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Casein Proteins as Molecular Chaperones

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Under conditions of stress, such as elevated temperature, molecular chaperones stabilize proteins from unfolding, aggregating, and precipitating. We have investigated the chaperone activity of the major milk proteins α_S-, β-, and κ-casein with reduced insulin and the milk whey proteins, α-lactalbumin and β-lactoglobulin, and compared it with that of the mammalian small heat shock protein (sHsp), α-crystallin, and clusterin. α_S-Casein exhibited different chaperone behavior under reduction and heat stresses, i.e., chaperone activity increased with increasing temperature (as observed with α-crystallin), but under reduction stress, its chaperone activity increased at lower temperatures. β- and κ-casein had comparable chaperone ability with each other but were less effective than α_S-casein. Under molecular crowding conditions, precipitation of stressed protein was accelerated, and α_S-casein was a poorer chaperone. Furthermore, at slightly alkaline pH values, α_S-casein was a less effective chaperone than at neutral pH. Detailed fluorescence, size exclusion chromatography, and real-time NMR studies studies indicated that the casein proteins underwent conformational changes and stabilized the partially unfolded whey proteins prior to formation of high molecular weight soluble complexes. These results are consistent with casein proteins acting as molecular chaperones in a manner similar to sHsps and clusterin.

KEYWORDS: Milk proteins; α_S-casein; β-casein; κ-casein; molecular chaperones; stability of milk proteins

INTRODUCTION

Milk is a heterogeneous mixture containing a plethora of components (amino acids, carbohydrates, lipids and minerals etc.), all of which contribute to its nutritional value. The components can be divided into two portions, the whey and casein fractions (*1*). Casein is perhaps the best characterized milk protein and constitutes over 70−80% of total bovine milk protein (*2*). In its native state in milk, casein exists as large macromolecular associates in colloidal dispersion. These "micelles" are $\sim 10^8$ Da in mass and $\sim$200 nm in size (*3*) and comprise four different casein subunits and calcium phosphate with few dissociated casein subunits (*4*). The relative proportion of these subunits, α_{S1}-, α_{S2}-, β-, and κ-casein, varies among species (*5*). In bovine milk, 65% of the casein fraction is comprised of the α_{S1}- and α_{S2}- subunits of 23.6 and 25.2 kDa in mass (known collectively as α_S-casein) that are not sequence related (*6*). S1 is the dominant α_S subunit. Structurally, caseins are unusual in that they are neither strictly globular nor fibrous proteins (*7*) and do not possess well-defined secondary or tertiary structure (*6*). Dunker et al. (2002) has classified α_S-casein as an intrinsically disordered protein implying that the function of the protein arises from its unstructured state (*8*).

The food industry has employed casein and caseinate products for their nutritional and functional value for many years (*9*). However, the observation that α_S-casein acts as a molecular chaperone under conditions of stress, e.g., elevated temperature, whereby it stabilizes other proteins (including milk proteins) to prevent their aggregation and potential precipitation (*6*), provides new possibilities for the use of casein proteins as well as further insights into the properties and stability of milk and milk products.

Molecular chaperone proteins are involved in the folding, transport, assembly, degradation, and stabilization of proteins (*10*). Several properties of α_S-casein are similar to those of other molecular chaperones, in particular the small heat shock proteins (sHsps) and clusterin (*11*, *12*). sHsps are ubiquitous, intracellular proteins that have a variable quaternary arrangement and little overall sequence similarity among their members (*13*). They recognize, stabilize, and bind to non-native states of proteins that result from cellular stresses (e.g., heat, oxidation, reduction) and thereby prevent their aggregation and precipitation. Clusterin is an extracellular mammalian protein that shares little sequence

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similarity to sHsps but similarly functions as a chaperone (*12*). The precise mechanism of chaperone action is not known for sHsps and clusterin. Most likely, hydrophobic interactions with partially folded target proteins are primarily involved in complex formation with hydrophobicity of the complex being offset by the solubilizing action of highly mobile hydrophilic regions (*11*, *12*). The tendency of the caseins to associate into large heterogeneous aggregates or micelles is similar to the behavior of many sHsps (*14*) and clusterin (*12*). The heterogeneous and dynamic nature of sHsps and clusterin is believed to be an important factor in their chaperone function (*11*, *12*).

Bhattacharyya and Das (*6*) reported that α_S-casein is able to prevent the aggregation of whey proteins and unrelated target proteins under various stress conditions. This promiscuous nature of chaperone action is another common feature of molecular chaperones. It was also shown that α_S-casein solubilized aggregated proteins and had enhanced chaperone action at lower temperatures (*6*). In addition, Matsudomi and co-workers recently showed that α_s-casein suppresses the heat-induced aggregation of egg white ovotransferrin. (*15*) The mechanism by which α_S-casein stabilizes and prevents the precipitation of other proteins in milk (e.g., β- and κ-casein, α-lactalbumin and β-lactoglobulin) is of particular interest to the dairy industry as a possible means of developing an alternative method for long-life milk treatment. In addition, a better molecular understanding of the stabilization and aggregation processes in milk are potential avenues to create new milk based products with novel textures and other organoleptic properties (*16*).

In this study, we have investigated the chaperone action of α_S-casein under a variety of experimental conditions including heat, reduction stress, pH and molecular crowding. The other casein proteins, β and κ, were also examined for potential chaperone ability. The formation of target-chaperone complexes between α_S-casein and a range of proteins was explored spectroscopically and chromatographically, and the conformation of the target protein, reduced α-lactalbumin, as it interacts with α_S-casein was investigated by real-time ^{1}H NMR spectroscopy. Direct comparisons of the chaperone ability of α_S-casein with that of the mammalian sHsp, α-crystallin (*17*), were also made.

MATERIALS AND METHODS

Materials. General reagents such as dibasic and diprotic sodium phosphate, sodium chloride, ethylenediamine tetraacetic acid (EDTA), sodium azide, 8-anilino-1-naphthalenesulfonic acid (ANS), dithiothreitol (DTT), and dextran (average molecular mass 78 kDa) were obtained from Sigma-Aldrich (St. Louis, U. S.A) and were of reagent grade. The bovine milk proteins, α-, β-, and κ-casein, α-lactalbumin, and β-lactoglobulin were also obtained from Sigma-Aldrich (St. Louis), as was insulin from bovine pancreas. α-Crystallin was purified from bovine lenses as previously described (*18*). Deuterated water (D_2O) and deuterated dithiothreitol (DTT) were obtained from Cambridge Isotope Laboratories (Andover, MA.).

Visible Absorption Spectroscopy. All UV−vis spectroscopic experiments for the various conditions described were performed in duplicate. Experiments examining the ability of α_S-casein to prevent the aggregation and precipitation of reduced apo and holo α-lactalbumin (2 mg mL^{-1}) and insulin (0.25 mg mL^{-1}) were performed at both 25 and 37 °C using a Spectramax 250 microplate reader (Molecular Devices, Sunnyvale, CA). Experiments were also performed with insulin to examine the chaperone action of β- and κ-casein. Experiments with apo α-lactalbumin and insulin as target proteins were undertaken in 50 mM sodium phosphate buffer, pH 7.1, 0.1 M NaCl, 2.5 mM EDTA, and 0.02% NaN_3. Experiments with holo α-lactalbumin as the target protein were performed in 50 mM imidazole buffer, pH 7.1, 0.1 M NaCl, 5 mM $CaCl_2$, and 0.02% NaN_3, and those with apo α-lactalbumin or insulin as the target protein were performed in 50 mM sodium phosphate buffer, pH 7.1, 0.1 M NaCl, 2.5 mM EDTA, and 0.02% NaN_3.

The ability of α_S-casein and α-crystallin to prevent the aggregation and precipitation of β-lactoglobulin (5 mg mL^{-1}) was investigated by monitoring light scattering at 360 nm and 70 °C using a Cary 500 Scan spectrophotometer (Varian, Palo Alto). Precipitation of β-lactoglobulin in 50 mM sodium phosphate buffer, 0.2 M NaCl, 2.5 mM EDTA, and 0.02% NaN_3 was heat-induced and was monitored by an increase in light scattering at 360 nm for up to 8 h. Control experiments were also performed in which both chaperones (each at 5 mg mL^{-1}) were heated at 70 °C for the same duration in identical buffer and examined for any increase in light scattering at 360 nm.

The chaperone ability of α_S-casein at different pH conditions was also investigated with heat-stressed β-lactoglobulin as described above. Chaperone and target protein concentrations of 5 mg mL^{-1} in 800 μL of 50 mM sodium phosphate buffer, 0.2 M NaCl, 2.5 mM EDTA, and 0.02% NaN_3 were employed at pH values of 7.0, 7.5, and 8.0. Light scattering measurements at 360 nm were taken for 10 hours.

Aggregation and precipitation of α-lactalbumin and insulin were initiated by addition of dithiothreitol (DTT, Sigma-Aldrich, St. Louis) to a final concentration of 20 mM and monitored by an increase in light scattering at 360 nm over time (6 h for α-lactalbumin and 2 h for insulin). Comparison was also made, in selected cases, between the relative chaperone ability of α_S-casein and α-crystallin, β-casein and κ-casein, and a mixture of α- (60%), β- (25%), and κ-casein (15%) to mimic the approximate proportions found in milk (*2*, *19*). The effects of combining α_S-casein and α-crystallin upon chaperone ability of each protein were also examined.

Effects of Molecular Crowding. Effects of molecular crowding on the chaperone action of α_S-casein were examined by the addition of 1−20% (w/v) dextran (average molecular mass 78 kDa) to reduced α-lactalbumin and insulin, under the same conditions used for the visible absorption spectroscopy experiments.

