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Chapter 3

Alpha-Casein as a Molecular Chaperone

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1. Introduction

The caseins are a heterogeneous group of dairy proteins constituting 80% of the protein content of bovine milk. The operational definition of casein is that proportion of total milk protein which precipitates on acidification of milk to a pH value of 4.6 [1]. The remaining dairy proteins, known collectively as whey proteins, do not precipitate. Caseins are synthesised in the mammary gland and are found nowhere else among the plant and animal kingdoms [2]. The casein family of proteins comprises α- , β- and κ-caseins, all with little sequence homology [3]. As their primary function is nutritional, binding large amounts of calcium, zinc and other biologically important metals, amino acid substitutions or deletions have little impact on function. The caseins also lack well-defined structure and as a result their amino acid sequence is less critical to function than in many 'classic' globular proteins. As a result, the caseins are one of the most evolutionarily divergent protein families characterised in mammals [2]. Alpha-casein, also known as α_S-casein, is in fact two distinct gene products, α_{S1}- and α_{S2}-casein, with the 'S' denoting a sensitivity to calcium. Of all the caseins, α_{S1}- and β-casein are predominant in bovine milk, representing 37 and 35% of whole casein respectively, whereas α_{S2}- and κ-casein make up 10 and 12% of whole casein, respectively [2].

1.1. Key structural features of the casein proteins

The casein proteins are important nutritionally not just because of their high phosphate content which allows them to bind significant quantities of calcium, but because they are high in lysine. The constituent proteins of α_S-casein, α_{S1}- and α_{S2}-casein, possess 14 and 24 lysines, respectively [2]. Lysine is an essential amino acid in humans and one in which many plant sources are lacking, therefore casein extracts form an effective nutritional supplement for cereals [2].

In addition to the variability inherent in their amino acid sequences, each of the caseins exhibit significant variability as a result of their degree of post-translational modification,

disulfide bonding, genetic polymorphism and the manner in which they are hydrolysed by the milk protease, plasmin. In terms of the extent of phosphorylation, each of the four caseins may have various numbers of phosphate groups attached via their serine or threonine residues [4]. For example, α_{S1}-casein may have 8 or 9, α_{S2}-casein 10, 11, 12 or 13, β-casein may have 4 or 5 and κ-casein, 1, 2 or 3 [5]. It is not known whether this variability results from the action of casein kinases phosphorylating to different degrees or by phosphatases dephosphorylating to a greater or lesser extent [5]. The predominant caseins, α_{S1}- and β-casein, contain no disulfide bonds; however α_{S2}- and κ-casein contain two cysteine residues which form intra- or intermolecular disulfide bonds under normal conditions. In the absence of a reducing agent, α_{S2}-casein exists as a disulfide-linked dimer and κ-casein can adopt dimeric to decameric forms, depending on the pattern of intermolecular disulfide bonding. κ-Casein is also the only casein which is glycosylated and the degree of glycosylation varies so that ten different molecular forms of κ-casein are possible on this basis alone [4].

Genetic polymorphism is yet another source of variability in the caseins. This phenomenon was first described in 1955 in relation to β-lactoglobulin [6] and exists when the same protein exists in a number of forms, differing from one another in just a few amino acids. This has since been shown to occur in all dairy proteins. The milk of one animal may contain one polymorphic form alone, or both, and the occurrence of particular polymorphs is breed-specific [7, 8] . Genetic variants are indicated by a latin letter i.e. α_{S1}-casein has been shown to be present in bovine milk as α_{S1}-caseinA – D; α_{S2}- caseinA – D; β-caseinA^1, A^2, A^3 – E and κ-caseinA and B [9]. With the combined variability between the caseins themselves, contributed by low sequence homology, glycosylation and disulfide bonding and within individual caseins due to the degree of phosphorylation and genetic polymorphism, this is a very interesting family of proteins. As will be described in greater detail in this chapter, the caseins have created even greater intrigue with the recent discovery of their chaperone abilities.

Structurally, the caseins are classified as 'intrinsically or natively disordered' proteins under physiological conditions [10, 11]. This disordered structure, which is present to some extent even in globular proteins, is different to random coil conformation. In natively disordered proteins, conformations of these regions are still relatively fixed with respect to the ϕ and ψ angles of the peptide bonds, as opposed to true 'random coil' polypeptide chains, which exhibit greater and more rapid fluctuation in bond angles [4]. The lack of well-defined structure in the casein proteins is believed to facilitate proteolysis and therefore ready absorption of amino acids and small peptides in the gut [2], but is another likely factor in the unwillingness of the caseins to crystallise to provide a 3D crystal structure [12]. Physical characterisations of caseins in solution and predicted 3D models have shown that the caseins have relatively little tertiary structure, but possess some secondary structure, similar to the classic 'molten globule' states described in [13]. The greatest degree of secondary structure exists in α_{S2}- and κ-casein, mainly in the form of β-sheets and β-turns rather than α-helix [14-16]. The formation of higher proportions of secondary and tertiary structural

elements in the caseins is likely to be inhibited by high numbers of proline residues which distort protein folding into α-helices and β-sheets [2].

Each of the casein proteins has a high degree of hydrophobicity as a result of containing approximately 35-45% non-polar amino acids (e.g. Val, Leu, Phe, Tyr, Pro), but this does not preclude them from being quite soluble in aqueous solvents due to the presence of high numbers of phosphate and sulfur-containing amino acids, and in the case of κ-caseins, carbohydrates [2]. These hydrophobic regions are likely exposed in the caseins as a result of their flexible and relatively unfolded structure. The hydrophobicity tends to occur in patches along the sequence of the caseins, however, and is interspersed with hydrophilic regions. It is this feature that is credited with making the caseins good emulsifying agents – a property exploited in the food industry. The clustered exposed hydrophobicity is also thought to be a major feature of the molecular chaperone action of the caseins [17] as discussed later in this chapter.

1.2. Self-association, fibril formation and micellar arrangement of the caseins

Although relatively small in size with molecular masses of 23.6 and 25.2 kDa for α_{S1}- and α_{S2}-casein, respectively, α-caseins readily associate with one another and with the other caseins (β- and κ-casein) to form large aggregates up to 1.4 MDa in size [18]. This tendency to form multimeric assemblies is likely to be another reason why it has not been possible to obtain crystal structures for the caseins thus far. In the presence of calcium, associations between the various caseins can lead to the formation of micelles [2]. These micelles are composed of approx. 94% protein and approx. 6% low molecular weight species such as calcium, phosphate, citrate and magnesium which together form 'colloidal calcium phosphate' [2] or amorphous calcium phosphate; APC [19]. Evolutionarily, it is thought that the formation of micelles has served as a means by which to increase the calcium concentration in milk over many millennia to satisfy its nutritional function without compromising physical stability [19]. The makeup of the micelle, which is roughly spherical in shape and has a radius of approx. 600 nm or less, comprises the amorphous calcium phosphate, more recently referred to as 'nanoclusters', bound to specific phosphorylated sequences in the surrounding α_{S1}- , α_{S2}- and β-casein chains [19]. The major protein constituent of casein micelles, accounting for 65% of protein is α_S-casein [4]. The function of κ-casein, present at the surface of the micelle, appears to be related to limiting the size of the micelle [19].

The C-terminal region of κ-casein is strongly hydrophilic, whereas the N-terminal region is strongly hydrophobic [4]. Such amphipathic qualities have been shown to be of great importance for molecules residing at the interface between hydrophobic and hydrophilic environments in various biological contexts (e.g. the phospholipid cell membrane, the assembly of lipoprotein particles) and are no doubt also important in the stabilisation of the micelle in the aqueous environment of milk. Further evidence for the localisation of κ-casein at the surface of the micelle forming a diffuse outer region [20] was provided by the discovery that in the formation of cheese, the more hydrophilic C-terminal portion is the one

cleaved by the action of rennet [2]. Recent Cryo-TEM and TEM studies have shown that the small electron-dense regions consistent with calcium phosphate nanoclusters are evenly distributed throughout the micellar structure rather than being sequestered within the core of the micelle [21-24] and that these structures, linked together by chains of caseins, were continuous throughout the entire micelle [25]. The sequestration of calcium phosphate, which accounts for 7% of the solute mass of bovine casein micelles, within a phosphoprotein matrix in this way is critical to maintaining the stability of these potentially very insoluble minerals in milk which would otherwise precipitate, compromising lactation [19]. It is the light scattering caused by these large (10^3 to 3×10^6 kDa) casein micelles in a colloidal dispersion that is thought to give milk its characteristic white colour [2, 25, 26]. The caseins are also very stable at high temperatures, a property thought to be associated with their high phosphate content [2] and lack of well-defined structure [11] which makes them resistant to denaturation by heat and chemical agents. Milk may be heated at 100°C at its native pH (~6.7) for 24 hours without coagulating and will withstand 140°C temperatures for 20 min. The current ultra high heat treatments applied to milk and milk products are made possible by the extreme thermostability of the caseins [2]. Our studies have shown that solutions of individual α_{S1}- and α_{S2}-caseins are stable at 70°C for a period of at least 8 hours at a concentration of 5 mg/mL [18].

Interestingly, both of the disulfide bond-containing caseins, κ- and α_{S2}-casein, have been shown to form amyloid fibrils under physiological conditions [27-30]. It has been suggested that casein proteins may have a propensity to form amyloid fibrils because they possessed similar structural features to the amyloid forming proteins tau, α-synuclein and amyloid β [31]. The similarity lies in the tendency of all four casein proteins to adopt a flexible and relatively unfolded conformation but also their possession of significant amounts of poly-L-proline (PPII) helix structure which likely arose from the relatively uniform distribution of proline residues [2, 31]. In contrast to α-helix, PPII helix is more open, flexible and extended and the conversion of this to antiparallel β-sheets, the precursor to amyloid fibril, is a highly energetically-favourable one [32]. As suggested in [33], other factors in the formation of amyloid fibrils must also be important , as only two of the four caseins (namely κ- and α_{S2}-casein) form fibrils under physiological conditions. Whole casein does not form fibrils under the same conditions as κ- and α_{S2}-casein and this is thought to be related to the inhibitory action of other caseins present. It has been shown previously that both α_{S1}- and β-casein are able to inhibit fibril formation by κ-casein [28].

