Casein proteins: investigating their chaperone activity and amyloid fibril formation.

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Abstract

Molecular chaperones are a diverse group of proteins that interact and stabilise partially folded proteins, thereby preventing improper or incorrect interactions that would result in their misfolding and aggregation under conditions of cellular stress, e.g. elevated temperature. There are two alternative and distinct routes by which the aggregation of the protein may proceed, i.e. via the formation of disordered, amorphous aggregates or ordered amyloid fibrils. The latter is of considerable interest to researchers because of its intimate association (e.g. via the formation of proteinaceous deposits or amyloid plaques) with a wide range of debilitating diseases, including Alzheimer’s disease and type II diabetes. Amyloid-like plaques have also been identified in the mammary gland of various species within calcified stones known as *corpora amylacea* (CA). While the composition of the protein(s) involved in formation of these amyloid deposits has not been determined conclusively, immunoblotting and sequence analysis of peptides obtained from mammary CA indicate that fragments of several milk proteins, in particular caseins, are present.

*In vitro* studies have shown that αs-, β- and κ-caseins, the major proteins in milk, are molecular chaperones, as they are able to stabilise heat-, light- and chemically-stressed target proteins by inhibiting their aggregation and precipitation. Casein chaperone-like activity is of biological importance since two of the four casein proteins, i.e. αs2- and κ-casein, assemble into amyloid fibrils under physiological conditions, *in vitro*, which is inhibited by the chaperone action of the other milk caseins, αs1- and β-casein. The chaperone-like activity of αs- and β-casein is of commercial interest due to their ability to stabilise other proteins during food processing, e.g. the heat treatment of milk during pasteurisation and the production of milk-related products.
The work described in this thesis has two overall aims: (i) to further investigate caseins’ chaperone-like ability and (ii) to examine the propensity of the caseins to form amyloid fibrils. As such, αs- and β-casein, were dephosphorylated to determine the effect of phosphate groups on the ability of these caseins to act as molecular chaperones. Dephosphorylation of αs- and β-casein resulted in a decrease in the chaperone efficiency against both heat- and reduction-induced amorphously aggregating target proteins. Circular dichroism and fluorescence spectroscopic data indicated that the loss of negative charge associated with dephosphorylation led to an increase in ordered structure of αs- and β-casein (Chapter 2). The binding site of β-casein with reduced, partially folded α-lactalbumin, a milk whey protein, was explored using limited proteolysis and mass spectrometry to give insight into the mechanism of β-casein chaperone interaction with target proteins. It was concluded that the hydrophobic C-terminus of β-casein, from Ala^{91} to Trp^{143}, is involved in binding to reduced α-lactalbumin (Chapter 3).

Amyloid fibrils were formed from reduced and carboxymethylated κ-casein and αs2-casein, and the amyloidogenic regions of both these proteins were identified using limited proteolysis and mass spectrometry. The residues from Tyr^{25}-Lys^{86} and Ala^{81}-Lys^{181} were determined to be incorporated into the core of κ-casein and αs2-casein fibrils respectively (Chapter 4). The oxidation of methionine residues is linked to the pathogenesis of several amyloid diseases. As such, the two methionine residues in κ-casein (Met-95 and Met-106) were oxidised and its effect on κ-casein structure and fibril-formation was investigated. Oxidation increased κ-casein’s fibril forming propensity and cellular toxicity. In addition, β-casein, which readily inhibits κ-casein fibril-formation in vitro, was less effective at suppressing fibril formation of oxidised κ-casein. As milk exists in an oxidative environment, this observation may have implications in vivo (Chapter 5).
Declaration

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution to Tomas Koudelka and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