Intrinsic Fluorescence. The intrinsic fluorescence of the two C-terminal tryptophan residues in α_S-casein was selectively monitored as a function of temperature in the presence and absence of insulin and/or DTT (20 mM) in 50 mM sodium phosphate buffer, pH 7.1, 0.1 M NaCl, 2.5 mM EDTA and 0.02% NaN_3. Control experiments containing combinations of insulin and/or α_S-casein, both in the presence and absence of DTT, were also performed. Solutions of α_S-casein at 0.3125 mg mL−1 and insulin at 0.625 mg mL−1 were incubated in 200 μL volumes of buffer at 15, 25 or 37 °C for 1 h. To avoid any signal quenching by DTT, samples were diluted to 2 mL with buffer immediately before fluorescence measurements were performed (giving final concentrations of 0.03125 mg mL^{-1} α_S-casein and 0.0625 mg mL^{-1} insulin). Samples were excited at 295 nm, and fluorescence emission was measured from 300 to 400 nm, using a Hitachi F-4500 fluorescence spectrophotometer (Hitachi, Tokyo, Japan). Slit widths of 5.0 and 10.0 nm, were used for excitation and emission, respectively.

Changes in the wavelength of maximum intrinsic fluorescence of α_s-casein with temperature (15, 25 and 37 °C) were also carried out at a higher α_S-casein concentration of 0.25 mg mL^{-1} after dilution. Slit widths of 2.5 and 5.0 nm, were used for excitation and emission, respectively.

ANS Binding Fluorescence. The increase in fluorescence of the hydrophobic probe 8-anilino-1-naphthalenesulfonic acid (ANS) upon binding to α_S-casein, in the presence and absence of insulin with and without DTT, was measured using identical sample buffers and protein concentrations as for visible absorption spectroscopy. Samples were prepared with or without 20 mM DTT in a final volume of 200 μL and were incubated for 1 h at 15, 25, or 37 °C. To avoid signal quenching by DTT, samples were diluted to 2 mL prior to fluorescence measurement, and the solutions were equilibrated at the temperature of analysis for five minutes. Aliquots (3 μL) of stock ANS (10 mM) were added to samples and then stirred for 30 s before a fluorescence reading was taken. Sequential aliquots of ANS were added until a plateau of maximum fluorescence was reached (usually after addition of 24−30 μL of stock ANS). ANS fluorescence was measured with an excitation wavelength of 387 nm and emission of 479 nm. Slit widths for excitation and emission were 5.0 and 10.0 nm, respectively. Changes in fluorescence from ANS itself were found to be negligible over the range 15−37 °C.

Size-Exclusion HPLC. The formation of a complex between apo α-lactalbumin and α_S-casein and between β-lactoglobulin and α_S-casein was investigated using size-exclusion HPLC. A Phenomenex BioSep SEC S4000 column (Phenomenex, Torrance, CA) was used with a mobile phase of 50 mM sodium phosphate buffer, pH 7.2 with a GBC/ICI LC 1150 pump and 1440 systems organizer (ICI, London) for apo α-lactalbumin and α_S-casein samples. For α_S-casein and β-lactoglobulin samples, the same column and mobile phase conditions were used with a Shimadzu LC-10AT liquid chromatograph with SCL-10A system controller.

A mixture of apo α-lactalbumin (2.5 mg) and α_S-casein (5 mg) was prepared in 250 μL of 50 mM sodium phosphate buffer, pH 7.2, 2.5 mM EDTA, 0.1 M NaCl and stressed by incubation with 20 mM DTT at 25 °C for 2 h. A mixture of β-lactoglobulin (2.5 mg) and α_S-casein (5 mg) was also prepared in 250 μL of 50 mM sodium phosphate buffer, pH 7.2, 2.5 mM EDTA, 0.2 M NaCl and stressed at 70 °C for 3 h. Samples of unstressed β-lactoglobulin, α_S-casein and apo α-lactalbumin (2.5 mg each) were prepared in 250 μL of 50 mM sodium phosphate buffer, pH 7.2, 2.5 mM EDTA, 0.1 M NaCl for the purposes of comparison. Additional controls containing a mixture of β-lactoglobulin and α_S-casein that were not subjected to heat stress and heat stressed β-lactoglobulin alone (prepared in the same manner as described above) were also included. All samples were centrifuged (Beckman Microfuge E) at 14 000 rpm for 5 min before 10 μL aliquots were injected onto the column. The mobile phase flow rate was 0.5 mL min^{-1} and peaks were detected at 280 nm on a GBC/ICI SD 2100 or Shimadzu SPD-10A UV−vis detector. A set of molecular mass standards (blue dextran, bovine thyroglobulin, catalase, bovine serum albumin (BSA), and ovotransferrin) were prepared under identical conditions at a concentration of 10 mg mL^{-1} and chromatographed as described above.

Real-Time ^{1}H NMR Spectroscopy. ^{1}H NMR spectroscopy was performed at 400 MHz on a Varian Unity-400 NMR spectrometer at 37 °C. Samples containing α-lactalbumin (10 mg) in the presence and absence of α_S-casein (20 mg) were prepared in 50 mM sodium phosphate buffer, pH 7.2 with 0.1 M NaCl, 2.5 mM EDTA, and 0.02% (w/v) NaN_3. The final sample volume was 700 μL in 99.9% D_2O. A series of 1D ^{1}H NMR spectra were collected with time immediately following the addition of deuterated DTT to a final concentration of 20 mM. Spectra were acquired over 16 000 data points with a spectral width of 4500 Hz. A total of 16 scans were collected for each experiment with a line broadening of 1 Hz applied prior to Fourier transformation. Solvent suppression was achieved via presaturation.

RESULTS

Chaperone Action of αs, β, and κ-Caseins with Reduced Insulin. Bhattacharyya and Das (*6*) have previously shown that over a range of temperatures, α_S-casein is able to act as a chaperone in preventing the aggregation and precipitation of reduced insulin arising from its B-chain. Under the conditions used in our experiments, α_S-casein was found to have similar chaperone action to that reported by Bhattacharyya and Das (*6*) at 25 and 37 °C (**Figure 1a,d**). The chaperone ability of α_S-casein at 25 °C was slightly better than that at 37 °C. When compared to α-crystallin, α_S-casein was a more efficient chaperone under the same experimental conditions at 37 °C. Complete suppression of aggregation was observed at a 0.48:1 molar subunit ratio of α_S-casein:insulin (2:1 w:w ratio) (**Figure 1d**), whereas a 1:1 molar subunit ratio of α-crystallin:insulin was required to prevent precipitation of the target protein (3.5:1 w:w ratio) (*20*).

Experiments examining the ability of β- and κ-casein to prevent DTT-induced insulin aggregation showed that these casein subunits also possess chaperone action at both 25 °C (**Figure 1b,c**) and 37 °C (**Figure 1e,f**). In contrast to α_S-casein, the chaperone ability of β- and κ-casein was similar at 25 and 37 °C. Both β- and κ-casein provided somewhat less protection than α_S-casein on a w:w (and molar) basis and were quite similar to each other in their effectiveness. The chaperone action of a combination of α- (60%), β- (25%), and κ-casein (15%) was also examined in order to mimic approximately the relative concentrations of these casein subunits found in milk (*19, 21*). On a w:w basis, this combination of caseins had very similar chaperone action to α_S-casein alone at both 25 and 37 °C (panels a and d, respectively, of **Figure 1**) when the total protein concentration was compared with that of the individual casein subunits; i.e., 1:1 (w:w) ratios of casein mixture:insulin and $\alpha_S$casein:insulin completely suppressed insulin B-chain precipitation in both cases (data not shown).

Intrinsic Fluorescence of α_s-Casein and Its Mixtures with Reduced Insulin. The bovine α-casein subunits, α_{S1} and α_{S2}, each contain two tryptophan residues in their C-terminal regions, at residues 164 and 199 in α_{S1}-casein and 109 and 193 in α_{S2}-casein (*2*). Changes in fluorescence intensity, and the wavelength at which maximum fluorescence intensity occurs, make it possible to determine whether there are any alterations in the polarity of the environment of tryptophan residues during chaperone interaction with a target protein. Reduced insulin was used as the target protein. Since it contains no tryptophan residues, changes in fluorescence of a mixture of the two proteins could be attributed solely to the tryptophan residues in α_S-casein. Also, as α_{S1}-casein is the predominant subunit of α_S-casein (in a ratio of approximately 4:1 (*19*)), α_S-casein tryptophan fluorescence can be attributed largely to the two C-terminal tryptophan residues of the α_{S1} subunit. An increase in maximum fluorescence intensity accompanied by a shift of the wavelength of maximum fluorescence to shorter wavelengths indicates that the polarity around these tryptophan residues has decreased, and vice versa (*22*).

The intrinsic fluorescence of a range of solutions containing insulin and/or α_S-casein, both in the presence and absence of DTT, was measured at 15, 25, and 37 °C (**Figure 2**). The relative fluorescence intensities of all of the combinations of