1.3. Separation and purification of α_{S1}- and α_{S2}-casein

Caseins can be separated from whey proteins in milk by a variety of methods that are effective for large scale applications such as the acid precipitation already described [2]. The individual casein proteins can also be separated from one another by classical methods based on solubility differences and more recently via gel chromatography (summarised in [4]). In order to study α_{S1}- and α_{S2}-caseins separately, these proteins were purified from their associated form, α_S-casein, by successfully employing the method previously described in reference [34] using a Q-Sepharose column with some minor modifications [17]. Subsequent investigation of the chaperone activities

of individual α_{S1}- and α_{S2}-casein proteins was then possible. As shown in Figure 1, purification gave an initial smaller peak corresponding to the predominant form of α_{S2}-casein, α_{S2}A-casein 11P (25.2 kDa with 11 phosphate groups attached), and a second, much larger peak corresponding to the predominant form of α_{S1}-casein, α_{S1}B-casein 8P (23.6 kDa; 8 phosphate groups attached). It would be logical to expect that the areas under these peaks to be roughly representative of the 4:1 ratio of α_{S1}:α_{S2}-caseins present in bovine milk, however, due to their differing amino acid compositions α_{S1}- and α_{S2}-casein have quite different specific absorbances of 10.1 and 14.0 A1%1 cm, respectively [2]. The smallest peak visible in Figure 1 is representative of a small amount of dimeric α_{S2}-casein eluting first from the column. This species had an approximate mass of 50 kDa due to the presence of intermolecular disulfide bonds between cysteine residues at positions 36 and 40 [17].

Successful separation and purification of the constituent α_S-casein proteins was confirmed by SDS-PAGE and ESI-MS, which gave a major peak at 23,619 kDa (Figure 2). This closely corresponded to the major variant of α_S-casein, α_{S1}B-casein 8P. Unfortunately it was not possible to obtain a similar spectrum for α_{S2}A-casein 11P, most likely as a result of the greater hydrophobicity of this casein and its propensity for amyloid fibril formation [17].

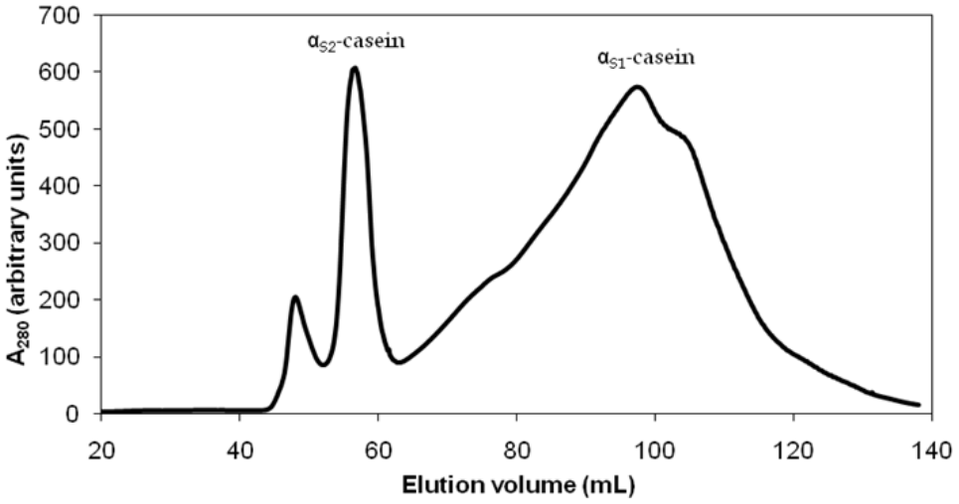

Figure 1. Purification of α_{S1}- and α_{S2}-casein from total α_S-casein. Reprinted with permission from [17]. Copyright (2011) Elsevier Inc.

1.4. Characterisation of purified α_{S1}- and α_{S2}-casein proteins

Biophysical characterisation of the purified α_S-casein proteins showed that the proteins possessed a similar degree of secondary structure to that expected from literature values [3, 35-38]. The far-UV CD spectra of purified α_{S1}-, α_{S2}- and α_S-casein proteins (Figure 3) show a minimum at approximately 202 nm for α_{S1}-casein, 203 nm for α_S-casein and 205 nm for α_{S2}-casein and a second minimum for all three proteins at 222 nm. Deconvolution of the far-UV CD data for α_{S1}- and α_{S2}-casein shown in Figure 3 was performed using SELCON software via the DICHROWEB database [39-43]. These data are summarised in Table 1. Deconvolution of data for α_S-casein could not be performed due to the presence of a 4:1 ratio of α_{S1}:α_{S2}-casein, each with a different number of amino acid residues.

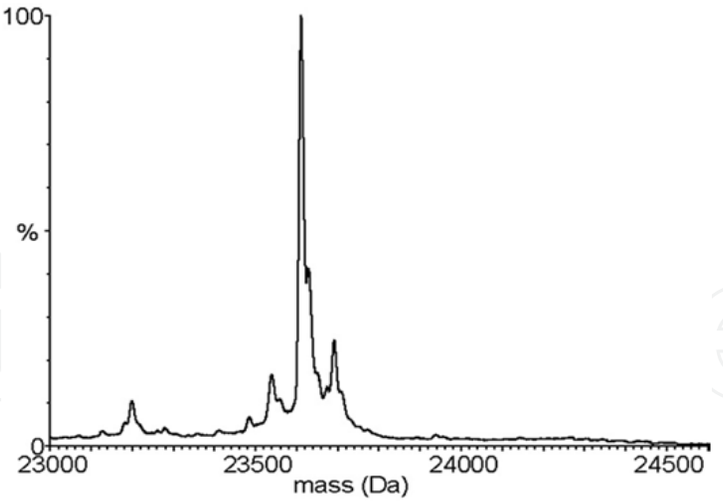

Figure 2. ESI-MS spectrum of purified α_{S1}-casein with a major peak occurring at 23, 619 kDa corresponding to α_{S1}B-casein 8P. Reprinted with permission from [17]. Copyright (2011) Elsevier Inc.

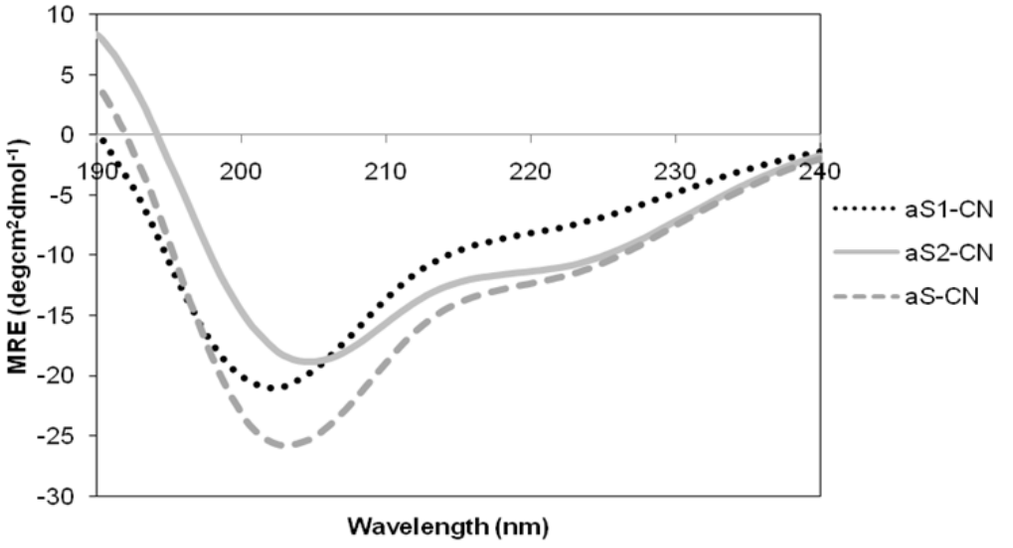

Figure 3. Far-UV CD spectrum of purified α_{S1}-, α_{S2}- and α_{S}-casein at 0.20 – 0.28 mg/mL in prefiltered 10 mM sodium phosphate buffer, pH 7.4. Reprinted with permission from [17]. Copyright (2011) Elsevier Inc.

Protein	α-helix (%)	β-sheet (%)	β-turns (%)	Disordered structure (%)
α_{S1}-casein	14	28	22	35
α_{S2}-casein	18	26	22	33

Table 1. Secondary structural elements of α_{S1}- and α_{S2}-casein as determined by deconvolution of far-UV CD spectra [39-43].

2. Introduction to molecular chaperones

2.1. The perils of protein folding

Early experiments on the folding of ribonuclease *in vitro* revealed that all of the information required for a protein to fold into its native 3D conformation was contained within the

primary sequence of that protein i.e. from the characteristics of amino acids and their positions in the polypeptide chain [44]. However, it also became evident that the folding of large, multi-domain proteins was complicated by incorrect intermolecular interactions involving the folding of the polypeptide chain [45] which prevented a proportion of proteins from reaching their native state, both *in vitro* [46, 47] and *in vivo* [48]. The process of protein folding, especially in the case of large proteins, appears to occur via a limited number of pathways. These pathways involve distinct intermediately-folded states known as 'molten globule' states [49]. This term is used to refer to a partially folded but compact state of a protein that has substantial amounts of secondary structure, but little or no tertiary structure [50]. In contrast to the natively folded state which is rigid and constrained, the molten globule state is not a single conformation but is instead a range of multiple conformations. These conformations are dynamic and rapidly interconvert with one another in response to the external environment [51] and they are also in equilibrium with more unfolded states [52]. Theoretical models have predicted a major loss of hydrophobic contacts in the molten globule compared to the natively folded state [50]. These intermediately folded states transiently expose previously buried hydrophobic areas on their surfaces and it is this characteristic that makes them prone to intermolecular association with other partially folded proteins, leading to aggregation and potential precipitation into insoluble aggregates [53, 54].

The currently accepted theory of protein folding is that natively folded proteins exist in equilibrium with less 'ordered' molten globule states. This 'folding/unfolding' pathway is reversible and slow, therefore it is possible for a protein that has partially unfolded but remains soluble to adopt a native state again provided it does not begin the process of aggregation. Once a molten globule state has begun to aggregate (either through interactions with other proteins or other molecules of the same protein), it has entered the fast and irreversible 'off-folding' pathway which ends with precipitation [55]. Within the cell, where proteins are present in high concentrations (e.g. 340 g/L in *E. coli*) and rates of protein production can be extremely high, folding of nascent proteins is further challenged by molecular crowding. Under such conditions, there are a great number of opportunities for inappropriate protein interactions. As the three-dimensional structure of a protein largely determines its function, the incorrect folding of proteins has the consequence of loss of function. It is not surprising therefore that abnormalities in protein folding form the basis of many human pathologies such as prion diseases and amyloidoses [56, 57].

