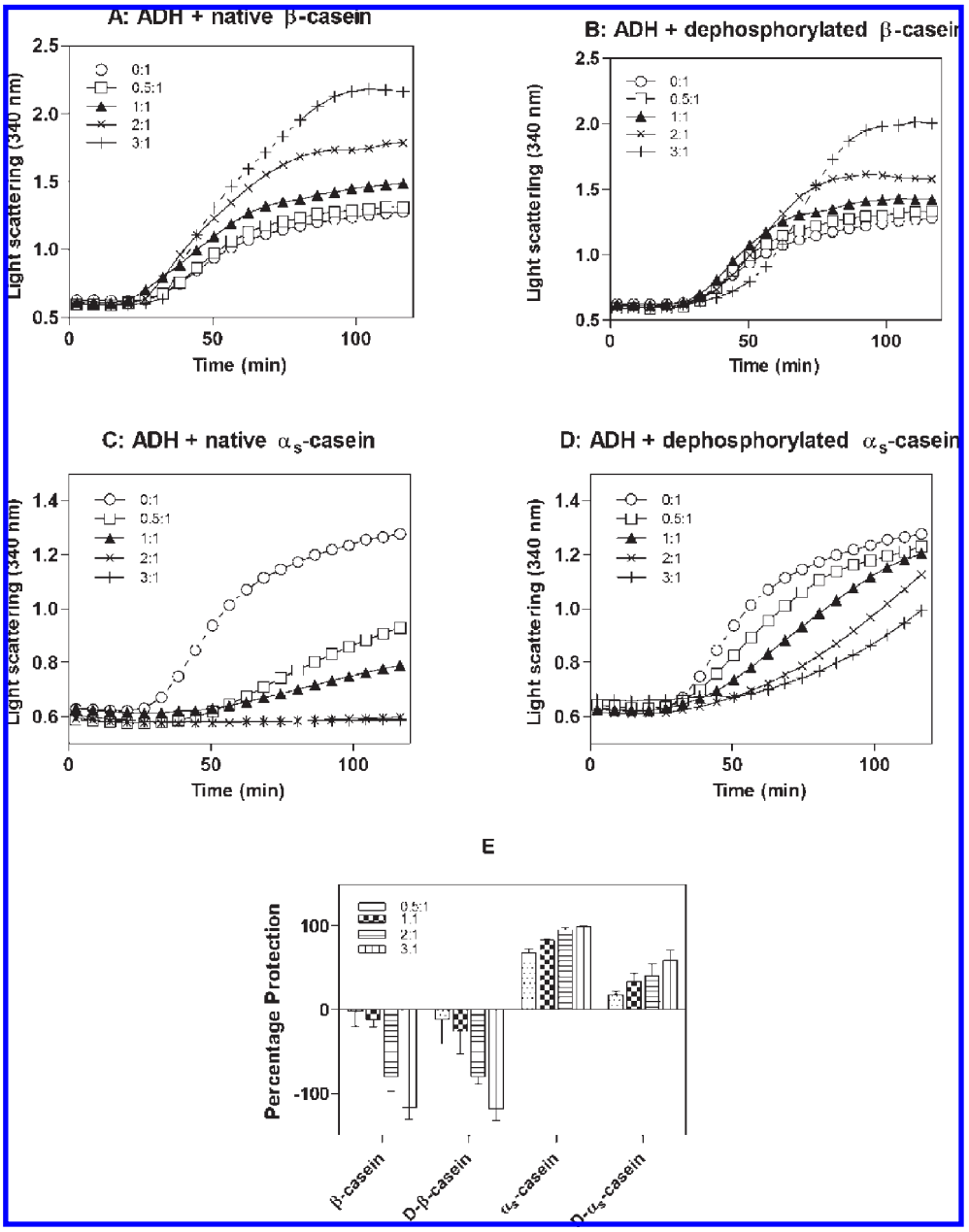

Figure 5. Thermal aggregation at 42 °C of ADH (1 mg/mL) in 50 mM phosphate buffer (pH 7.4, 2 mM EDTA) in the presence of increasing amounts of (A) β-casein, (B) dephosphorylated β-casein, (C) native α_s-casein, and (D) dephosphorylated α_s-casein. (E) Chaperone activity of native and dephosphorylated α_s- and β-caseins was quantified by comparing the initial rate of aggregation with and without chaperone present. Molar ratios are indicated. Error bars represent the standard error of at least three independent experiments.

aggregation means its interaction with α_s- or β-casein may not be dependent upon the phosphorylation state of the chaperone proteins. In contrast, amorphous aggregation of target proteins is much slower than RCM κ-casein fibril formation from its monomeric state, which would enable optimal interaction with the chaperones (α_s- and β-caseins) and hence be dependent upon their phosphorylation state. A marked variation in chaperone efficiency between fibril formation and amorphous target protein aggregation has been observed with the sHsp, αB-crystallin (*31*, *32*). Furthermore, Ecroyd et al. (*32*) observed variation in efficiency of chaperone ability for αB-crystallin when interacting with different fibril-forming target proteins. For αB-crystallin, these observations may reflect a different mechanism of chaperone action against amorphous and fibrillar target protein aggregation (*33*). By analogy, similar behavior may be occurring with α_s- and β-caseins.