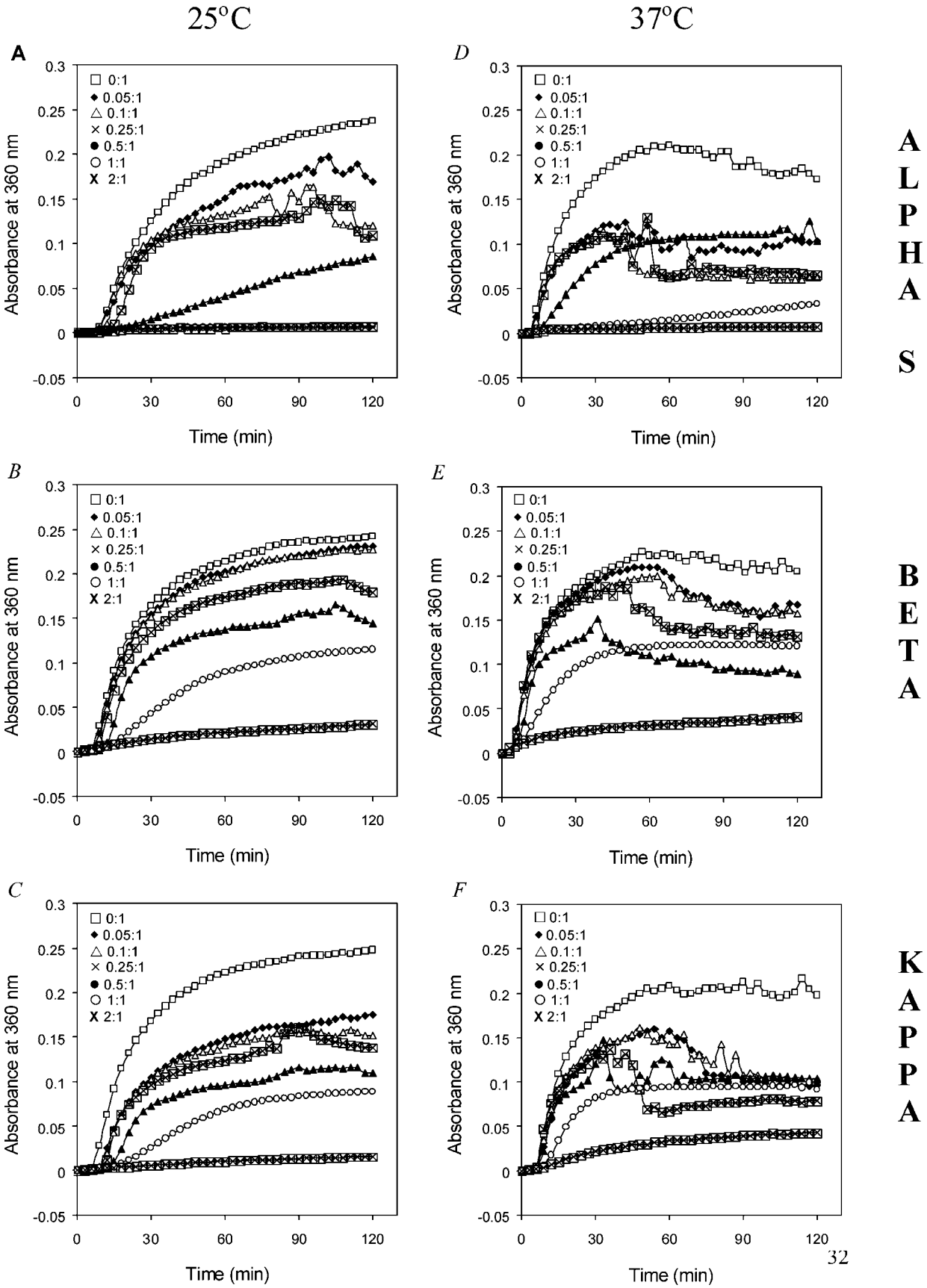

Figure 1. Prevention of DTT-induced aggregation of insulin by α-, β- or κ-casein. Insulin (0.25 mg mL^{-1}) was incubated at 25 °C (a–c) and 37 °C (d–f) for 120 min in the presence of increasing ratios (w:w) of α- β- or κ-casein. Aggregation of the insulin was induced by the addition of DTT to a final concentration of 20 mM at the commencement of the experiment. (A) α_S-casein at 25 °C; (B) β-casein at 25 °C; (C) κ-casein at 25 °C; (D) α_S-casein at 37 °C; (E) β-casein at 37 °C; (F) κ-casein at 37 °C. Ratios are given as the ratio (w:w) of casein:insulin, and in each case the 0:1 ratio corresponds to target protein aggregation in the absence of chaperone. The decrease in light scattering observed at longer time periods for some solutions arises from settling of the aggregates to the bottom of the individual wells. Results shown are representative of at least two independent experiments.

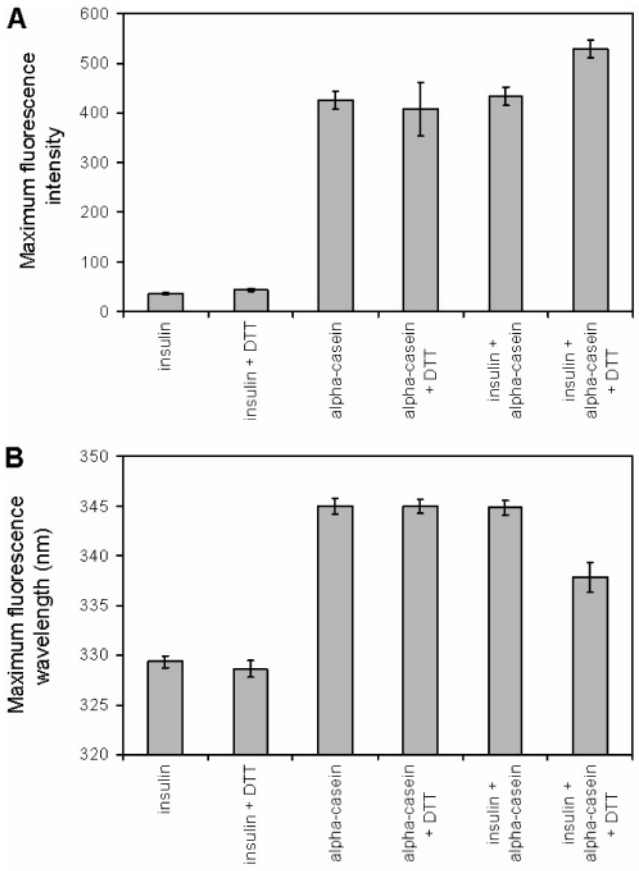

Figure 2. Intrinsic fluorescence measurements of α_S-casein in the presence and absence of insulin and/or DTT. Insulin (final concentration 0.625 mg mL^{-1}) and α_S-casein (final concentration 0.3125 mg mL^{-1}) were incubated separately or together in the presence and absence of 20 mM DTT for 60 min at 15 °C, in 50 mM sodium phosphate buffer, pH 7.1, 0.1 M NaCl, 2.5 mM EDTA, and 0.02% NaN_3. The total incubation volume was 200 μL, but prior to fluorescence measurements, samples were diluted to 2 mL with buffer. Intrinsic fluorescence measurements used an excitation wavelength of 295 nm, and emission spectra were collected over the range 300–400 nm. The peak in the range of 320–350 nm was analyzed for (A) maximum fluorescence intensity and (B) wavelength of maximum fluorescence intensity. Results shown are the mean of at least three independent experiments with the error bars given being the standard error of the mean.

insulin and α_S-casein with and without DTT exhibited the same trends (thus, only the data at 15 °C are shown in Figure 2). The low intrinsic fluorescence of insulin, both in the presence and absence of DTT, arises from its lack of tryptophan **residues (Figure 2a)**, whereas, as expected, α_S-casein exhibited much greater fluorescence. Upon addition of DTT to α_S-casein, the intensity and wavelength of maximum fluorescence intensity was not affected significantly at 15, 25, and 37 °C; however, the insulin and α_S-casein samples containing DTT displayed an increase in maximum fluorescence of approximately 20%, suggesting a reduced polarity of the environment of the tryptophan residues when these proteins interact under chaperone conditions (**Figure 2a**). Consistent with this, there was a concomitant decrease in the wavelength of maximum fluorescence (from 345.2 to 339.0 nm) when α_S-casein was incubated with insulin in the presence of DTT (**Figure 2b**). Taken together, these data suggest that a conformational change occurs in the C-terminal region of α_S-casein when it interacts with insulin as a molecular chaperone under reduction stress. In doing so, this region is in a more nonpolar environment, possibly as a result of interaction of the bound (hydrophobic) insulin B chain with the C-terminal tryptophan residues of αs-casein.

ANS Binding to Mixtures of αs, β, and κ-Casein with Reduced Insulin. ANS is a hydrophobic dye which binds to clustered regions of hydrophobicity on exposed surfaces of proteins and fluoresces strongly. It can therefore be used to indicate conformational changes as previously buried areas of hydrophobicity become exposed to solution. ANS binding experiments with α_S-casein and insulin at a 0.5:1 (w:w) ratio of α_S-casein:insulin showed a marked increase in fluorescence when they were incubated together for 1 h in the presence of DTT, at all temperatures examined, compared to controls (**Figure 3**). In addition, ANS fluorescence intensity decreases with increasing temperature, as expected. These data suggest that significant protein conformational changes occur to one or both proteins during chaperone interaction between α_S-casein and the B-chain of insulin leading to an increase in exposed hydrophobicity. The other casein subunits, β- and κ-casein, exhibited very similar increases in measurable hydrophobicity when incubated with insulin in the presence of DTT (data not shown), implying that similar interactions were occurring during chaperone action. Significantly, the ANS fluorescence of the combination of α_S- (60%), β- (25%), and κ-casein (15% w:w) was considerably increased in the presence of insulin and DTT, suggesting that some synergistic effect may be occurring between the casein subunits (**Figure 3**).

Effect of α_s-Casein and α-Crystallin on Each Other's Chaperone Ability. The chaperone action of a combination of α_S-casein and α-crystallin was investigated at both 25 °C (**Figure 4a**) and 37 °C (**Figure 4b**), by the ability of the proteins to prevent DTT-induced insulin B-chain aggregation. The chaperone action was compared at each temperature using the concentrations of α_S-casein and α-crystallin needed to give approximately 50% protection against insulin precipitation after 120 min in the presence of DTT. The results show that the chaperone action of these two proteins combined is not additive at either temperature. For example, at 25 °C, a 0.1:1 (w:w) ratio of α_s-casein:insulin gives approximately 50% protection against precipitation, and a 2:1 (w:w) ratio of α-crystallin:insulin gives approximately 38% protection. When these two are combined, precipitation of insulin is only decreased by approximately 40% (**Figure 4a**). The results of the experiments performed at 37 °C are similar to those performed at 25 °C with a combination of 0.5:1 (w:w) ratio of α_S-casein:insulin and a 1:1 (w:w) ratio of α-crystallin:insulin, giving only approximately 6% more protection than given by the same ratios of each chaperone individually (Figure 4b). The lack of additive chaperone action with the combination of α_S-casein and α-crystallin is in contrast to that observed with the combination of α_S-, β-, and κ-casein, whose chaperone action with reduced insulin was comparable to that of α_S-casein implying that the three casein proteins do not interfere with each other's chaperone action. By contrast, the presence of both α_S-casein and α-crystallin leads to an abrogation of one or both of the two proteins' chaperone action. This may arise by mutual association between the two chaperones that leads to a reduction in each other's chaperone ability. Similar behavior has been observed between α-B-crystallin and α-synuclein (*23*), where weak association occurs between the two chaperone molecules.