2.2. The role of molecular chaperones

Molecular chaperones are a diverse group of proteins that act to prevent 'improper' interactions between other proteins that may result in aggregation and precipitation [58]. They ensure high fidelity protein folding and assembly without becoming part of the natively folded structure. In doing so, chaperones perform important roles in the stabilisation of many other proteins both intra- and extracellularly. Proteins from many unrelated families have been identified as possessing molecular chaperone function [59]. There are four key features that must be exhibited by a protein in order for it to be classified

as a molecular chaperone: 1) suppression of aggregation during protein folding, 2) suppression of aggregation during protein unfolding, 3) influence on the yield and kinetics of folding and 4) effects exerted at near stoichiometric levels [60]. Many molecular chaperones have been identified in the eukaryotic cell, particularly in the endoplasmic reticulum and cytosol where they ensure correct folding, transport and biological activity of countless proteins [61]. Some chaperones act in sequence with others, passing on intermediately folded proteins to continue the folding process [62, 63]. Other chaperones, such as the small heat-shock proteins which are explained in greater detail later in this chapter, specifically interact with proteins on the off-folding pathway only. These intermediates, more prone to aggregation, are the ones recognised by the chaperone and stabilised against precipitation [58, 64].

2.3. Molecular chaperones are also heat-shock proteins

The expression of molecular chaperones is markedly increased (10 to 20 fold) under conditions of physiological stress (e.g. heat, reduction, oxidation stresses) - a feature which explains their other moniker as 'heat-shock' proteins (Hsps). It has been demonstrated that both the protein coding sequences [65, 66] and regulatory sequences [67] of some heat-shock genes have been highly conserved [68, 69].

2.4. The sHsps and clusterin

There are several classes of heat shock proteins, and the accepted nomenclature is based on their approximate molecular mass on SDS-PAGE i.e. Hsp60 and Hsp70 are 60 and 70 kDa, respectively. A subset of the Hsps is called the small heat-shock proteins, or sHsps, with molecular masses of monomers ranging from 15-30 kDa. These chaperones act in an ATP-independent manner, which unlike Hsp60 and Hsp70 do not actively refold in the presence of ATP. Instead, they interact with partially unfolded or 'stressed' proteins, stabilising them in a soluble, high molecular weight complex to prevent their precipitation from solution [70]. They do not interact with natively folded proteins, nor with those that have already aggregated [71]. Rather than simply serving as a one-way 'sink' for denatured proteins, however, previous studies have shown that in addition to their ability to interact with and stabilise stressed proteins, some sHsps such as Hsp25 [58], α-crystallin [72] and clusterin [73] act co-operatively with ATP-dependent chaperones (e.g. Hsp70) to refold the stressed protein when the stress is removed and normal cellular conditions are restored. Studies on α-crystallin have shown that the presence of ATP causes the sHsp to undergo a conformational change whereby the stressed target protein is released facilitating refolding by chaperones such as Hsp70 [74]. Members of the sHsp family have several features in common, including size and amino acid sequence homology within the C-terminal domain [55]. There have been 10 human sHsps identified thus far, including α_A- and α_B-crystallin (discussed below), Hsp27 and its murine equivalent, Hsp25 [75, 76].

The sHsps are also able to exist as monomeric and dimeric species which associate with one another to form large multimeric complexes, somewhat akin to the behaviour of the casein

proteins both within and without the casein micelle. Our studies have shown that α_S-casein, and more specifically, α_{S1}- and α_{S2}-casein, also act as molecular chaperones and do so in a manner that is similar to the sHsps and an extracellular chaperone, clusterin. Clusterin is a secreted mammalian chaperone present in bodily fluids such as blood and semen. Like the sHsps, clusterin is highly conserved and is upregulated in many cell types under conditions of stress and in protein misfolding diseases such as Alzheimer's disease [77]. It is a disulfide-linked dimer which is 75 – 80 kDa in size and highly glycosylated [73, 77]. Clusterin has been shown to aggregate in aqueous solution and at physiological pH values is present in monomeric, dimeric and multimeric states [78]. Like the sHsps, clusterin is an ATP-independent molecular chaperone capable of stabilising a wide array of target proteins [73, 77]. Stoichiometrically, clusterin has been shown to protect stressed target proteins at levels consistent with the concentration range of clusterin normally found in extracellular fluids (50 – 370 μg/mL in human serum and 2.1 – 15.0 mg/mL in human seminal fluid). In addition, clusterin displays greater chaperone activity with smaller target proteins versus larger ones [77] indicating that it has the ability to interact differently with various target proteins depending on the conditions. This variability of chaperone action has also been demonstrated extensively in studies on sHsps such as α-crystallin which have been shown to exhibit increased or decreased chaperone activity depending on the mode of aggregation of a target protein [79-81].

α-Crystallin is a member of the small heat-shock protein family and has for some time been known to play a major role in stabilising other crystallin proteins in the eye lens. It was identified some time ago that α-crystallin performed an important structural role in the lens, as a member of the crystallin family of proteins which also comprises β- and γ-crystallin [82]. In its normal state, the lens is transparent despite the high concentrations of these proteins in the cell cytoplasm (33% in the human lens and 50% in rat and bovine lenses [83]). Such a high concentration of proteins would ordinarily cause a significant degree of light scatter, but these highly homologous proteins adopt a critical short-range order that allows them to exist in a dense glass-like liquid resulting in unimpeded transmission of light through the lens [84, 85]. Their structural integrity therefore is of prime importance and disruption to their three-dimensional arrangement as a result of chemical modification, for example, has been shown to result in increased light scattering manifesting as cataract [86]. Due to the lack of protein turnover in the lens over the lifetime of an organism, the occurrence of α-crystallin in the lens allows it to perform a second, equally important role as a molecular chaperone. Numerous studies have shown that under conditions of cellular stress, α-crystallin interacts with not only other crystallins but with a plethora of other proteins, stabilising them against precipitation. Its wide tissue distribution and localisation in various disease states associated with protein misfolding also provides strong evidence for the role of α-crystallin as a molecular chaperone outside the lens. Like α_S-casein, α-crystallin is made up of two distinct gene products designated α_A- and α_B-crystallin, so named because of their relatively acidic and basic properties. The individual subunits of α-crystallin are present in a 3:1 ratio (α_A:α_B) in the human lens, are each ~20 kDa in size and these proteins readily associate with one another to form dimeric and multimeric species. These multimers can be

up to 1.2 MDa in size and exhibit dynamic subunit exchange. As such, α-crystallin, like αS-casein has been resistant to crystallisation and a precise picture of its mechanism of action is still to be elucidated. Almost 100 years after being identified in the lens, individual αA- and αB-crystallin subunits were found in non-lenticular tissues [87-91]. These proteins and other sHsps have been identified in the brain where they are associated with neurodegenerative diseases such as Alzheimer's, Creutzfeldt-Jakob and Parkinson's diseases (summarised in [92]).

2.5. Putative mode of action of sHsps and clusterin

As reviewed in [93], current theories of the chaperone action of both sHsps and clusterin centre around their ability to expose hydrophobic regions that interact with partially folded target proteins, also known as 'disordered' molten globules, forming soluble, high molecular weight complexes and preventing them from precipitation. It has been shown that sHsps do not interact with target proteins that are natively folded, completely denatured, or those in stable molten globule states [55, 94-97]. The stoichiometry of sHsp and clusterin interaction with target proteins suggests that one oligomer of chaperone can bind to and stabilise many molecules of stressed target protein, in fact, CryoEM and X-ray solution studies on stressed α-lactalbumin and αB-crystallin showed that the target protein coated the exterior surface of the αB-crystallin oligomer upon formation of the chaperone-target protein complex [98, 99].

The relatively hydrophobic nature of the more globular portion of the sHsp is balanced by the adjoining flexible, dynamic C-terminal extension which is solvent exposed and hydrophilic and is thought to play a major role in ensuring the solubility of the huge complex formed upon chaperone interaction [100, 101]. It has also been demonstrated that the C-terminal extension in α-crystallin is critical for oligomeric assembly [79]. Altered 'spacing' of chaperone molecules resulting from modification of the C-terminal sequence results in abnormally sized oligomers with perturbed structure, physical stability and chaperone function [79]. The large, oligomeric forms of sHsps are thought to exist in dynamic equilibrium with smaller species which rapidly interchange with the oligomer [102, 103]. This equilibrium of subunit exchange is believed to be key to sHsps broad target protein specificity [97, 103-105], and although clusterin shares many features with the sHsps in terms of its chaperone action, the potential importance of subunit exchange for it has not been described to date [106].

3. αS-Casein as a molecular chaperone

The casein proteins and their derivatives have been used by the food industry as important nutritional and stabilising proteins for many years [4]. Early studies showed that whole casein (i.e. αS-, β- and κ-casein) prevented heat induced aggregation of whey proteins, even in calcium-containing systems [107, 108]. This stabilising action of the caseins on heat-denatured target proteins was proposed to occur through non-specific interactions and opened up a new avenue of uses for casein proteins in stabilising both milk and non-milk proteins and thereby contributing to novel properties of milk products. It was then

demonstrated in 1999 that individual αs-casein possessed molecular chaperone activity [109]. Since then, β- and κ-casein have both been shown to also act as molecular chaperones [18, 33, 110-112]. The presence of high numbers of phosphate groups in the casein proteins appears to be important for chaperone action against amorphously aggregating target proteins under both reduction and heat stress, with studies showing that removal of these in αs- and β-casein reduced their ability to prevent the aggregation of target proteins [113, 114]. Previous work on αs-casein showed that it prevented the stress-induced aggregation of natural target proteins such as the whey proteins β-lactoglobulin and bovine serum albumin, but also of unrelated proteins such as alcohol dehydrogenase and carbonic anhydrase [109]. In acting as a molecular chaperone under these conditions, αs-casein was able to interact with partially unfolded target proteins and prevent their incorporation into insoluble aggregates which would then have formed precipitates. Furthermore, when added to partially aggregated reduction-stressed insulin, αs-casein not only prevented further aggregation of insulin but facilitated its re-solubilisation when present at a 2:1 (w:w) ratio [109].

As shown in Figure 4, the putative mode of action of αs-casein is based on a similar model proposed for sHsps [70] where a natively folded protein (N) unfolds via a number of intermediately folded states (I_1, I_2 etc.) or 'molten globule' states on its way to the unfolded state (U). This folding and unfolding is fast and reversible and involves the exposure of hydrophobic regions normally buried in the interior of the protein.