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Abbreviations

ACN      acetonitrile
ADH      alcohol dehydrogenase
Aβ       amyloid-β
AD       Alzheimer’s disease
ANS      8-anilino-1-napthalene sulphonate
CA       corpora amylacea
CD       circular dichroism
DHB      2,5-dihydroxy benzoic acid
DTT      1,4-dithiotheritiol
FPLC     fast protein liquid chromatography
Glu-fib  glul-fibrinopeptide B
MTT      methylthiazolyldiphenyl-tetrazolium bromide
MALDI    matrix-assisted laser desorption/ionisation
NMR      nuclear magnetic resonance
OT       ovotransferrin
PC       pheochromocytoma
RCM      reduced and carboxymethylated
SEC      size-exclusion chromatography
sHsps    small heat shock proteins
TEM      transmission electron microscopy
ThT      thioflavin T
TOF      time of flight
TFA      trifluoroacetic acid
Chapter 1

Introduction and literature review
1.1 Caseins

Milk, in terms of nutrition, is one of the most complete single foods available, containing carbohydrates, amino acids, fatty acids, vitamins, inorganic elements, and is free from toxins and anti-nutritional factors (Fox and McSweeney, 2003). In the US, Australia, and New Zealand about 30% of dietary protein is supplied by milk and dairy products (Fox and McSweeney, 2003). Caseins and whey proteins are the two main types of milk proteins, with casein constituting approximately 80% of total bovine milk protein (Table 1.1) (Swaisgood, 1992). The heterogeneous proteins, $\alpha_{s1}$ and $\alpha_{s2}$-casein, collectively known as $\alpha_{s}$-casein, because of their close association, contribute approximately 50% of the casein fraction, with S1 the dominant subunit. $\beta$-Casein contributes approximately 35% of total casein content, while $\kappa$- and $\gamma$-casein (the C-terminal peptides resulting from plasmin proteolysis of $\beta$-casein) make up a smaller portion of casein fraction (Table 1.1). The caseins are all phosphorylated to various extents which contributes to their calcium binding properties and other metals enabling a high concentration of calcium phosphate to be carried in milk in a ‘soluble’ form to the neonate (Holt, 1994). They are also very open and flexible structures making them very susceptible to proteolysis; which facilitates their natural function to supply the neonate with essential amino acids (Fox and McSweeney, 2003). While the caseins are relatively small proteins, ranging in molecular mass from about 19-25 kDa, they are not usually found as individual species since they each exhibit a strong tendency to associate with each other through hydrophobic and electrostatic interactions (Swaisgood, 1992). It is this tendency to associate, along with calcium and other ions present in milk, that bovine milk caseins exist as large stable micelle with an average diameter of ~150 nm and mass of ~$10^8$ Da (Fox and McSweeney, 2003).
1.2 Casein Micelle

The structure of the casein micelle has received the attention of scientists for a considerable time. Knowledge of the micelle structure is important as reaction undergone by casein micelles are central to many dairy processing operation, e.g. cheese manufacture, the stability of sterilised, sweetened-condensed and reconstituted milks and frozen products (Fox and McSweeney, 2003). The casein micelle occupies a unique position among biological systems because of the various different models that have been proposed for its structure. This situation has probably developed due to the relatively large size of the casein micelles, which prohibits a direct and explicit determination of the structure (Phadungath, 2005). Moreover, the majority of the models were based on experimental data covering a limited range of micellar properties (Rollema, 1992). Over several decades, a variety of models have been proposed to depict the structure of bovine casein micelles. These models have generally fallen into three categories: coat-core models, subunit models, and internal structure models. Each of these categories was first proposed in the 1960s, and the original models were either abandoned or modified as supplementary information about casein micelles was obtained by subsequent researchers (Phadungath, 2005).

1.2.1 Coat-core model

The first coat-core model was proposed by Waugh and Nobel in 1965 (Phadungath, 2005). This model is based originally on their studies of the casein solubility in Ca$^{2+}$ solutions. The model depicts the formation of low weight ratio complexes of α$s_1$- and κ-caseins with the absence of calcium (Wong, 1988). Upon addition of calcium, the α$s_1$- and β-caseins, depicted as monomers with a charged loop that begin to aggregate to a limiting size (the caseinate core). Precipitation of the caseinate is prevented by the formation of a monolayer of low weight α$s_1$-κ-casein complexes (Wong, 1988) (Figure 1.1). This coat complex has the κ-casein monomers spread out completely on the surface; thus, the size of micelle is dictated by
the amount of the κ-casein available. In the absence of κ-casein the αs1- and β-caseins cores agglutinate and precipitate from solution. Waugh's model is able to explain the lyophilic nature of the colloidal casein complex and also the ready accessibility of the κ-casein to the enzyme chymosin (Phadungath, 2005, Garnier, 1973).