Chaperone Ability of α_s-Casein with Reduced α-Lactalbumin. α-Lactalbumin is a small (14 kDa), monomeric whey milk protein that binds a calcium ion and has four disulfide bonds. In the presence of a chelating agent such as EDTA, the calcium ion is removed to give the apo form of α-lactalbumin,

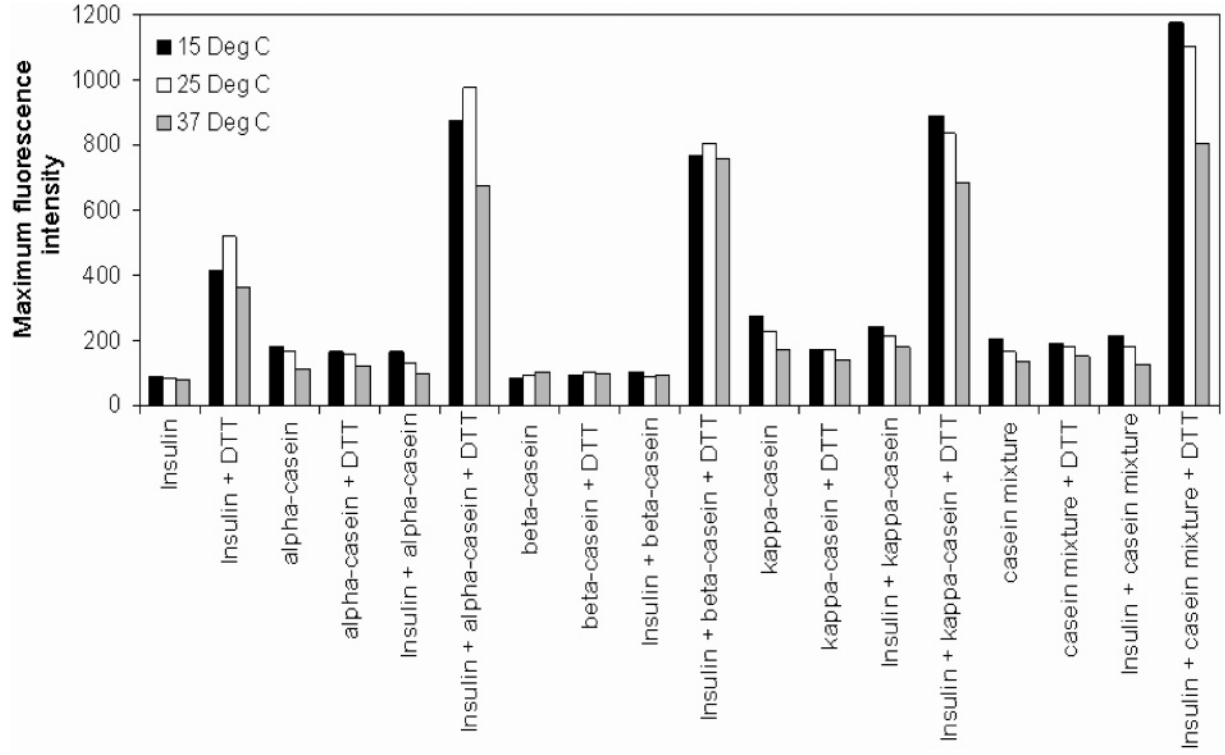

Figure 3. ANS fluorescence of casein subunits in the presence and absence of insulin and DTT. Insulin (0.625 mg mL^{-1}) and α_S-, β-, or κ-casein (0.3125 mg mL^{-1}), or a combination of α_S- (60%), β- (25%), or κ-casein (15%) (0.3125 mg mL^{-1} total protein concentration), were incubated in the presence and absence of 20 mM DTT for 60 min at 15, 25, and 37 °C, in 50 mM sodium phosphate buffer, pH 7.1, 0.1 M NaCl, 2.5 mM EDTA, 0.02% NaN_3. Total incubation volume was 200 μL, but prior to fluorescence measurements, samples were diluted to 2 mL with buffer. Aliquots (3 μL) of stock ANS (10 mM) were added to samples, which were then stirred for 30 s before a fluorescence reading was taken. Sequential aliquots of ANS were added until a plateau of maximum fluorescence was reached (usually after addition of 24–30 μL of stock ANS). ANS fluorescence was measured with an excitation wavelength of 387 nm and emission of 479 nm. Slit widths for excitation and emission were 5.0 and 10.0 nm, respectively. Results shown are mean of at least three independent experiments.

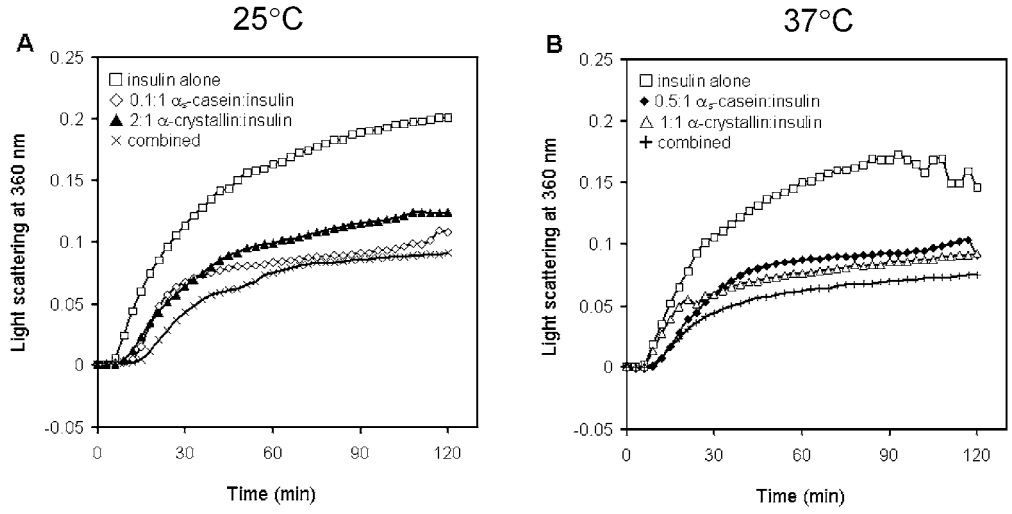

Figure 4. The chaperone action of a combination of α_S-casein and α-crystallin when preventing DTT-induced insulin aggregation is not additive. Insulin aggregation assay was performed as detailed in Figure 1 caption and in the text. Experiments were performed at (A) 25 and (B) 37 °C for 120 min. Results shown are representative of at least two independent experiments.

and its structure is destabilized such that its disulfide bonds are more accessible to reduction by DTT (*24*). α-Lactalbumin has been studied extensively as a model for protein folding/unfolding pathways as it adopts a variety of partially folded intermediates or molten globule states (*24*−*34*). For example, completely reduced apo α-lactalbumin forms a "disordered" molten globule which subsequently aggregates and precipitates over a relatively slow time period which is ideal for monitoring by real-time spectroscopic methods. In the presence of calcium and in the absence of a chelating agent, α-lactalbumin forms the holo species and its disulfide bonds are reduced at a slower rate than the apo form (*24*). In this study, the chaperone interaction of α_S-casein with reduced holo and apo α-lactalbumin at 25 and 37 °C was compared with the sHsp, α-crystallin, whose protein chaperone stabilizing characteristics have been extensively investigated (summarized in (*35*)).

At 25 °C, holo α-lactalbumin precipitation commenced approximately 60 min after the addition of DTT. The addition