Structural Studies. Natively unfolded proteins, such as caseins, are characterized by having numerous uncompensated charged groups, resulting in a large net charge at neutral pH and a low overall content of hydrophobic amino acid residues (*34*, *35*). The caseins have a high net negative charge arising from mainly phosphoserine residues and are not exceptionally hydrophobic (*1*). The high net charge leads to charge−charge repulsion and low hydrophobicity results in less driving force for a compact structure (*3*). The removal of phosphoserine residues upon dephosphorylation significantly reduces the negative charge on the protein and may lead to significant structural alteration. Accordingly, intrinsic fluorescence, extrinsic fluorescence (ANS binding), and CD spectroscopy were used to investigate changes in the structure of α_s- and β-caseins upon dephosphorylation.

The intrinsic fluorescence spectra of native and dephosphorylated α_s- and β-caseins were acquired at neutral pH and 37 °C.

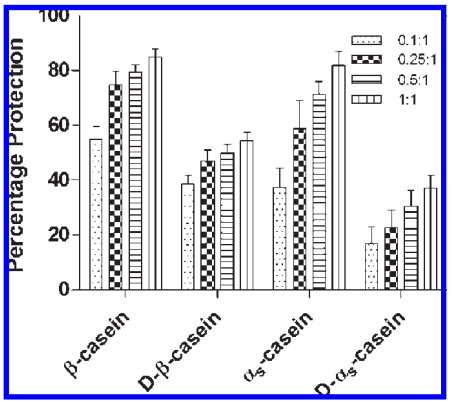

Figure 6. Reduction of α-lactalbumin (2 mg/mL) induced by the addition of 20 mM DTT in 50 mM phosphate buffer (pH 7.1, 2 mM EDTA and 0.1 M NaCl) at 37 °C and monitored by light scattering at 340 nm over 360 min in the presence of increasing amounts of native and dephosphorylated α_s- and β-caseins. The chaperone activity of native and dephosphorylated α_s- and β-caseins was quantified by comparing the initial rate of aggregation with and without chaperone present. Molar ratios are indicated. Error bars represent the standard error of at least three independent experiments.

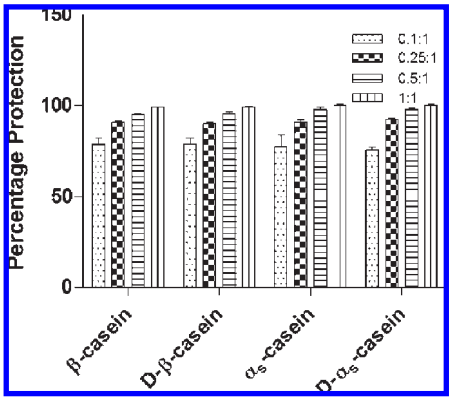

Figure 7. Fibril formation of RCM κ-casein (1 mg/mL) was induced by incubation at 37 °C in 50 mM phosphate buffer at pH 7.4 and was monitored over 20 h in the presence of increasing amounts of native and dephosphorylated α_s- and β-caseins. The chaperone activity of native and dephosphorylated α_s- and β-caseins was quantified by comparing the initial rate of aggregation with and without chaperone present. Molar ratios are indicated. Error bars represent the standard error of at least three independent experiments.

The fluorescence of free L-tryptophan under the same conditions was used to compare to that of the protein-bound tryptophan. The fluorescence of tryptophan residues is highly dependent upon their environment (*36*). α_{s1}- and α_{s2}-caseins each contain two tryptophan residues at positions 164 and 199 and 109 and 193, respectively, while β-casein has one tryptophan residue at position 143 (*1*). Accordingly, native and dephosphorylated β-casein exhibits lower fluorescence intensity compared to native and dephosphorylated α_{s1}- and α_{s2}-caseins (**Figure 8**). Native β-casein has a wavelength of maximum fluorescence at 346 nm, while dephosphorylated β-casein has a wavelength of maximum fluorescence at 342 nm and higher fluorescence intensity (**Figure 8**). For native α_s-casein, a wavelength of maximum fluorescence was observed at 348 nm, while removal of the phosphate groups resulted in a red shift to 341 nm and an increase in the fluorescence intensity (**Figure 8**). Thus, the intrinsic fluorescence spectra indicate that the tryptophan residues of both α_s- and β-caseins