1.2.2 Sub-micelle model

The first sub-micelle model was proposed by Morr (1967). This model was based on the results obtained from a study of the influence of urea and oxalate treatment on the disruption of casein micelles (Morr, 1967). Morr stated that αs1-, β- and κ-casein monomers formed small uniform sub-micelles. The sub-micelles, estimated by sedimentation velocity studies, are stabilized by hydrophobic interactions and calcium caseinate bridges, and the sub-micelles believed to be linked together by colloidal calcium phosphate (CCP) with a micelle structure covered by αs1- and κ-casein (Phadungath, 2005). The structure of the sub-micelle remains a contentious issue with variations to the model proposed by Waugh et al., (1970), Rose (1969), Slattery and Evard (1973) and Schmidt (1980, 1982). However, the most commonly accepted model in this category was proposed by Walstra and Jenness (1984). This model suggests that casein the composition of the sub-micelles is variable and the size is in range 12-15 nm in diameter, and each sub-micelle has 20-25 casein molecules. The sub-micelles are kept together by hydrophobic interactions between proteins, and by calcium phosphate linkages. There are two main types of sub-micelles; one mainly consisting of αs- and β-caseins, and another type consisting of αs- and κ-caseins, which is more hydrophilic because of the sugar residues on κ-caseins (Walstra, 1999, Walstra and Jenness, 1984). The κ-caseins are located near the outside of the micelle with the hydrophilic C-terminal region of κ-casein protruding from the micelle surface, forming a layer 5-10 nm thick and giving the micelles a hairy appearance (Fox and McSweeney, 2003). The hairy layer is responsible for micelle stability, preventing further aggregation of micelles by steric and electrostatic repulsion (Walstra, 1999,
Walstra and Jenness, 1984) (Figure 1.2). Although the sub-micelle casein model as extended by Walstra (1999) has been widely accepted, two alternative models, which fall into the internal structure category, have been proposed by Holt (1992) and by Horne (1998).

1.2.3 Internal structure models

All the models in this category are based on the properties of the isolated casein constituents, causing or directing the formation of the internal structure of the casein micelle. The first internal structure model was proposed by Rose (1969). He used the known endothermic polymerization of β-casein as the foundation for his micelle structure. Therefore, he assumed that β-casein monomers begin to self associate into chain-like polymers (Phadungath, 2005). Subsequently, α\textsubscript{s1}-caseins molecules are attached to the β-casein polymers, while κ-caseins interact with α\textsubscript{s1}-caseins, forming aggregates of limited size. Upon forming the micelle structure, colloidal calcium phosphate acts as a stabilizing agent and cross-links the network (Rollema, 1992, Wong, 1988).

Holt (Holt, 1992, Holt, 1994) depicted the casein micelle as a tangled web of flexible casein molecules forming a gel-like structure in which microgranules of CCP are an integral feature and from the surface of which the C-terminal region of κ-casein extends, forming a hairy layer (Figure 1.3). The surface of the micelle is thought to be partially covered with κ-casein which is distributed heterogeneously on the surface (Dalgleish, 1998). This surface coverage provides steric stabilisation against the approach of large particles, such as other micelles, but the small-scale heterogeneities and the gaps between κ-casein molecules provide relatively easy access for molecules with dimensions of individual proteins or smaller (Dalgleish, 1998). This allows enzymes in rennet, such as chymosin and pepsin to penetrate the hairy layer to find the rennet-sensitive Phe-Met bond in κ-casein, or other enzymes to permeate the surface layer to attack the other caseins, the former of which is a key step in the manufacture of most
cheese varieties (Swaisgood, 1993). The hairy layer of κ-casein is also thought to be sufficiently diffuse to permit the passage of the individual molecules of β-casein upon cooling or the formation of disulfide bonds between micellar κ-casein and β-lactoglobulin (β-LG) from serum when milk is heated (Dalgleish, 1998).