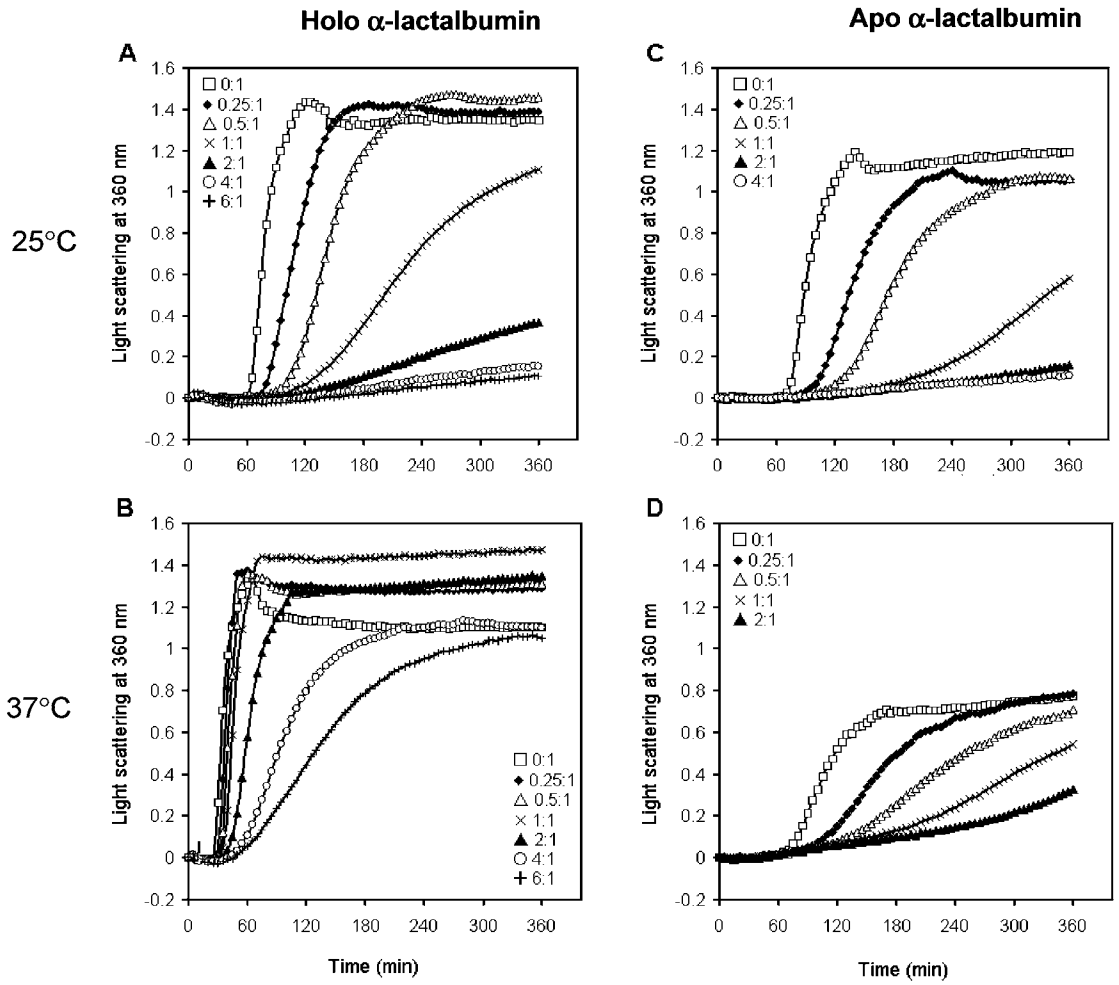

Figure 5. Prevention of DTT-induced aggregation of α-lactalbumin by α_S-casein. α-Lactalbumin (2 mg mL^{-1}) was incubated at 25 or 37 °C in 50 mM imidazole buffer, pH 7.1, 0.1 M NaCl, 5 mM $CaCl_2$, and 0.02% NaN_3 (holo α-lactalbumin; graphs A and B), or 50 mM sodium phosphate buffer, pH 7.1, 0.1 M NaCl, 2.5 mM EDTA, and 0.02% NaN_3 (apo α-lactalbumin; graphs C and D) for 360 min in the presence of increasing ratios (w:w) of α_S-casein. Aggregation of α-lactalbumin was induced by the addition of DTT to a final concentration of 20 mM at the commencement of the experiment. (A) Holo α-lactalbumin at 25 °C; (B) holo α-lactalbumin at 37 °C; (C) apo α-lactalbumin at 25 °C; (D) apo α-lactalbumin at 37 °C. Ratios are given as the ratio (w:w) of α_S-casein:α-lactalbumin, and in each case the 0:1 ratio corresponds to target protein aggregation in the absence of chaperone. Results shown are representative of at least two independent experiments.

of a 6:1 w:w (10:1 molar subunit) ratio of α_S-casein:α-lactalbumin provided complete protection from precipitation (**Figure 5a**). In contrast, a 12:1 w:w (17:1 molar subunit) ratio of α-crystallin:α-lactalbumin did not give complete protection from precipitation (R. A. Lindner, unpublished results), showing α_S-casein to be a more efficient chaperone under these conditions. However, when these experiments were repeated at 37 °C, the chaperone action of α_S-casein was considerably reduced, with a 10:1 (molar subunit) ratio of α_S-casein:α-lactalbumin able to reduce the rate, but not the extent, of precipitation (**Figure 5b**). This is consistent with the results of the chaperone action of α_S-casein with reduced insulin (**Figure 1**). By contrast, α-crystallin is a more effective chaperone at this elevated temperature, with a 17:1 (molar subunit) ratio of α-crystallin:α-lactalbumin providing almost complete protection from precipitation (*24*). The increased chaperone ability of α-crystallin at higher temperatures has been well characterized (*36*).

By way of comparison, at 25 °C, precipitation of apo α-lactalbumin after the addition of DTT occurred more slowly than for the holo form with increasing light scattering commencing at approximately 75 min. The addition of a 2:1 (w:w) ratio of α_S-casein: α-lactalbumin gave complete suppression of precipitation (**Figure 5c**). In contrast, 4:1 w:w (2.8:1.0 molar subunit) α-crystallin:apo α-lactalbumin gave no protection from precipitation. At 37 °C, a 2:1 w:w (1.4:1.0 molar subunit) ratio of α_S-casein:apo α-lactalbumin gave approximately 50% protection from precipitation as indicated in Figure 5d by having only 50% of the intensity of scattered light, whereas a 1:1 (w:w) ratio of α-crystallin:apo α-lactalbumin gave complete protection from precipitation (*24*).

Real-Time ^{1}H NMR Spectroscopy of α_s-Casein Interaction with Reduced α-Lactalbumin. The well-described molten globule states of α-lactalbumin (*24*−*34*, *37*, *38*) provide an ideal model for the investigation of protein folding/unfolding in real-time. Molten globule states of α-lactalbumin, e.g., of the reduced apo form, are intermediately folded species characterized by relatively compact structure in which a significant proportion of native secondary structure exists with concomitant loss of tertiary structure (*39*). These molten globules states also expose regions of hydrophobicity to solution (*28*) which are buried in the native state, and it is primarily this feature that is thought to cause them to interact with themselves or with molecular chaperones (*40*, *41*). Reduced α-lactalbumin forms a molten globule state that is structurally similar to α-lactalbumin at pH 2.0 (*32*) and is inherently unstable such that it readily aggregates and precipitates from solution. It is possible to monitor this

process in real-time via acquisition of a series of 1D ^{1}H NMR spectra (*24*, *33*).

The formation of the apo α-lactalbumin molten globule was followed in real-time by ^{1}H NMR spectroscopy in the presence and absence of a 2-fold (w:w) excess of α_S-casein. The aromatic region of the^{1}H NMR spectra of mixtures of α-lactalbumin and α_S-casein show resonances arising primarily from the monomeric target protein, as those from α_S-casein are relatively broad by comparison. Resonances in this region of the spectrum of reduced apo α-lactalbumin are broad and lack chemical shift dispersion, features which arise from dynamic structures undergoing rapid alterations in conformation (*20*, *24*, *42*) and are characteristic of the molten globule state. In this region is an isolated resonance arising from the tyrosine^{3,5} ring protons (at 6.8 ppm), which can be used as an indicator of the extent of molten globule formation in α-lactalbumin (*24*).

As reported previously by us (*24*), in the absence of α-crystallin, the molten globule state of reduced apo α-lactalbumin at 2.0 mg/mL aggregates rapidly as indicated by a loss of the tyrosine^{3,5} ring proton resonance a rate of 1.50 (±0.04) $\times$ 10 $^{-3}$ s^{-1}. In the presence of bovine α-crystallin, however, this rate is reduced to 8.00 (±0.53) $\times$ 10 $^{-4}$ s^{-1}, as interaction between the two proteins occurs and the molten globule form of α-lactalbumin is stabilized by the chaperone action of α-crystallin such that precipitation does not occur (*24*).

In the absence of α_S-casein at 37 °C (**Figure 6a**), an initial buildup of the signal arising from the tyrosine^{3,5} protons is observed from 0 to approximately 200 s after the addition of DTT. This phase corresponds to complete reduction the disulfide bonds of α-lactalbumin (*24*). Subsequently, the intensity of the tyrosine^{3,5} resonance was rapidly lost at a rate of 2.70 (±0.11) $\times$ 10 $^{-3}$ s^{-1}, with maximum aggregation of the protein occurring approximately 1000 s after the addition of DTT (Figure 6c). This rate is considerably faster than that reported by Carver et al. (*24*), but the increased rate observed here was most likely due to the much greater concentration of α-lactalbumin in the sample (14.3 mg/ mL). In the presence of a 2-fold (w:w) excess of α_S-casein, however (**Figure 6b**), the loss of resonance intensity (corresponding to the interaction of the disordered molten globule state of α-lactalbumin with α_S-casein) was approximately 50% slower (1.25 (±0.08) $\times$ 10 $^{-4}$ s^{-1}), indicating that α_S-casein interacted with and stabilized this molten globule state of apo α-lactalbumin in a chaperone-like manner as was observed with α-crystallin (*24*, *33*).

Effect of Molecular Crowding on the Chaperone Action of α_s-Casein. To investigate the chaperone properties of α_S-casein under similar conditions to those found in milk where many other proteins and macromolecules are present, aggregation assays were performed in the presence of the experimental crowding agent, dextran. Dextran (average molecular mass 78 kDa) was selected as it is an uncharged, inert polymer with excluded volume properties that have been shown to be independent of pH, salt concentration, protein concentration, and degree of its polymerization (*43*). Insulin and α-lactalbumin were used as target proteins and the assay conditions were identical to those performed in the absence of crowding.

The addition of dextran to reduced α-lactalbumin and insulin resulted in significant increases in the rate of protein aggregation and precipitation over that of α-lactalbumin or insulin in the absence of dextran (**Figure 7**). The addition of 1−20% (w/v) dextran to DTT-reduced α-lactalbumin resulted in greater and faster aggregation with increasing dextran concentration. For example, for reduced holo α-lactalbumin, aggregation was complete in under 30 min at 10% (w/v) dextran concentration at both 25 and 37 °C, whereas this process took between 60 and 100 minutes in the absence of dextran (cf. **Figure 7a,d** to **Figure 5a,b**). The same acceleration of precipitation was observed for reduced apo α-lactalbumin (**Figure 7b,e**). The addition of 1% or 2% (w/v) dextran to DTT-reduced insulin resulted in little enhancement of aggregation, however the presence of 10% (w/v) dextran caused enhanced insulin aggregation at both 25 and 37 °C (cf. **Figures 7c,f** to **Figure 1a,d**). The chaperone action α_S-casein was markedly reduced in the presence of dextran. For example, in the presence of 10% dextran 10:1 (w:w) α_S-casein:apo α-lactalbumin did not completely prevent the aggregation of reduced α-lactalbumin whereas a 2:1 ratio prevented aggregation in the absence of dextran. The effect of dextran on α_S-casein chaperone action with reduced insulin was not as great. Thus, the suppression of insulin precipitation by α_S-casein at 25 °C required a 2:1 (w:w) ratio of α_S-casein:insulin for complete suppression in the presence of 10% dextran (**Figure 7c**) whereas in the absence of dextran, a 1:1 ratio (w:w) ratio of α_S-casein:insulin was required to achieve the same level of suppression (Figure 1a).