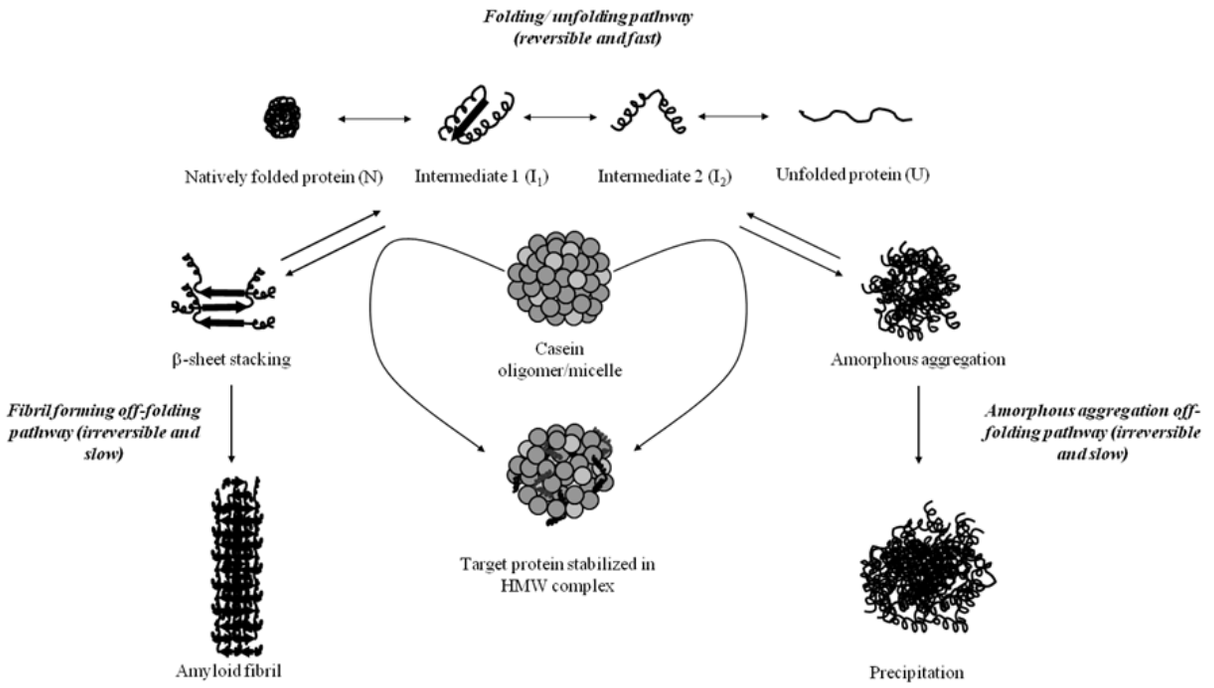

Figure 4. Putative mechanism of action of αs-casein showing its interaction with target proteins on the folding and off-folding pathways. See text for explanation. Reprinted with permission from [33]. Copyright (2009) Dairy Industry Association of Australia.

Under conditions of cellular stress, when intermediately folded states are present for longer periods, self-association is promoted by the prolonged exposure of hydrophobic

surfaces. When self-association occurs, the intermediately folded states enter the off-folding pathways which are slow and irreversible and may lead to either amorphous aggregation as shown on the right hand side of the figure or to fibril formation as shown on the left. Amorphous aggregates result from disordered aggregation and lead to the formation of insoluble protein precipitates. Conversely, the ordered amyloid pathway leads to highly ordered β-sheet stacking giving cross β-sheet fibrils. Casein micelles or oligomeric forms of αs- or β-casein are able to interact with partially folded proteins and stabilise them against aggregation and precipitation by forming a soluble high molecular weight (HMW) complex [33].

3.1. Assessment of molecular chaperone activity

Assessment of molecular chaperone activity traditionally involves an *in vitro* assay in which a target protein is subjected to a form of stress similar to what would be encountered under physiological conditions (e.g. heat, oxidation, reduction etc.) in the presence and absence of different amounts of chaperone. These assays are not complicated by the aggregation of the chaperone itself which is stable under these conditions, and suppression of aggregation has been shown to be specific to the action of molecular chaperones as substitution of these with non-chaperones (e.g. ovalbumin) has been shown to have no effect on the extent of target protein precipitation [73]. For the assessment of amorphous aggregation, light scattering of the proteins in solution at 360 nm is monitored over time, whereas for fibril forming proteins, amyloid formation is monitored by increasing Thioflavin T (ThT) fluorescence. These values increase to a maximum over the timeframe of the experiment and are used to estimate % protection provided by the chaperone as per the following formula:

$$\% \text{ Protection } = \frac{100 * \left(\Delta I - \Delta I_{\text{chaperone}}\right)}{\Delta I} \quad (1)$$

(1) Calculation of percentage protection of a target protein by molecular chaperone. In this formula, ΔI and $\Delta I_{chaperone}$ represent the change in light scattering (for amorphously aggregating target proteins) or in Thioflavin T (ThT) fluorescence (for amyloid forming target proteins) in the absence and presence of the chaperone, respectively [79].

3.2. αs-Casein stabilises proteins *via* formation of soluble high molecular weight complexes

Consistent with the well-characterised properties of a molecular chaperone, it has been shown that αs-casein (and β-casein) form soluble, high molecular weight (HMW) complexes with stressed target proteins that can be identified by size-exclusion chromatography. These complexes, formed between αs-casein and heat-stressed β-lactoglobulin or apo-α-lactalbumin eluted from the column at a retention time correlating to an approximate mass of 1.8 MDa [18]. Control experiments showed that in the absence of a chaperone, heat stressed β-lactoglobulin also formed large, multimeric species but these were largely insoluble [18]. Mixtures of the individual whey proteins and αs-casein that were not exposed

to heat stress eluted as separate peaks with elution times corresponding to the individual proteins i.e. there was an absence of a target protein-chaperone or HMW complex. Additional experiments showed that the elution time of αs-casein was not altered in any discernible way by the addition of heat stress [18].

The molten globule states of a natural target protein of αs-casein, α-lactalbumin, have been well characterised [13, 55, 115-122]. Intermediately folded states of the apo- form of α-lactalbumin provide an ideal model for the investigation of protein folding and unfolding and therefore the action of molecular chaperones when present in solution. The molten globule states of apo-α-lactalbumin exhibit a relatively compact structure in which a secondary structure is largely preserved, but tertiary structure is lost [123]. A characteristic of these states is that they expose significant amounts of hydrophobicity to solution as a result of their being 'uncovered' from the interior of the previously natively folded protein and it is these exposed hydrophobic areas that appear key to their interaction with molecular chaperones [124, 125]. Destabilisation of α-lactalbumin upon removal of its calcium ion by a chelating agent such as EDTA induces a conformational change that further exposes the disulfide bonds of the protein to reduction with DTT [122]. Under this reduction stress, α-lactalbumin adopts a molten globule state that is structurally unstable and similar to that formed at pH 2.0 [13]. This state, in the absence of a molecular chaperone, such partially unfolded proteins will readily aggregate and precipitate. When monitored via real-time ^{1}H NMR spectroscopy, the formation of the molten globule state, its aggregation and eventual precipitation can be visualised. The aromatic protons in the region of the spectrum from 6-8 ppm can be attributed almost exclusively to the signals of protons in the target protein, which is small (14.4 kDa) and monomeric. Resonances arising from aromatic protons in αs-casein are relatively broad by comparison, so that even in the presence of added chaperone, the structural alterations in the target protein are easily observed. Isolated resonances arising from tyrosine 3,5 ring protons at 6.8 ppm are therefore a reliable indicator of molten globule formation and stabilisation in α-lactalbumin [18, 122, 126]. The well-resolved resonances visible at Time 0 are representative of the native state and are quickly lost with the addition of DTT. In the absence of αs-casein (Figure 5A), an initial increase in resonance arising from the Tyr (3,5) protons can be observed from ~ 0-200 s following the addition of DTT. This period represents the reduction of the disulfide bonds in α-lactalbumin by DTT, giving the molten globule state which in the absence of chaperone is prone to aggregation then precipitation [122] after a period of approximately 1000 s [18]. In the presence of a 2:1 (w:w) ratio of αs-casein to apo-α-lactalbumin (Figure 5B) , signals arising from the aromatic protons are preserved, indicating that the molten globule state of α-lactalbumin is stabilised by the interaction between the two proteins. As shown in Figure 5C, in the absence of αs-casein the rate of decay of the Tyr (3,5) resonance of apo-α-lactalbumin is rapid, occurring at rate of 2.70 (± 0.11)× 10^{-3} s^{-1} whereas in the presence of αs-casein it is approximately 50% slower at 1.25 (± 0.08)× 10^{-3} s^{-1} [18].

Extrinsic fluorescence studies have shown that upon formation of a high-molecular weight complex between αS-casein and reduction stressed insulin, a conformational change in one or both proteins resulted in an increase in clustered, exposed hydrophobicity. The other

casein proteins, β- and κ-casein also exhibited similar increases in hydrophobicity in the presence of reduction stressed insulin, implying that similar interactions were occurring during formation of the chaperone-target protein complex [18]. A mixture of the three caseins (αs-, β- and κ-casein) combined according to their approximate proportions in bovine milk (60%, 25% and 15%, respectively), exhibited considerably increased exposure of clustered hydrophobic areas, indicating a synergy between the various subunits during stabilisation of stressed insulin [18]. Intrinsic (tryptophan) fluorescence studies showed that the tryptophan residues in the C-terminal region of αs-casein were exposed to a more non-polar environment as a result of the interaction with the reduced insulin B-chain, and may indicate involvement of this region of the chaperone with the hydrophobic (bound) target protein [18].