Recently, the dual bonding model of Horne (1998) suggests that the proteins in casein micelles are bound together by two types of bonding; attractive hydrophobic interactions and electrostatic repulsion (Horne, 1998). Hydrophobic interactions are the driving force for the formation of casein micelles, while electrostatic repulsions limit the degree of polymerization. The conformation of αs1- and β-caseins when they are adsorbed at hydrophobic interfaces form a train-loop-train and a tail-train structure, respectively (Horne, 1998). Through hydrophobic interactions both caseins are able to self-associate and polymerize (Figure 1.4). Calcium phosphate nanoclusters are considered to be one of the linkages between the casein proteins. They also neutralize the negative charge of the phosphoserine residues, consequently, electrostatic repulsion is reduced and hydrophobic interaction between caseins is still dominant, resulting in more associations of proteins (Phadungath, 2005). κ-Caseins acts as a propagation terminator, as they do not have a phosphoserine cluster to bind calcium or another hydrophobic point to prolong the chain (Figure 1.5).

Numerous models for the structure of casein micelles have been proposed in the past four decades. Based on the chemical and physical properties of micelles, these models fit into three main categories; the models of coat-core structure, sub-micelles and internal structure. Neither of the models proposed by Waugh (1965), Walstra (1999), Holt (1992, 1994) and Horne (1999) are conclusive; with the micelle structure still a matter of ongoing debate. The true micelles might have a structure, which is intermediate between the models from Walstra, Holt and Horne (Phadungath, 2005). Nevertheless, all the models are helpful in explaining the
formation, structure, and properties of the casein micelle and its reaction to changing conditions during milk processing (Phadungath, 2005).

1.3 Phosphate groups

The caseins have significant post-translational modification, with all of them being phosphorylated to varying extents at serine, and occasionally threonine residues. In addition, κ-casein is also glycosylated resulting in extreme heterogeneity (a total of 19 variants make-up this protein family) (Swaisgood, 1993). The genetic variants of αs1-, αs2-, β- and κ-caseins contain a characteristic number of phosphorylated residues; 8 or 9 (αs1-), 10-13 (αs2-), 4 or 5 (β-) and 1-3 for κ-casein (Whitney, 1988). The clustering of the phophoseryl residues into phosphate centres is perhaps the most unique feature of the primary structure of the Ca-sensitive caseins. The caseins contain various amounts of phosphate centres (Table 1.2) which are required to form a stable link to calcium phosphate and casein (De Kruif and Holt, 2003). A phosphate centre requires at least three but usually four phosphorylated residues together with at least two and mostly more, other types of acidic residues to occur within a fairly short sequence (Aoki et al., 1992, Greenberg et al., 1984). The cluster sequence Glu-PSer-X-PSer-PSer-Glu-Glu, where X is Ile or Leu and PSer is phosphoserine, occurs in all known variants of αs1- and β-caseins. Similar sequences, namely PSer-PSer-PSer-Glu-Glu and PSer-PSer-PSer-Glu-PSer-Ala-Glu, occur in αs2-caseins (Swaisgood, 1992). Sequences such as these, which are present in the polar domains of the calcium-sensitive caseins, apparently are encoded by a number of short exons, thus allowing for their duplication and leading to the rapid evolution of these proteins (Bonsing and Mackinlay, 1987). Owing to their high content of phosphate groups, which occur in clusters, αs1-, αs2- and β-caseins have a strong tendency to bind metal ions which is essential for the stability of the casein micelle. The various phosphate binding centres in αs1-, αs2- and β-casein (Table 1.2) create casein polypeptide cross-linking by calcium phosphate. More specifically, αs1-, αs2-caseins which have more
than one phosphate centre act as links between calcium phosphate core structures, cross-linking the micelle (Holt, 1992). Therefore, the calcium-sensitive caseins are cemented into the micelle structure by strong linkages to calcium phosphate and explain why the micelle structure is preserved upon heating, or disrupted upon removal of the colloidal calcium phosphate, e.g. by the addition of EDTA. The calcium-sensitive proteins, which represent approximately 85% of total casein, are insoluble at calcium concentrations greater than ~6 mM. Since bovine milk contains a calcium concentration of approximately 30 mM, one would expect that the caseins would precipitate under the conditions prevailing in milk. However, κ-casein, which predominantly contains only one organic phosphate group, binds calcium weakly and forms the outer layer of the casein micelle which solubilises the micelle and bound $\text{Ca}^{2+}$ (Swaisgood, 1992). This solubility is imparted by the highly hydrophilic C-terminal region of κ-casein, which is extensively glycosylated, providing solubility and polarity to the outer region of the micelle (Swaisgood, 1992).