Chaperone Action of α_s-Casein with Heat-Stressed β-Lactoglobulin. The thermal aggregation of the major whey protein, β-lactoglobulin, has been extensively studied, including examining the effects of salts, calcium, pH, and temperature on the rate of aggregation (*44*). Initial experiments utilized the well-known characteristic that the addition of calcium considerably enhances the rate of thermal aggregation of β-lactoglobulin (*45*). However, the propensity of α_S-casein to precipitate in the presence of calcium is well-known, with the "s" referring to calcium "sensitivity" (*19*), thus renders these experimental conditions inappropriate. Studies by Verheul et al. (*44*) showed that aggregation of β-lactoglobulin at 68.5 °C was enhanced in the presence of high concentrations of NaCl, therefore experiments investigating the chaperone ability of α_S-casein with heated β-lactoglobulin contained 0.2 M NaCl. Under these conditions, aggregation of a 5 mg mL^{-1} solution of β-lactoglobulin commenced after approximately 25 min, and was complete after approximately 360 min (**Figure 8**). Addition of a 0.5:1 (w:w) ratio of α_S-casein:β-lactoglobulin resulted in a significant reduction in the extent of aggregation of β-lactoglobulin. However, increasing the α_S-casein concentration above this ratio did not suppress any further aggregation.

The ability of α_S-casein to prevent precipitation of heat stressed β-lactoglobulin at various pH values was also investigated. As with the previous thermal aggregation experiments, β-lactoglobulin was stressed at 70 °C in the presence of different ratios of the chaperone. These experiments showed that at pH 7.0, α_S-casein was an efficient chaperone with almost complete suppression of aggregation at a 1:1 (w:w) ratio of α_S-casein to β-lactoglobulin (data not shown). When the same experiment was repeated at pH 7.5, α_s-casein was less effective in suppressing the aggregation of β-lactoglobulin with substantial aggregation seen even in the presence of a 1:1 ratio of α_S-casein to β-lactoglobulin. At pH 8.0, α_S-casein proved to be a poor chaperone with very little suppression of β-lactoglobulin at ratios up to 1:1 α_S-casein:β-lactoglobulin. For example, the extent of suppression of β-lactoglobulin precipitation by α_S-casein at a 0.25:1 (w:w) ratio decreased from 73% at pH 7.0 to 33% at pH 7.5 and, finally, 19% at pH 8.0 after 450 min. In control experiments (data not shown), it has been demonstrated that α_S-casein is unaffected by the stress conditions employed in these assays. In addition, similar experiments conducted with clusterin have shown that the ability to suppress aggregation of target proteins is specific to molecular chaperones as their

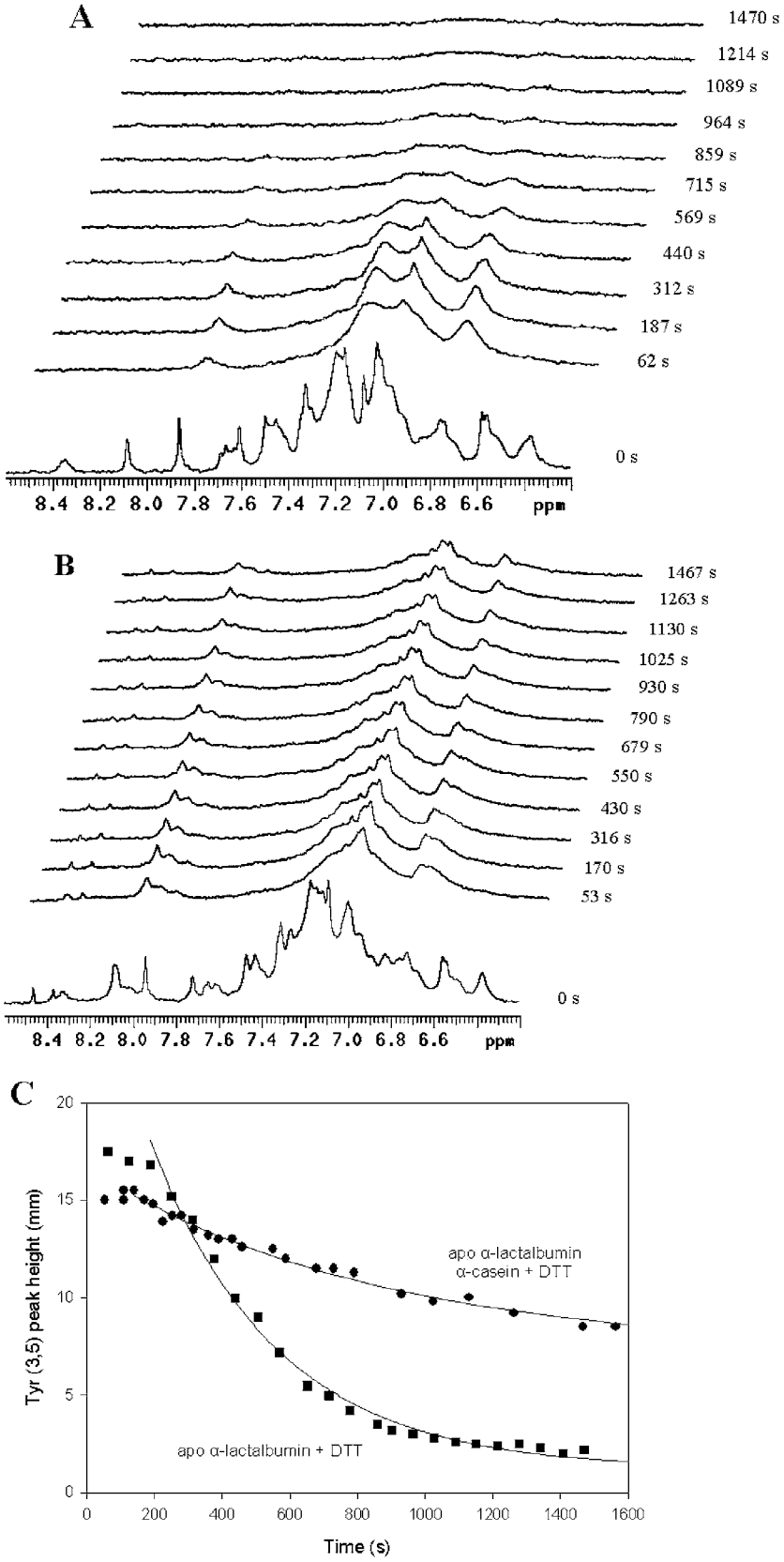

Figure 6. Real-time ^{1}H NMR spectroscopy of apo α-lactalbumin reduction by DTT at 37°C in the presence and absence of α_S-casein. Stacked plots of NMR spectra of molten globule formation in apo α-lactalbumin in (A) the absence of and (B) the presence of α_S-casein at a 2:1 (w:w) ratio. The first-order decay of the resonance arising from the tyrosine (3,5) aromatic ring protons at 6.8 ppm in both cases is represented by a plot of peak height (mm) against time (s) (C) from which rates were calculated. Results shown are representative of at least two independent experiments and were very reproducible.

substitution with nonchaperones, e.g., ovalbumin, has no effect on the extent of target protein precipitation (*46*).

Size-Exclusion HPLC of Mixtures of α_S-Casein with Stressed Target Proteins. Experiments showed that soluble