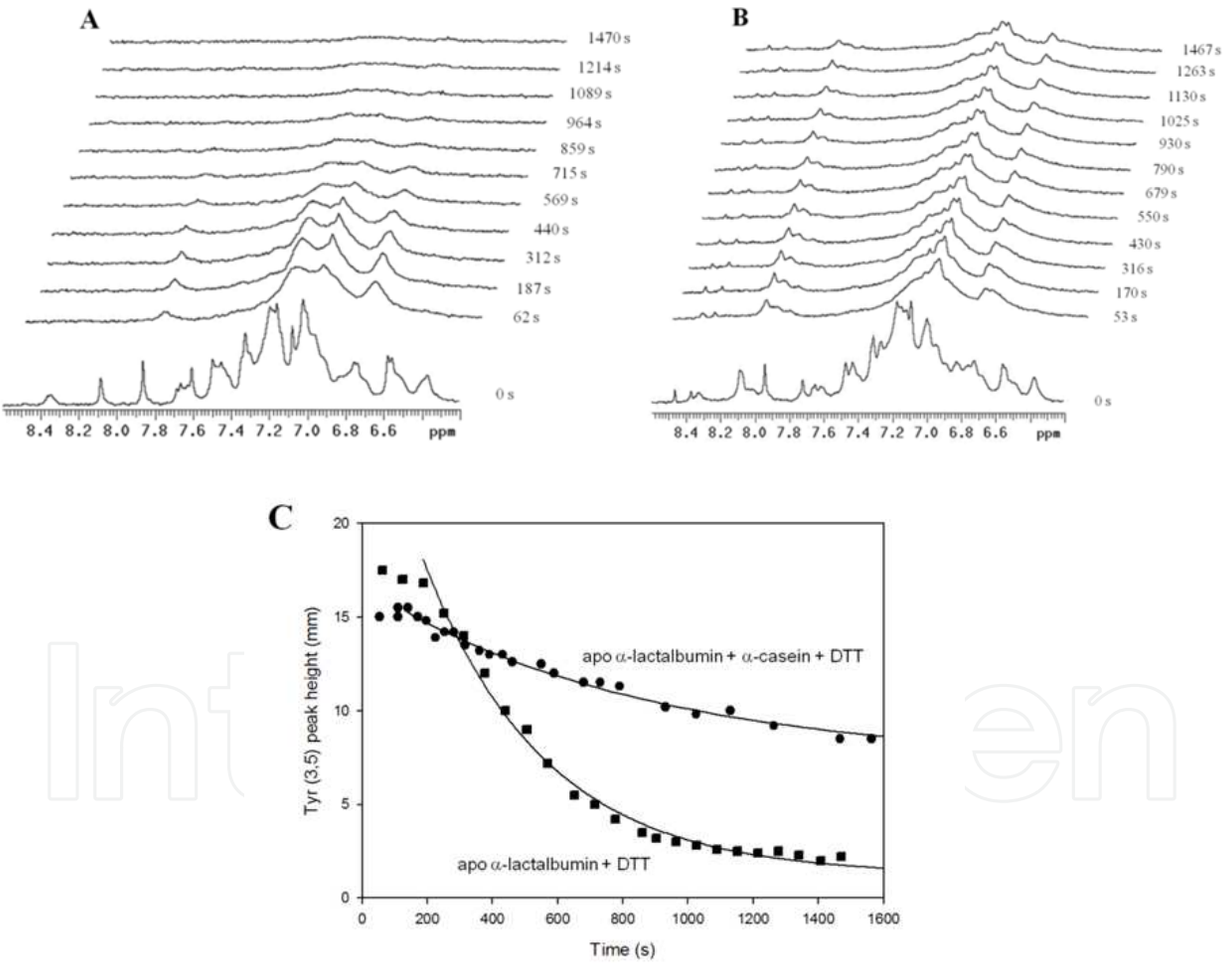

Figure 5. Real-time ^{1}H NMR spectroscopy of apo-α-lactalbumin under reduction stress at 37°C in the presence and absence of αs-casein. Stacked plots of 1D ^{1}H NMR spectra show molten globule formation by reduced apo-α-lactalbumin in A) the absence and B) the presence of αs-casein at a 2:1 (w:w) ratio. First-order decay of the resonance arising from the tyrosine (3,5) ring protons at 3.5 ppm in both cases (C) was used to calculate rates i.e. peak height (mm) v time (s). Reprinted with permission from [18]. Copyright (2005) American Chemical Society.

3.3. Effects of temperature, pH and molecular crowding

As shown in [18], αs-casein displayed temperature dependence in preventing aggregation of the reduced insulin B-chain, with comparatively better chaperone ability observed at 25°C compared to that at 37°C. This was in keeping with previous studies that showed that αs-casein was a considerably better chaperone at lower temperatures, giving 52% protection of insulin aggregation at 27°C compared to almost complete protection at 18°C [109]. This 'cold shock protein' characteristic is quite different to the temperature effects of other molecular chaperones such as α-crystallin and tubulin, which display enhanced chaperone activity at increased temperatures [127-129]. At temperatures above 30°C, α-crystallin undergoes structural transitions that involve rearranging and/or increasing hydrophobic surfaces, enhancing hydrophobic interactions between the chaperone and the target protein [124, 125]. Conversely, clusterin's chaperone ability appears to be quite independent of temperature effects [130]. The properties of αs-casein that allow it to chaperone at lower temperatures are reflected in the fact that greater amounts of αs-casein have been identified in mammals inhabiting low temperature zones compared to those in more temperate climates [131, 132] which may indicate an important stabilising role in physiological adaptation to low temperatures. Both β- and κ-casein also display chaperone activity, but in contrast to αs-casein, the chaperone action of these proteins does not appear to be as dependent on temperature as αs-casein [18]. In stabilising insulin, apo- and holo-α-lactalbumin as target proteins, β- and κ-casein did not provide as much protection against precipitation as αs-casein on a w:w and molar basis, but were similar to each other in their efficacy [18]. This was in contrast to previous reports which described greater chaperone action in β-casein compared to αs-casein in stabilising heat stressed catalase and reduction stressed lysozyme [110], whereas others found that αs-casein was better than β-casein at preventing the aggregation of heat-stressed ovotransferrin [113]. This apparent disparity in the relative chaperone performances of the casein proteins is likely to be related to their broad target protein specificities. The same observations have been made in relation to the chaperone action of sHsps and chaperone efficiency has been shown to be dependent upon the size of the target protein and the rate and mode of aggregation, as previously discussed [79-81]. A combined form of the casein proteins (60% αs-, 25% β- and 15% κ-casein) was shown to possess similar chaperone activity to that of αs-casein alone [18].

In the investigation into the mechanism of chaperone action of αs-casein, experiments with its natural target protein, β-lactoglobulin, have been performed under heat stress at 70°C [18, 109]. Obviously this level of heat stress is not physiologically appropriate, but the ability of αs-casein to stabilise other dairy proteins under extreme heat stress is of importance to the dairy industry which employs treatment processes such as pasteurisation and ultra-high temperature treatment [18]. In these studies, it was found that pH had a major effect on the chaperone's ability to suppress the aggregation of the target protein. The ability of αs-casein to suppress the aggregation of β-lactoglobulin at a 0.25:1 (w:w) ratio decreased from 73% at pH 7.0 to 33% at pH 7.5 and 19% at pH 8.0 after 450 min [18]. This is likely to be related to the rate at which β-lactoglobulin aggregates at higher pH values and may also be related to changes in the chaperone ability of αs-casein at more alkaline pH values. At slightly alkaline

pH values, the histidine residues of αs-casein deprotonate (αs1-casein has five histidines and αs2-casein, three) and it is feasible that this results in a loss of electrostatic contacts between the subunits, disrupting their ability to effectively bind the target protein [18].

A pH dependence is also exhibited by clusterin, which is in fact a better chaperone at more acidic pH values [130]. This is believed to be due to the increased dissociation of clusterin from its larger multimeric state at acidic pH values [130]. Conversely, α-crystallin is less effective as a chaperone at slightly acidic pH values [130, 133] but this is thought to be due to subtle structural changes in the protein with a change in pH rather than a change in aggregation state [134]. It may also be that at increased pH values αs-casein is less effective as a chaperone against β-lactoglobulin aggregation because the nature of the aggregation changes at more alkaline pH values. Greater intermolecular disulfide bond formation produces oligomers of β-lactoglobulin which then polymerise to form an aggregate. It is likely that αs-casein is similar to α-crystallin in that it cannot interact with and stabilise these forms of aggregated proteins as effectively as those that aggregate in a nucleation-dependent manner [135]. Other factors that have been shown to influence the nature and rate of target protein aggregation also have an impact on αs-casein's chaperone action. One of these is molecular crowding. Experiments were conducted in the presence of 10% dextran in order to simulate the protein-rich environment of milk and a greater rate of target protein (insulin, apo- and holo-α-lactalbumin) precipitation was observed under these conditions [18]. This increased rate of aggregation affects αs-casein's chaperone ability in the same way as described previously for α-crystallin, other sHsps and clusterin, which are all poorer chaperones with rapidly aggregating target proteins [94, 96, 122, 136, 137].

3.4. αs-Casein is unable to protect enzymes from loss of function due to heat stress

As previously discussed, it is known that molecular chaperones such as sHsps interact with and bind partially unfolded target proteins in molten globule-like states, but not once they have begun to aggregate [71]. The chaperone behaviour of αs- and β-casein appears different in this regard, with both having been shown to re-solubilise aggregated DTT-treated insulin [109, 110]. According to reference [138], one of the features of a protein used to classify it as a molecular chaperone is that it be able to aid in the recovery of lost biological activity. Like the sHsps and clusterin, however, αs-casein is unable to prevent the loss of activity in enzymes induced by heat stress. Our experiments with catalase, GST and ADH show that the presence of αs-casein at a 1:1 (w:w) ratio does not protect these enzymes to any significant extent against heat-induced loss of function (Figure 6).