1.4 Casein structure

The caseins are unique in their structure as they are neither globular, nor fibrillar (De Kruif and Holt, 2003) and instead belong to a group of proteins termed ‘natively disordered’ since they adopt little ordered structure under physiological conditions (Uversky et al., 2000). Due to their flexible and dynamic nature, it has been suggested that the caseins occur naturally in a molten globule-like state (Farrell et al., 2003a). This molten globule state is characterised by a somewhat compact structure, a higher degree of hydration and side chain flexibility, a significant amount of native secondary structure but little tertiary fold (Farrell et al., 2003a). The inability of caseins to form stable structures is due mainly to their high content of the structure-breaking amino acid proline, especially in β-caseins (17%), which interrupt α-helical and β-sheet structures and usually occur in β-turns (Swaisgood, 1993, Vanhoof et al., 1995). Another characteristic of molten globule states shared by caseins, and accounting for much of
their propensity for association, is extensive regions of solvent-exposed and clustered hydrophobicity (Kuwajima, 1996). Globular proteins tend to fold so that their hydrophobic residues are buried deep within the core of the molecule with hydrophilic regions exposed to the solvent (Treweek et al., 2003). In contrast, caseins which owing to their lack of stable tertiary structures, have their hydrophobic residues exposed to solution (Fox and McSweeney, 2003). This lack of stable tertiary structure means that the caseins are not denatured to a more disordered structure upon heating. Their very high heat stability makes it possible to produce heat-sterilised dairy products with little change to physical properties (Fox and McSweeney, 2003).

Due to the extended, flexible nature of their polypeptide chains and their dynamic and heterogeneous nature, caseins have never been successfully crystallised, and elucidation of their structure in detail by X-ray crystallography is not likely to be realised (Alaimo et al., 1999). Only predicted three-dimensional molecular models are available (Kumosinski et al., 1991, Kumosinski et al., 1993a, Kumosinski et al., 1993b). As expected, theoretical calculations indicate that caseins have little tertiary structure but, like the molten globule state, caseins possess some degree of secondary structure. Table 1.3 summarises the assignments of secondary structure elements to the individual caseins, based on various spectroscopy techniques (CD, Raman, Fourier transform infra-red (FTIR)), with the highest belonging to $\alpha_s$- and $\kappa$-casein. Interestingly, even though the same molecule is doing the vibrating, bending and stretching, the various techniques give different results (Horne, 2002). For example, $\beta$-casein shows the amount of $\alpha$-helix assigned as ranging from 13 to 29% and the contribution from irregular structure ranging from 72 to 4% (Table 1.3). Similarly the $\alpha$-helix content in $\kappa$-casein can differ by a factor of two (9 to 17%), using different techniques (Table 1.3). Unfortunately, there is no independent means of checking these assignments as the caseins have not been, nor are likely to be, crystallised and NMR structural studies have
so far extended only to peptides where analysis is complicated by extensive self-association (Alaimo et al., 1999). In addition, the assignments of secondary structure in these spectra are made on the basis of prior appearance of such absorbances in the spectra of other proteins and confirmation of the corresponding conformation generally by X-ray crystallography (Horne, 2002). Since, the majority of such crystallographic studies have been carried out on globular proteins, the assignments of secondary structure are inevitably biased and non-representative of all the conformational structures that might be possible in proteins (Horne, 2002). Moreover, neither far-UV CD nor NMR is able to distinguish between an extended conformation of the poly-L-proline II (PPII) type and the truly random coil conformation, so both are classed as ‘other’ in many quantitative analysis algorithms (De Kruiif and Holt, 2003).