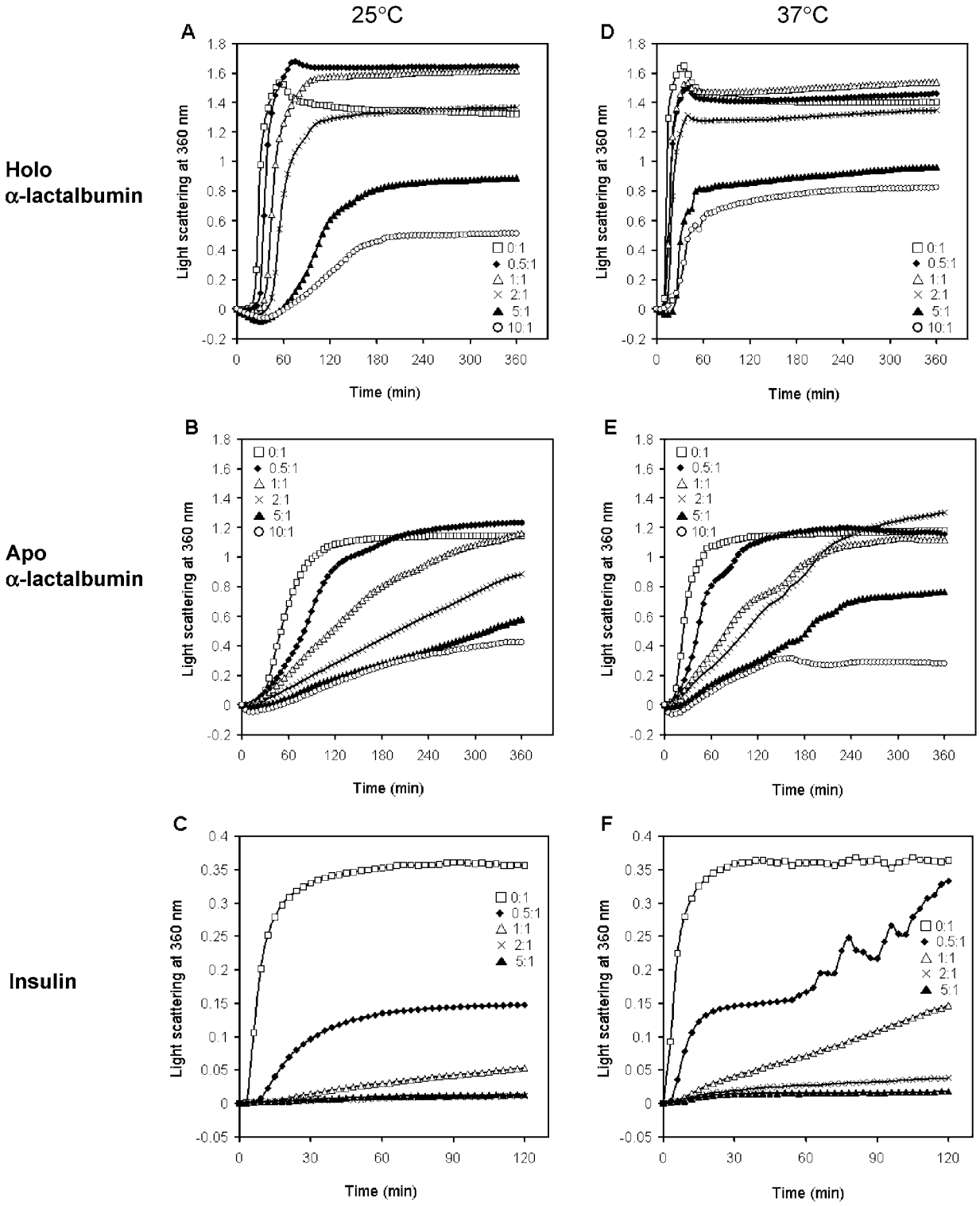

Figure 7. Enhancement of DTT-induced aggregation of holo- and apo α-lactalbumin and insulin by the addition of the molecular crowding agent dextran and the prevention of aggregation by the addition of α_S-casein. Holo- and apo α-Lactalbumin (2 mg mL^{-1}) were incubated at 25 °C and 37 °C for 360 min in the presence of 10% (w/v) dextran and increasing ratios (w:w) of α_S-casein. Insulin (0.25 mg mL^{-1}) was incubated at 25 and 37 °C for 120 min in the presence of 10% (w/v) dextran and increasing (w:w) ratios of α_S-casein. Aggregation of α-lactalbumin and insulin was induced by the addition of DTT to a final concentration of 20 mM at the commencement of the experiment, and was detected by measuring the change in absorbance at 360 nm. (A) Holo α-lactalbumin, 25 °C; (B) apo α-lactalbumin, 25 °C; (C) insulin, 25 °C; (D) holo α-lactalbumin, 37 °C; (E) apo α-lactalbumin, 37 °C; (F) insulin, 37 °C. In each case, 0:1 ratios given are representative of target protein aggregating in the absence of chaperone protein and results shown are representative of at least two independent experiments.

complexes of very large mass were formed between α_S-casein and the milk-derived target proteins, α-lactalbumin and β-lactoglobulin, when mixtures of these proteins were stressed (**Figure 9**). A mixture of α_S-casein and β-lactoglobulin (in a ratio of 2:1 (w:w) at 10 mg mL^{-1} β-lactoglobulin) was subjected to heat stress at 70 °C for 3 h. Visible absorption data had shown that at this temperature in the absence of chaperone, significant precipitation of β-lactoglobulin occurred after 3 h (**Figure 8**).

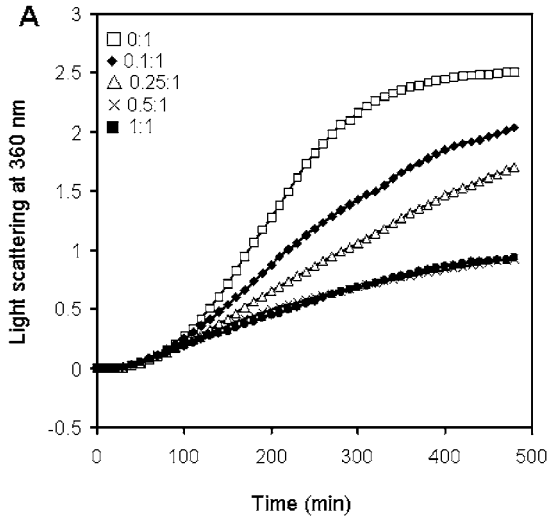

Figure 8. Prevention of thermally induced aggregation of β-lactoglobulin by α_S-casein as measured by light scattering at 360 nm. Samples were heated at 70 °C for 480 min in 50 mM sodium phosphate buffer, pH 7.1, 0.2 M NaCl, 2.5 mM EDTA, and 0.02% NaN_3. Ratios of α_S-casein:β-lactoglobulin are given on a w:w basis with 5 mg mL^{-1} β-lactoglobulin + varying amounts of α_S-casein. The 0:1 ratio represents target protein aggregating in the absence of chaperone protein and results shown are representative of at least two independent experiments.

In the presence of α_S-casein at a 2-fold w:w excess, this solution appeared opaque but did not contain a precipitate.

Chromatographic separation of this mixture (**Figure 9a**) showed a high molecular weight (HMW) peak with a retention time of 4.2 min—considerably earlier than either of the component proteins when chromatographed individually. Thus, α_S-casein and β-lactoglobulin had retention times of 6.7 and 7.2 min, respectively, which corresponded to proteins of approximately 54 and 40 kDa in mass, respectively. Bovine β-lactoglobulin is known to exist as a noncovalently linked dimer of approximately 37 kDa at physiological pH (*19*, *47*). The predominant α_S-casein subunit, α_{S1}, is a monomer of 23 kDa but can aggregate to form tetramers approximately 113 kDa in mass with the degree of polymerization being dependent on temperature and protein concentration, whereas α_{S2}-casein exists as a dimer under native conditions due to its intermolecular disulfide bonding giving a molecular mass of approximately 50 kDa (*19*). Thus, the broad HPLC peak at a retention time of 6.7 min. arising from α_S-casein (**Figure 9**) is a result of these heterogeneous aggregated states. The high molecular weight (HMW) aggregates in the first peak eluting at 4.2 min. arise from species of approximately 1.8 MDa in mass. The closely overlapping peaks arising from noncomplexed α_S-casein and β-lactoglobulin eluted later than the HMW peak with the expected elution times of 6.7 and 7.2 min, respectively. Additional controls show that stressed β-lactoglobulin also forms large aggregates when heat-stressed in the absence of a chaperone; however, these species are largely insoluble and formed a precipitate which was removed by centrifugation. An unstressed mixture of α_S-casein and β-lactoglobulin gave rise to two closely overlapping peaks with similar retention times for to those given for the individual proteins with no high molecular weight species being observed. In additional experiments, the retention time of α_S-casein alone was not affected to any significant extent by heat stress under the same conditions (data not shown).

The results of precipitation assays showed that under reduction stress, α_S-casein was a better chaperone at lower temperatures (**Figures 1** and **5**). A mixture of apo α-lactalbumin and α_S-casein (in a ratio of 2:1 (w:w)) was therefore subjected to reduction stress at 25 °C and formed HMW species of approximately 1.7 MDa in mass eluting at 4.4 min (Figure 9b). The HMW peak under these conditions was even broader than that for the heated α_S-casein and β-lactoglobulin mixture, indicating that the aggregates formed between the two proteins are very polydisperse (**Figure 9b**). Size-exclusion studies also showed that there was no evidence for interaction between these two proteins when incubated in the absence of DTT with both α-lactalbumin and α_S-casein eluting at the same time as when chromatographed individually (data not shown). The peak arising from α_S-casein (retention time of 6.7 min) overlapped with that of apo α-lactalbumin (14 kDa) to a greater extent than in the presence of β-lactoglobulin (**Figure 9a**). This shift is most likely due to the reduction of the intermolecular disulfide bonds in α_{S2}-casein by DTT resulting in a mixture of α_{S1}- and α_{S2}-casein monomers of 23 and 25 kDa, respectively. Thus, for both target proteins under temperature and reduction stresses, α_S-casein interacted to form a soluble HMW complex to prevent

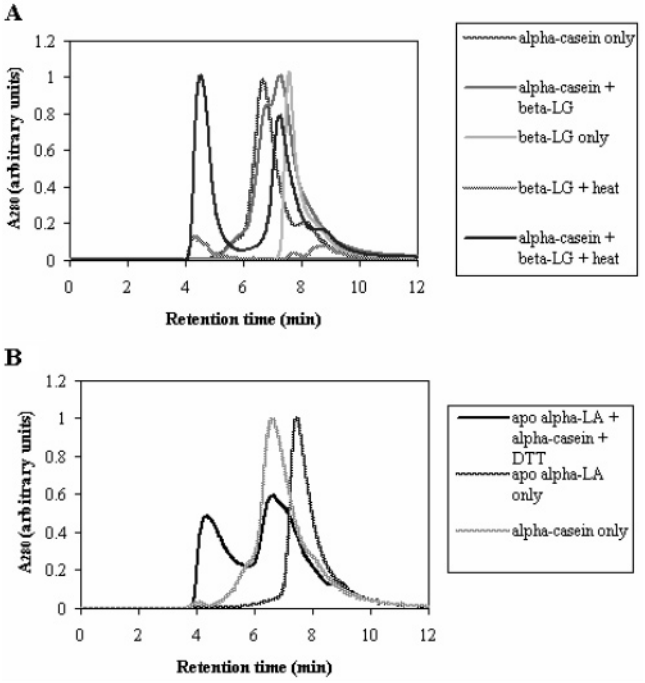

Figure 9. Size-exclusion HPLC of the complexes formed between α_S-casein and the whey proteins, α-lactalbumin, and β-lactoglobulin. A mixture of (A) β-lactoglobulin (2.5 mg) and α_S-casein (5.0 mg) was prepared in 250 μL of 50 mM $NaPO_4$, pH 7.2, 0.2 M NaCl, 2.5 mM EDTA, 0.02% NaN_3 and stressed at 70 °C for 180 min. Samples of both proteins were also prepared at the same concentrations as above and chromatographed separately as was a mixture of the two proteins that had not undergone heat stress. β-Lactoglobulin was also prepared at the same concentrations as stated above and subjected to heat stress in order to demonstrate its propensity to form large aggregates. A mixture of (B) apo α-lactalbumin (2.5 mg) and α_S-casein (5.0 mg) was prepared in 250 μL of 50 mM sodium phosphate buffer, pH 7.2, 0.1 M NaCl, 2.5 mM EDTA, 0.02% NaN_3 and stressed by the addition of a final concentration of 20 mM DTT at 25 °C for 2 h. Samples of both proteins were also prepared at the same concentrations as above and chromatographed separately. All samples were centrifuged at 14 000 rpm before 10 μL aliquots were injected onto a BioSep SEC S4000 column with a mobile phase of 50 mM $NaPO_4$, pH 7.2 at a flow rate of 0.5 mL min^{-1}. All samples were chromatographed in duplicate or triplicate and very similar results obtained in each case.

the target proteins from precipitating. This feature is also characteristic of sHsps and clusterin (*33*, *48*).