3.5. αs-Casein is ATP-independent in its chaperone action

In order to obtain further insight into the molecular mechanism of action of αs-casein, the effect of ATP on its chaperone function was also investigated [17; Treweek, Price & Carver, unpublished work]. Previous studies have shown that αs-casein acts in a similar manner to small heat shock proteins (sHsps) and clusterin [17, 18, 28, 29, 33]. A major characteristic of

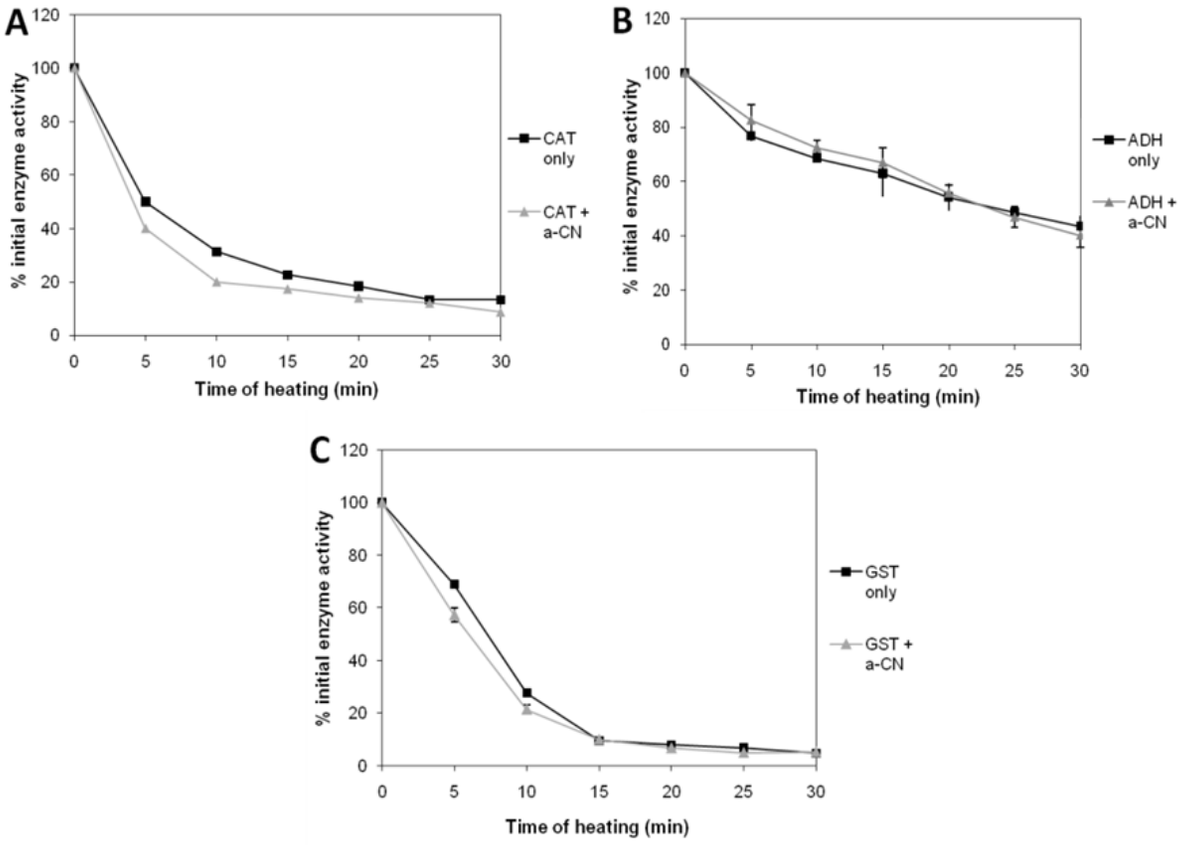

Figure 6. Loss of enzyme activity (catalase, CAT; ADH, alcohol dehydrogenase; GST, glutathione-S-transferase) with heat stress at 55°C and the effect of addition of αs-casein at a 1:1 (w:w) ratio. Values represented are means of independent triplicate measurements and error bars shown are standard deviations of the mean which in some cases are too small to be visible. Reprinted with permission from [17]. Copyright (2011) Elsevier Inc.

sHsps and clusterin is that they function in an ATP-independent manner consistent with their inability to refold stressed proteins [139-141]. ATP levels in milk are relatively low (5 μM) and as such it is likely to be non-essential to αs-casein's chaperone activity, however, it is an important mechanistic tool. A similar study on clusterin (which is also extracellular and as such experiences low physiological ATP levels) provided valuable insight into the ATP-independent action of the chaperone in binding the stressed enzymes catalase, ADH and GST [73]. Subsequent refolding of bound target proteins to clusterin and sHsps (e.g. Hsp25 and α-crystallin) is achieved via ATP-dependent chaperones such as Hsp70 [58, 72]. The presence of physiologically relevant levels of ATP on the ability of αs-casein to suppress aggregation of stressed target proteins (specifically catalase, ADH and insulin) was investigated and it was found that the chaperone action of αs-casein was unaffected by the presence of ATP [17; Treweek, Price & Carver, unpublished work]. In addition, the ATPase activity of αs-casein was assessed and it was found that αs-casein had no detectable ATPase activity either on its own or during chaperone action i.e. when interacting with heat stressed β-lactoglobulin in a chaperone complex (Figure 7) [Treweek, Price & Carver, unpublished work].

3.6. αs-Casein does not bind target proteins in a way that allows refolding by Hsp70

As previously mentioned, some sHsps such as Hsp25 [58], α-crystallin [72] and clusterin [73] act co-operatively with ATP-dependent chaperones such as Hsp70 to refold stressed proteins when the stress is removed and cellular conditions are restored [139]. Studies on α-crystallin have revealed that a region in the conserved α-crystallin domain of αB-crystallin undergoes structural modification upon binding of ATP [142] and that this conformational change causes α-crystallin to release stressed target proteins, facilitating their refolding by chaperones such as Hsp70 [74]. Although not a natural component of milk, Hsp70 was used to probe the mechanism of αs-casein's chaperone action and to allow comparisons with better characterised chaperones to be made.

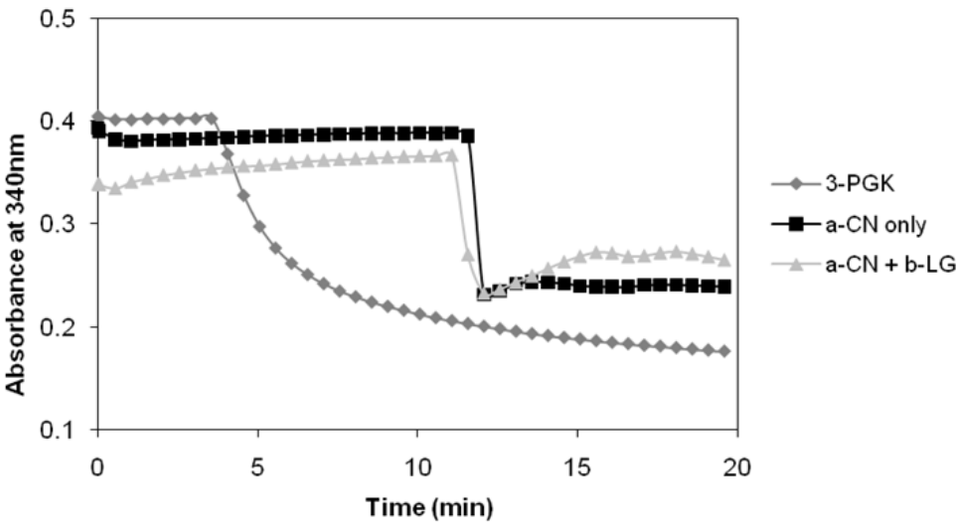

Figure 7. ATPase activity of αs-casein. The ability of αs-casein (a-CN) to hydrolyse 170 μM ATP was examined in the presence and absence of heat-stressed β-lactoglobulin (b-LG) at a ratio of 2:1 (w:w) in 50 mM sodium phosphate buffer containing 0.2 M NaCl, 2.5 mM EDTA and 0.02% NaN_3 at pH 7.2. Generation of ADP was monitored *via* NADH oxidation giving a decrease in absorbance at 340 nm. This can be seen with the addition of 21 nmol exogenous ADP at 11 min (black arrow) and the addition of 1.1 μmol 3-phosphoglycerate to 0.76 units of 3-phosphoglycerate kinase (3-PGK) at 4 min (grey arrow). All experiments were performed in triplicate at 37°C.

Recovery of enzyme activity was assessed using heat-stressed catalase and ADH [17, 73] in the presence and absence of 1:1 (w:w) ratios of αs-casein similar to the assays shown in Figure 6, with the addition of Hsp70 and ATP after a recommended 30 min 'recovery period' [17, 58]. In the presence of αs-casein, neither catalase nor ADH showed any significant recovery from heat stress at 55°C, with only 1(±5)% activity remaining 5 hours after the addition of Hsp70 and ATP. In contrast, control experiments showed 27(±5)% recovery of catalase activity over the same time period and under the same conditions, but with the addition of clusterin instead of αs-casein [17]. This effect of clusterin has been shown to be specific for the chaperone, as other proteins added in its place (e.g. lysozyme, myoglobin) have been shown to be unable to facilitate enzyme recovery to the same extent

[73]. It could be concluded, therefore, that the binding of Hsp70 to its target protein to achieve refolding and release, coupled with ATP binding and hydrolysis, is impeded by the manner in which αS-casein binds the destabilised target protein [17]. In stabilising the heat-stressed enzymes, αS-casein may hold its target more tightly or incorporate them into the complex in such a way that they are not accessible to Hsp70 to allow for the refolding that has been seen for target proteins stabilised by sHsps and clusterin [58, 72, 73, 139]. It is possible that the αS-casein-target protein complex is to some extent incorporated the casein micellar structure and this arrangement influences the accessibility of Hsp70 [17]. CryoEM and X-ray solution studies on sHsps have revealed that stressed α-lactalbumin molecules coat the exterior surface of the oligomeric form of αB-crystallin when the chaperone-target protein complex is formed [99, 143]. Further studies with spin-labelled melittin peptides have shown that there is a stoichiometry of approximately 1:1 [144] in the binding of the peptides to each monomer of α-crystallin and that these are relatively evenly spaced [145]. This regular arrangement of target protein binding to α-crystallin may be an important factor in the ability of Hsp70 to subsequently refold target proteins [17].

3.7. αS1- and αS2-Casein also act as molecular chaperones

The constituent proteins of αS-casein, αS1- and αS2-casein (separated and purified as described earlier in this chapter) have been shown to exhibit chaperone action independently of one another. Like αS-casein, the chaperone action of αS1- and αS2-casein has been described for a range of target proteins under various stress conditions [17]. Studies with reduced insulin as the target protein showed that at ratios of 0.25:1 and 0.5:1 (w:w casein: insulin) both αS1- and αS2-casein had comparable chaperone activity to αS-casein (summarised in Table 2).

α-CN:insulin (w:w)	αS-CN	αS1-CN	αS2-CN
0.1:1	66 (± 2)%	49 (± 2)%	64 (± 2)%
0.25:1	74 (± 0.6)%	69 (± 2)%	73 (± 1)%
0.5:1	96 (± 0.4)%	91 (± 1)%	91 (± 0.3)%
1:1	98 (± 0.9)%	98 (± 0.1)%	97 (± 0.4)%

Table 2. Summary of chaperone assay data with insulin under reduction stress in the presence of increasing amounts of αS-, αS1- or αS2-CN. Figures shown are % protection of stressed target protein by the chaperone. Percentage protection is calculated as previously described. Reprinted with permission from [17]. Copyright (2011) Elsevier Inc.

When assessed with catalase under heat stress, a 0.5:1 ratio of αS-casein to catalase provided 88 (± 2)% protection after 50 min. Under these conditions, αS2-casein was the better chaperone, giving 84 (± 4)% protection at the same ratio and time point, whereas αS1-casein provided only 64 (± 1)% at the same ratio and time point [17]. In another set of experiments that included 0.1 M NaCl in order to more accurately simulate the high salt conditions in milk, catalase aggregation occurred more rapidly and as a result, all of the α-casein proteins tested were less effective in preventing catalase aggregation and precipitation. These results are summarised in Table 3 and visible spectroscopy spectra are shown in Figure 8.