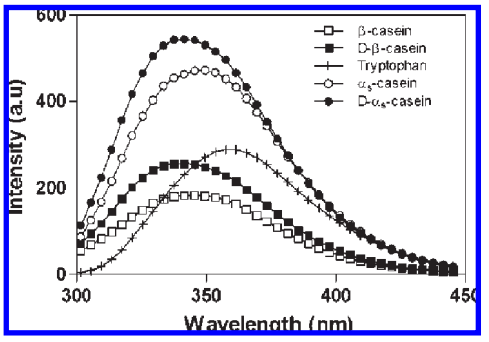

Figure 8. Intrinsic fluorescence spectra of native and dephosphorylated α_s- and β-caseins (0.5 mg/mL) and L-tryptophan in 50 mM phosphate buffer at pH 7.4 and 37 °C.

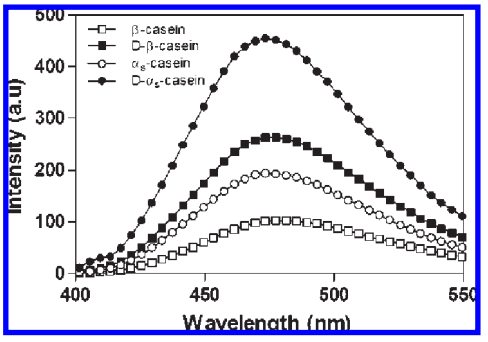

Figure 9. Extrinsic ANS fluorescence spectra of native and dephosphorylated α_s- and β-caseins (0.5 mg/mL in 50 mM phosphate buffer at pH 7.4 and 37 °C).

are less exposed to solution upon dephosphorylation and are in a more internal hydrophobic environment (*36*).

ANS is a fluorescent "hydrophobic probe" for examining proteins, which binds to nonpolar, exposed, and clustered hydrophobic regions of proteins and membranes and fluoresces (*37*, *38*). Aliquots of a stock solution of ANS were added to solutions of native and dephosphorylated α_s- and β-caseins 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. Native β-casein fluorescence intensity was approximately 100 au, which increased to 260 au upon dephosphorylation (**Figure 9**). Likewise, ANS bound to native α_s-casein had a maximum fluorescence intensity of 190 au, which increased to 450 au upon dephosphorylation (**Figure 9**). Thus, α_s- and β-caseins experienced a large increase in exposed clustered regions of hydrophobicity upon dephosphorylation. While ANS becomes fluorescent upon binding to hydrophobic sites, its binding affinity is considered to be a consequence of electrostatic and hydrophobic effects (*39*). ANS is an amphiphatic molecule, which contains an anionic sulfonate; thus, the removal of negatively charged phosphate moieties of α_s- and β-caseins upon dephosphorylation may also increase the ability of ANS to bind to the protein. The increase in extrinsic fluorescence observed may therefore not be entirely attributed to conformational changes within the protein upon dephosphorylation.

The far-UV CD spectra of native and dephosphorylated α_s- and β-caseins were used to probe the overall secondary-structural changes upon dephosphorylation. The CD spectra of α_s- and β-caseins were very similar to those in the literature (*40*–*42*) and were indicative of proteins that have significant regions of little ordered structure (panels **A** and **B** of **Figure 10**). The CD spectrum of α_s-casein upon dephosphorylation exhibited