DISCUSSION

The chaperone action of the milk casein proteins has been investigated here under a range of experimental conditions. Many of these studies used the primary whey proteins in bovine milk, α-lactalbumin and *β*-lactoglobulin, which are natural partners for the chaperone action of α_S-casein. α_S-Casein displayed enhanced chaperone action at lower temperatures with reduced α-lactalbumin and insulin as target proteins. *β*- and *κ*-casein did not exhibit such behavior with reduced insulin. For α_S-casein, these findings are consistent with previous data (*6*), but are in contrast to those of sHsps (e.g., α-crystallin) which have enhanced chaperone action at higher temperatures (*36*). On the other hand, clusterin exhibits very little temperature dependence in its chaperone ability (*49*). From these results, therefore, it would seem that α_S-casein has different temperature effects in its chaperone ability dependent on the stress involved. This may arise from the different chaperone ability of the reduced form of the α_{S2} subunit of α_S-casein compared to the dimeric oxidized species. Studies to explore this possibility are underway by examining the chaperone ability of the individual α_S-casein subunits. The ability of α_S-casein to chaperone effectively under temperature stress is of obvious importance in milk treatment processes such as pasteurization and ultrahigh-temperature treatment.

The observation that α_S-casein prevents the heat-induced precipitation of ovotransferrin (*15*) is consistent with our results and is further evidence for the general nature of α_S-casein chaperone action with a broad range of target proteins. Furthermore, Matsudomi et al. (*15*) observed that dephosphorylated α_S-casein was a poor chaperone in suppressing ovotransferrin aggregation. During the chaperone action of sHsps, exposed, flexible C-terminal regions of the protein act as solubilizing agents for the chaperone itself and the complex it forms with the target protein (*11*, *12*). By analogy, it is conceivable that the exposed phosphate groups in α_S-casein perform a similar solubilizing role for the hydrophobic α_S-casein-ovotransferrin complex. Thus, for both α_S-casein and sHsps, the presence of distinct flexible, polar polypeptide regions and hydrophobic regions is consistent with surfactant-like properties for both proteins, as has been demonstrated for α-crystallin (*50*).

β-Lactoglobulin comprises 50% of the whey component of bovine milk (*19*). Bhattacharyaa and Das (*6*) used a mixture of whey proteins as a target for thermal aggregation studies (at 70 °C) in the presence of a 1:1 (w:w) ratio of α_S-casein. They showed that at this ratio, α_S-casein was able to fully suppress whey protein aggregation. The extent of suppression of *β*-lactoglobulin alone by α_S-casein was approximately 70% with 1:1 (w:w) concentrations of both proteins at pH 7.1. At pH 7.0 and under the same buffer conditions, α_S-casein was a slightly more efficient chaperone than at pH 7.1 in preventing the aggregation of *β*-lactoglobulin. As the pH was increased (i.e., to pH 7.5 and 8.0), the chaperone ability of α_S-casein correspondingly decreased markedly. At these slightly alkaline pH values, histidine residues deprotonate (α_{S1}- and α_{S2}-casein have five and three histidines, respectively). It is feasible that, in addition to hydrophobic interactions, one or more of these histidine residues are involved in binding target proteins and their deprotonation disrupts electrostatic interactions between the two proteins so that they can no longer interact effectively to bind target proteins. Clusterin has a similar pH dependence in its chaperone action, i.e., acidic pH values favor enhanced chaperone action, which is associated with dissociation of clusterin from its aggregate (*49*). α-Crystallin, on the other hand, is a poorer chaperone at slightly acidic pH values (*49*, *51*) which is not accompanied by a change in aggregated state but arises from subtle structural changes in the protein (Brockwell, C.; Rekas, A.; Carver, J. A. Unpublished results, 2003). In addition, as pH is increased, greater intermolecular disulfide bond formation occurs between *β*-lactoglobulin molecules (*52*) to produce oligomers. As this type of aggregation is simple polymerization and is not nucleation dependent, α_S-casein may have a similar ability to α-crystallin in only being able to suppress the latter type of aggregating species (*53*). In summary, for heated *β*-lactoglobulin, pH significantly influences its aggregation behavior and thereby its interaction with α_S-casein (which itself may have pH-dependent chaperone ability).

In the presence of dextran, the crowded molecular environment of milk was simulated and a greater rate of precipitation of target proteins (apo and holo α-lactalbumin and insulin) was observed (e.g., cf. **Figure 5a** to **Figure 7a**). The increased rate of aggregation of target proteins is likely to be a major reason for the decreased chaperone action of α_S-casein under molecular crowding conditions. α-Crystallin behaves similarly in having reduced chaperone ability in the presence of crowding agents (Ghahghaei, A.; Rekas, A.; Carver, J. A. Unpublished results, 2003). sHsps and clusterin are very inefficient chaperones under conditions of relatively rapid target protein aggregation (*24*, *48*, *54*, *55*). Thus, reduced apo α-lactalbumin aggregates at a slower rate than the holo form (**Figure 5**), and α_S-casein and α-crystallin (*24*) are much more efficient chaperones with respect to the former species. Clusterin is similarly poor at preventing rapidly aggregating proteins from precipitating (*48*). In the case of milk where there is a high concentration of proteins, the presence of a large excess of casein chaperone proteins would ensure solubility under the variety of stresses associated with milk processing, e.g., heat, pressure, and shear.

The chaperone ability of α_S-casein was investigated in the presence of α-crystallin with reduced insulin in order to ascertain whether it was likely that these two chaperones could function in concert to suppress aggregation. It was found that at both 25 and 37 °C, a combination of α_S-casein and α-crystallin was only slightly more effective in preventing the precipitation of insulin than each chaperone when assayed individually. It can be concluded therefore that α_S-casein and α-crystallin, despite being similar in their mode of operation, have some degree of association that causes partial abrogation of each other's chaperone ability. This could arise from an interaction between the two, which blocks the chaperone binding site(s) of one or both proteins. Similar behavior occurs in the interaction between the two chaperone proteins, αB-crystallin and α-synuclein (*23*).

Tryptophan fluorescence studies of the interaction between reduced insulin and α_S-casein indicated that the C-terminal tryptophan environment of α_S-casein became more hydrophobic upon interaction with the insulin B-chain, which may result from a structural change in this region during its chaperone action; e.g., the C-terminal region of the protein is internalized to a greater extent upon binding insulin or brought into close proximity to exposed hydrophobic regions of the partially unfolded insulin B-chain. When a mixture of α_S-casein and insulin was incubated with DTT, a significant increase in ANS fluorescence was observed (**Figure 3**). This increase in fluorescence implies that in the presence of reduction stress, the

complex exposed increased hydrophobicity as a result of chaperone interaction between α_S-casein and the B-chain of insulin.

The conformational state of reduced apo α-lactalbumin as it interacts and is sequestered by α_S-casein was investigated in detail by real-time ^{1}H NMR spectroscopy. Under the conditions used, α_S-casein stabilized and interacted with a disordered molten globule form of reduced apo α-lactalbumin. Again, this mirrors the behavior of α-crystallin (*24*, *33*) and clusterin (*48*). By analogy with the behavior of α-crystallin with reduced α-lactalbumin (*24*, *33*), α_S-casein stabilized a monomeric form of this species to prevent it from entering the off-folding pathway which leads to aggregated and eventually precipitated species (*11*, *24*, *33*, *56*). Following stabilization, complexation between the two proteins occurred to form a HMW aggregate.

α_S-Casein also formed soluble HMW aggregates with thermally stressed β-lactoglobulin and reduced apo α-lactalbumin to prevent its stress-induced aggregation. The ability to form a soluble HMW complex in which the partially unfolded target protein is stabilized is a feature characteristic of sHsps and clusterin (*33*, *48*). They act as ATP-independent chaperones but do not refold the target protein following interaction. Instead, they incorporate the partially folded target protein into a soluble HMW complex to prevent it from precipitating. α_S-Casein acts in a similar manner. In the case of sHsps, other chaperones (e.g., Hsp70) may refold the target protein when cellular stress has abated (*57*). It is not known whether other chaperones with refolding ability exist in milk although clusterin (*58*) and another extracellular chaperone, haptoglobin (*59*), are present and may function in a synergistic chaperone manner with casein proteins to minimize protein aggregation.

From these data, it is apparent that the chaperone action of α_S-casein (and β- and κ-casein) bears strong functional similarities to that of sHsps and clusterin. Casein proteins have no sequence similarity to sHsps or clusterin but the proteins share many structural similarities. They are all dynamic and heterogeneous proteins that have extensive regions of little ordered structure (*8*, *12*). In sHsps and clusterin, these characteristics are crucially important for their chaperone function (*8*, *12*). It is conceivable, therefore, that the structural malleability of casein proteins facilitates their interaction with a broad range of destabilized proteins. Furthermore, subunit exchange has been implicated as an important factor in the chaperone action of sHsps and, probably, clusterin (*12*). The dynamic, micellular structure of caseins would facilitate chaperone action via this mechanism and subunit exchange of casein proteins may therefore be a regulator of chaperone action. Investigation of this aspect of casein proteins, along with other features of their chaperone action, is ongoing.

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