α-CN:cat (w:w)	αS-CN	αS1-CN	αS2-CN	αS-CN + salt	αS1-CN + salt	αS2-CN + salt
0.25:1	76 (± 1)%	44 (± 9)%	77 (± 3)%	15 (± 0.1)%	3 (± 0.8)%	16 (± 2)%
0.5:1	88 (± 2)%	64 (± 1)%	84 (± 4)%	27 (± 1)%	16 (± 2)%	26 (± 2)%
1:1	96 (± 2)%	80 (± 3)%	85 (± 6)%	57 (± 1)%	16 (± 3)%	18 (± 2)%

Table 3. Summary of chaperone assay data with catalase (cat) under heat stress in the presence of increasing amounts of αS-, αS1- or αS2-CN and in the presence and absence of 0.1 M NaCl. Figures shown are % protection of stressed target protein by the chaperone. Percentage protection is calculated as previously described. Reprinted with permission from [17]. Copyright (2011) Elsevier Inc.

Under conditions of added salt, the chaperone abilities of αS-, αS1- and αS2-casein were all greatly reduced, with a 0.5:1 ratio of αS-casein: catalase providing only 27 (± 1)% protection, compared with 88 (± 2)% for the same ratio in the absence of salt. At the same ratio and time point αS2-casein showed similar chaperone ability to αS-casein (26 (± 2)% protection), with the most profoundly affected protein being αS1-casein. Under these conditions, even a 1:1 ratio of αS1-casein: catalase provided only 16 (± 2)% protection. Analysis of the samples from these assays by SDS-PAGE showed that αS1-casein had actually formed high molecular weight complexes that failed to migrate through the gel matrix to any extent, but like the other chaperones, remained soluble [17]. Despite remaining in solution, the data show that this aggregated form of αS1-casein was no longer an effective chaperone. The self-association of αS1-casein in the presence of salt most likely occurs as a result of neutralisation of charged residues on the protein ('charge screening' [146]) by the interaction with sodium and chloride ions i.e. the early stages of isoelectric precipitation [12]. Aromatic residues in αS1-casein are also thought to play a major role in the hydrophobic interactions between αS1-casein molecules, which at increasing ionic strengths go from monomers to dimers, tetramers, hexamers, octamers, then higher order aggregates [147].

Molecular chaperones are known to stabilise amorphously aggregating proteins like those described above, but they also interact with and stabilise proteins destined to form fibrillar aggregates (refer to Figure 4). This property has been described for sHsps in suppressing amyloid fibril formation in β-amyloid peptide [148, 149], apolipoprotein C-II [150] and in α-synuclein, the protein present in the Lewy bodies of Parkinson's disease [151]. A form of κ-casein which is destabilised as a result of being reduced and carboxymethylated (RCM-κ-casein) has been shown to form fibrils at 37°C in the presence of DTT and has been widely used as a model for investigating chaperone action against fibrillar proteins [28, 33, 79, 152].

Studies have shown that the presence of a ~0.6:1 w:w ratio of αS-casein:RCM-κ-casein reduced the Thioflavin T fluorescence (an indicator of the probe's binding to forming fibrils) by 65%, and in the presence of a 2.5:1 w:w ratio of αS-casein:RCM-κ-casein, fibril formation was completely abrogated [17, 28]. In the presence of αS1- or αS2-casein at the same ratio, fibril formation was also completely suppressed. At lower ratios however i.e. ~0.6:1 w:w α-casein:RCM-κ-casein, αS1-casein was comparable to αS-casein in that it suppressed fibril formation by 96%, but αS2-casein was able to only provide 56% protection under the same conditions as shown in Figure 9 [17].

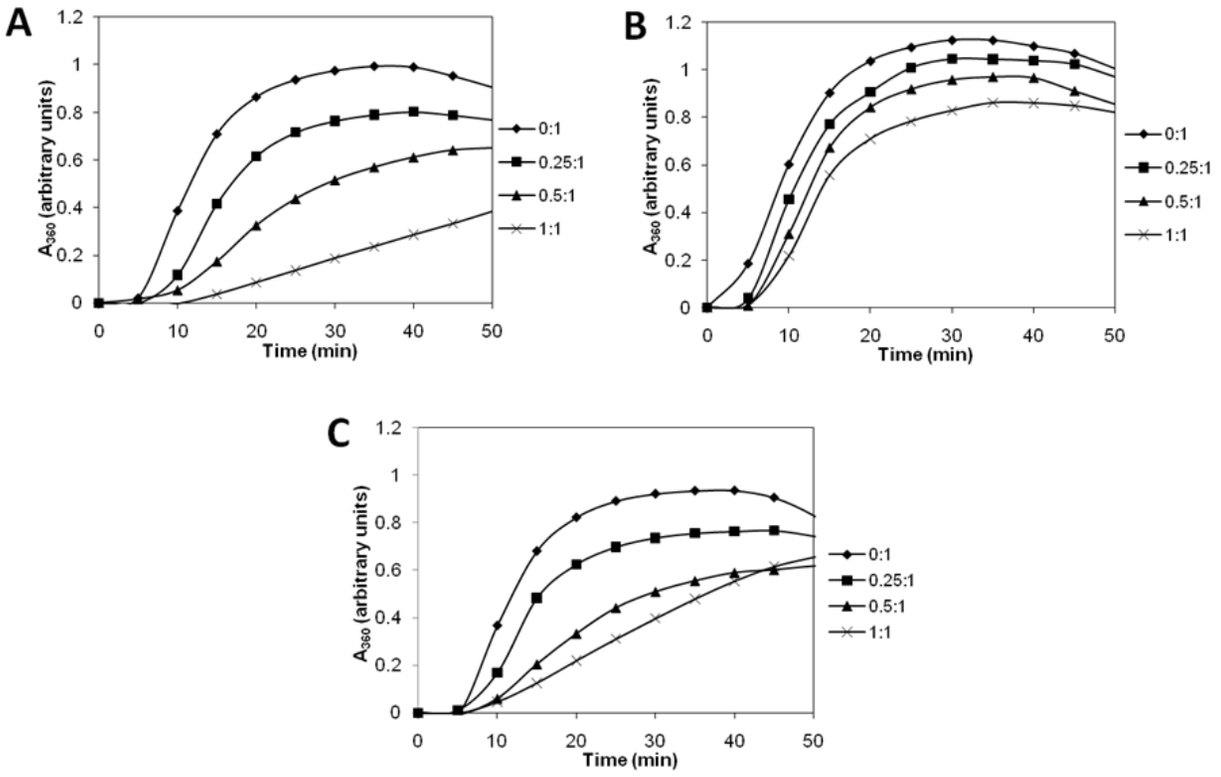

Figure 8. Chaperone activity of A) αS-, B) αS1- and C) αS2-casein against amorphously aggregating catalase, in the presence of 0.1 M NaCl and various w:w ratios as determined by light scattering at 360 nm. The 0:1 ratio in each assay represents catalase aggregation in the absence of α-casein i.e. no chaperone present. Reprinted with permission from [17]. Copyright (2011) Elsevier Inc.

The results of the fibril-forming experiments discussed above were further confirmed by TEM studies (Figure 10) which showed that the presence of a 1.25:1 ratio of αS1-casein resulted in reduced numbers of fibrils being formed by RCM-κ-casein (Figure 10B). In addition, those fibrils that were formed were shorter in length than those observed for RCM-κ-casein in the absence of chaperone (Figure 10A). Conversely, in the presence of the same ratio of αS2-casein, RCM-κ-casein fibrils were abundant and were associated with rounded aggregates 50–100 nm in diameter (Figure 10C) that may have contained one or both proteins. αS2-Casein, which forms characteristic twisted fibrils, was included as a control at the same concentration but in the absence of chaperone [17].

Previous studies have shown that both β- and αS-casein have the ability to inhibit the formation of fibrils by κ-casein [28], and this observation has led to the conclusion that amyloid formation in mixtures of casein (i.e. whole casein), namely by αS2- and κ-casein, is prevented by the action of the casein chaperones, β- and αS1-casein. As αS1-casein was a more potent inhibitor of fibril formation than αS2-casein under these conditions, it would be reasonable to assume that a large proportion of the fibril-preventing action of αS-casein is provided by αS1-casein. As previously discussed, the chaperone activity of αS1-casein against amorphously aggregating catalase in the presence of salt was a stark contrast, but provides the rationale that perhaps in milk, where salt concentrations are high, αS1-casein

has an important role in preventing fibrillar aggregation rather than amorphous aggregation [17].