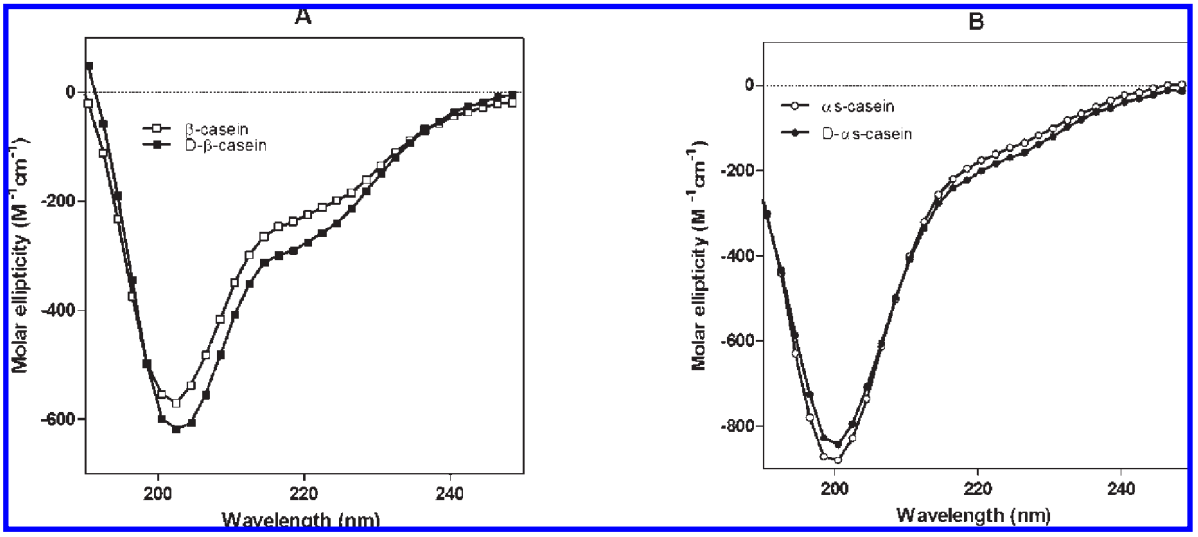

Figure 10. Far-UV CD spectra of (A) native and dephosphorylated β-casein and (B) native and dephosphorylated α_s-casein (0.2 mg/mL in 10 mM phosphate buffer at pH 7.4 and 25 °C).

a decrease in molar ellipticity at around 200 nm, which corresponds to a decrease in the random-coil structure. In contrast, the CD spectrum of β-casein exhibited a slight increase. Removal of the phosphate groups in both proteins resulted in an increase in molar ellipticity at 218 and 222 nm, corresponding to an increase in α-helix and β-sheet structure, respectively (panels **A** and **B** of **Figure 10**). It is concluded that secondary structure in α_s- and β-caseins is present to a greater extent in the dephosphorylated species, i.e., when the repulsion of strongly charged groups is decreased. Furthermore, the removal of bulky phosphate groups reduces steric hindrance in the vicinity of the serine residues, which may facilitate the enhancement of secondary structure. Molecular modeling simulations of the β-casein peptide (1−25) by Farrell et al. (*40*) indicated that the densely packed negative charges in this region cause the native peptide to be more rigid and stiff, while the dephosphorylated peptide assumes a much more flexible and dynamic structure (*40*), which may facilitate alterations to adopt greater secondary structure upon dephosphorylation. Consistent with our results, a study on structure induced by dephosphorylation of β-casein and the resultant effect on emulsion properties of the protein observed an increase in secondary structure of β-casein and its amphiphilic fragment [1−105/107] upon dephosphorylation (*43*).

The ability of α_s- and β-caseins to act as chaperones to prevent the aggregation of amorphously aggregating target proteins is reduced upon dephosphorylation. The CD and intrinsic fluorescence spectroscopic data are consistent with greater secondary structure being present for α_s- and β-caseins upon dephosphorylation. Although these species have some elements of enhanced structure, the large decrease in chaperone ability of α_s- and β-caseins upon dephosphorylation against amorphously aggregating target proteins is most likely because of a loss of charge and the amphiphilic nature of the proteins. α_s- and β-Caseins have exposed hydrophobic regions, which can bind to partially folded target proteins and solubilize them via their highly flexible phosphoserine-rich polar regions. The removal of phosphate groups reduces the hydrophilicity of α_s- and β-caseins and decreases their ability to solubilize unfolding target proteins upon binding. The lack of an effect on chaperone ability of α_s- and β-caseins upon dephosphorylation when interacting with the fibril-forming protein RCM κ-casein may reflect a different mechanism of chaperone action with amorphously aggregating and fibril-forming target proteins, as is observed with sHsps, and/or that the unique mechanism of RCM κ-casein fibril formation does not affect the interaction with native or dephosphorylated α_s- or β-casein.

ABBREVIATIONS USED

ADH, alcohol dehydrogenase; OT, ovotransferrin; RCM, reduced and carboxymethylated; DTT, 1,4-dithiotheritiol; ThT, thioflavin T; ANS, 8-anilino-1-napthalene sulfonate; CD, circular dichroism; sHsps, small heat-shock proteins.

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NOTE ADDED IN PROOF

Yousefi et al. (*Biopolymers* **2009**, *91*, 623−632.) have recently published a paper that draws similar conclusions to those presented herein with respect to the effect of dephosphorylation of β-casein on its chaperone activity. In the case of this work, however, a recombinant form of β-casein without the post-translational addition of phosphate groups was used to compare its chaperone action with that of the native β-casein.

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