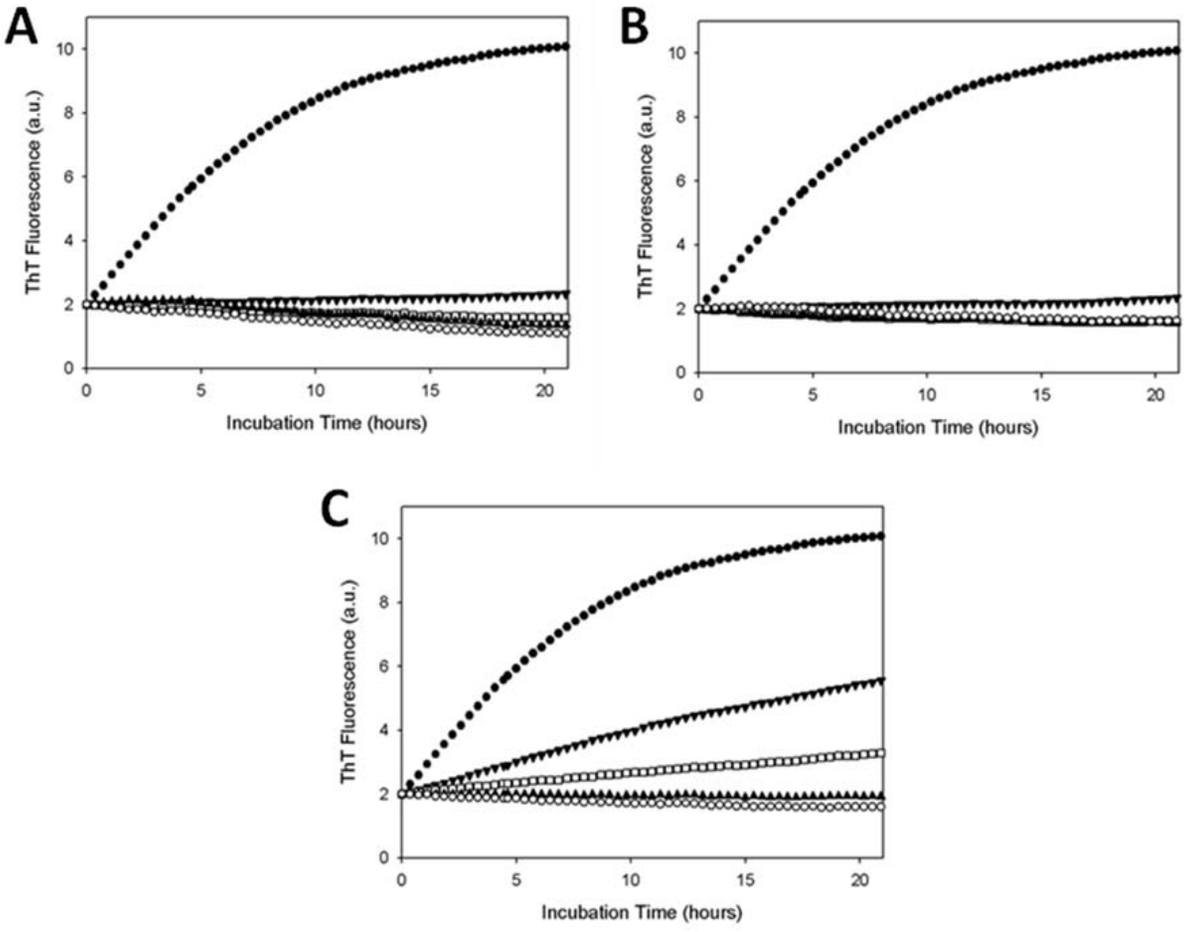

Figure 9. Chaperone activity of A) α_S-, B) α_{S1}- and C) α_{S2}-casein against amyloid forming RCM-κ-casein as determined by Thioflavin T (ThT) fluorescence. Ratios of chaperone:RCM-κ-casein equivalent to ~0.6:1, 1.25:1 and 2.5:1 (w:w) are represented by ▼, □ and ▲, respectively. Reprinted with permission from [17]. Copyright (2011) Elsevier Inc.

Investigations into the mechanism of α_S-casein's chaperone action are only in their infancy, and the precise nature of its chaperone function remains largely an enigma. Despite this, comparisons with the chaperone action of sHsps have provided several important insights. In sHsps, binding of a stressed target protein is thought to occur primarily via hydrophobic interactions between exposed hydrophobic regions on the chaperone and on the partially unfolded protein [139]. It has been well described that under conditions of heat stress, some sHsps undergo a conformational change which increases the extent of exposed hydrophobicity and these structural changes are accompanied by functional ones i.e. increased chaperone action [94, 125, 127, 153, 154]. Solubility of the large sHsp aggregates is maintained by hydrophilic regions of the chaperone that are dynamic and solvent-exposed, such as the flexible C-terminal extensions in α-crystallin [70, 93]. The presence of these polar regions is also believed to be important in maintaining the solubility of the target-protein-

chaperone complex once interaction between the two proteins has taken place, resulting in a high molecular weight complex [155]. It is plausible that αS-casein has a similar mode of action. The distribution of hydrophobic and hydrophilic residues in the caseins is not uniform and their lack of well-defined structure encourages self-association but also likely aids in chaperone action with partially unfolded target proteins. Within the predominant αS-casein subunit, αS1-casein, hydrophobic residues are clustered into three distinct regions (residues 1-44, 90-113 and 132-199) and the hydrophilic phosphoserine residues are also clustered in the polar domains (residues 41-80) [5]. It is possible that the structure of αS1-casein is similar to that of the sHsps whereby it has a predominant, relatively globular hydrophobic domain linked to a highly polar region akin to the flexible polar C-terminal extension of the sHsps [155-157]. Hypothetical structures for αS-casein subunits obtained through energy minimisation calculations are consistent with this model [15]. The most hydrophilic of the caseins is αS2-casein (present at a ratio of 4:1 αS1:αS2 in bovine milk) with only two areas of hydrophobicity arising from residues 160-207 and 90-120 [17]. The C-terminal region of αS2-casein possesses a high net charge despite being relatively hydrophobic [5].

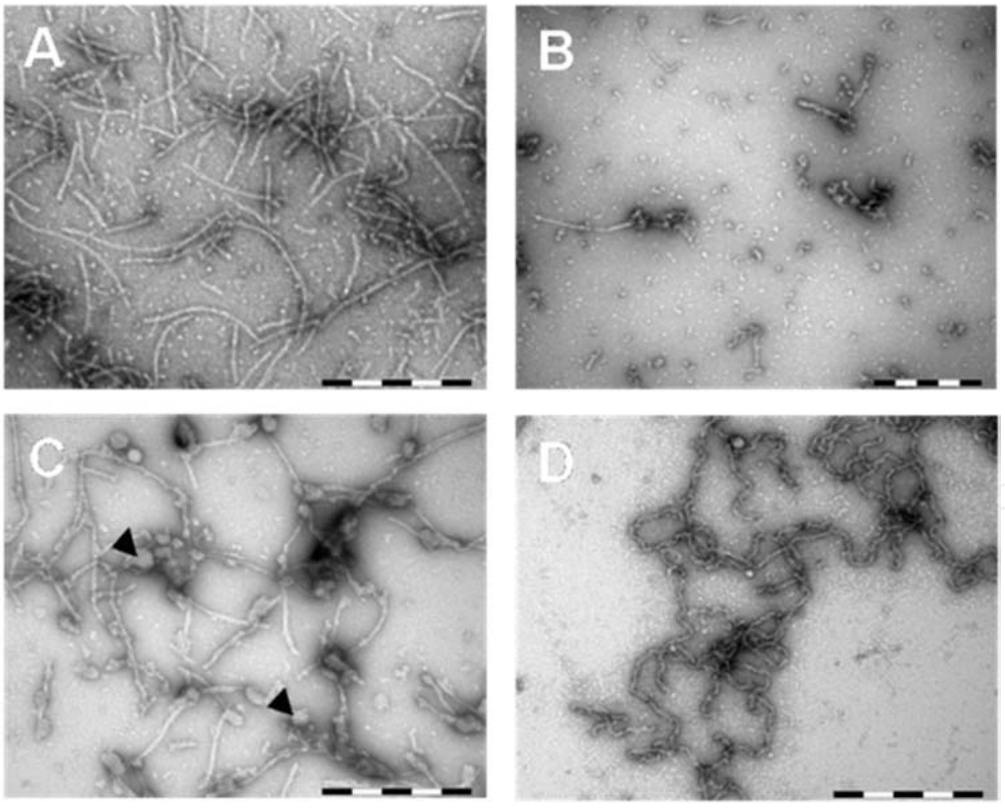

Figure 10. Electron micrographs of RCM-κ-casein incubated for 50 h at 37°C with and without αS-casein proteins. RCM-κ-casein incubated in the absence (A) or presence of ~1.0 mol:mol ratio (3.75 mg/mL) of either αS1-casein (B) or αS2-casein (C). αS2-Casein alone is also shown (D; 3.75 mg/mL). In (C), small, rounded aggregates are indicated by ◀. Scale bars represent 500 nm. Reprinted with permission from [17]. Copyright (2011) Elsevier Inc.

Size-exclusion HPLC studies have shown that αs-casein exists as a polydisperse aggregate [18] and it is likely that this heterogeneity arises from association of the two subunits. It is apparent from Figure 4 and from the previous discussion of the chaperone mechanism of sHsps that a crucial part of the process is the dynamic interaction between large, heterogeneous aggregates formed by αs-casein and smaller oligomers, which, in the case of the sHsps, are believed to be the active form of the chaperone [18, 110]. As the other casein proteins present in milk (β-, and κ-casein) have also been shown to possess chaperone activity [18, 33, 110-112] it is likely that these subunits also play an important role in dynamic subunit exchange and stabilisation of the casein aggregate. The ability of αs-casein to resolubilise aggregated target protein species is especially interesting in the context of the mechanism shown in Figure 4. The data on insulin resolubilisation by αs-casein indicates that an equilibrium certainly exists between intermediately folded states of target proteins (e.g. I_2) and their amorphous aggregates, but that in addition, αs-casein is capable of pushing this equilibrium back toward a more soluble and therefore more stable state (I_1 or I_2) following formation of the aggregate. Dynamic equilibrium between the αs-casein aggregate and smaller, dissociated species is likely to be important in this aspect of its chaperone function, but this remains to be elucidated.

4. Conclusion and future directions

The predominant milk protein, αs-casein, has been shown to possess molecular chaperone abilities with a range of target proteins, under different stress conditions. Like the sHsps, αs-casein is also ATP-independent in its chaperone action. As described in this chapter, αs-casein and its two constituent proteins, αs1- and αs2-casein are capable of interacting with and stabilising a range of physiological and non-physiological target proteins. This 'promiscous' nature is a feature of many other chaperones. Under a variety of stress conditions, αs-, αs1- and αs2-casein form high molecular weight complexes with partially unfolded target proteins, stabilising them against precipitation, whether this be via amorphous or fibrillar pathways.

Like the sHsps and clusterin, the α-casein proteins exhibit different degrees of chaperone activity depending on the mode of target protein aggregation (i.e. amorphous versus fibrillar), the rate of target protein aggregation, the size of the target protein, the conditions of stress applied and the presence of competing ions (e.g. salt). Unlike the sHsps (specifically Hsp25 and α-crystallin) and clusterin, however, αs-casein binds target proteins in a manner that does not allow subsequent interaction and reactivation by the ATP-dependent Hsp70.

The mechanism/s by which αs-casein stabilises and prevents the precipitation of other proteins in milk (such as the other caseins and whey proteins such as α-lactoglobulin and β-casein) is of interest to the dairy industry as it may provide an alternative method for long-life milk treatment [18]. It has been demonstrated that αs-casein, and indeed other caseins such as β-casein, interact with 'molten globule' states or folding intermediates of proteins. As suggested by others, processing treatments in dairy foods have the potential to transform

previously native structures into denatured or partially denatured states and that the presence of these states may either present a problem or offer opportunities for novel foods to be developed [123]. This is where the action of molecular chaperones may play an important role. Thus, a better understanding of the aggregation processes in milk and how these can be modified opens up potential avenues for new milk based products with novel textures and other organoleptic properties to be developed.

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