The Raman Optical Activity spectra of αS-, β-, and κ-casein are dominated by a strong positive band at ~1318 cm⁻¹, which is indicative of significant amounts of PPII helical conformation (Smyth et al., 2001, Syme et al., 2002). Other proteins with significant PPII helical structure include α-, β-, and γ-synuclein and tau (Syme et al., 2002). PPII helix consists of a left-handed extended helical conformation that is relatively open, flexible and lacks inter-chain hydrogen bonds (Blanch et al., 2000). PPII helix is thought to bestow a flexible and open character to the tertiary structure of proteins and is most often found in longer loops connecting secondary structure (Blanch et al., 2000). PPII helix has been identified as a favourable polypeptide conformation for molecular recognition and binding to proteins (Siligardi and Drake, 1995). The open, extended and flexible character of the PPII helix and the lack of intra-chain hydrogen bonds facilitate peptide backbone interactions with proteins (Siligardi and Drake, 1995). It may also be favourable for fibril formation as elimination of water molecules between extended polypeptide chains with fully hydrated backbone C=O and N-H groups to form β-sheet hydrogen bonds is a highly favourable
process entropically (Syme et al., 2002). Therefore, the PPII helix which bestows an open and flexible structure to these proteins also predisposes certain proteins, e.g. αS-casein, κ-casein and α-synuclein, to form amyloid fibrils (De Kruif and Holt, 2003).

1.5 Amyloid fibrils

To date, more than 25 proteins have been shown to assemble into amyloid fibrils in vivo with subsequent accumulation of these fibrils, either intra- or extracellularly, ultimately resulting in the development of diseases such as type II diabetes, Parkinson’s and Alzheimer’s disease (Dobson, 2003, Horwich, 2002, Kelly, 1998). The various peptides and proteins associated with amyloid diseases have no obvious similarities in size, amino acid composition, sequence or structure. Nevertheless, the amyloid fibrils into which they convert have marked similarities both in their external morphology and in their internal structure (Figure 1.6). Circular dichroism and Fourier transform infra-red spectroscopy both indicate a high content of β-structure in amyloid fibres, even when the native conformation of the monomeric peptide or protein is substantially disordered or rich in α-helical structure, for example, myoglobin (Fandrich et al., 2001). Investigation of the molecular structure of amyloid fibrils by electron and atomic force microscopy show that they are typically long, straight and unbranched. The fibrils are typically 6–12 nm in diameter and usually consist of two to six ‘protofilaments’, each of diameter about 2 nm, that are often twisted around each other to form supercoiled rope-like structures (Serpell et al., 2000). X-ray fibre diffraction data suggest that each protofilament in such structures appears to have a highly ordered inner core that consists of some or all of the polypeptide chain arranged in a characteristic ‘cross-β’ structure. In this structural arrangement, the core is composed of β-sheets in which strands run perpendicular to the fibril axis, resulting in β-sheets that propagate along the length of the fibril (Sunde and Blake, 1997). This results in a characteristic cross formed by the meridional and equatorial reflections in X-ray diffraction studies (the former of ~4.7 Å and latter of ~9–11 Å) (Figure
1.6C), which represent the hydrogen bonding distance between adjacent β-strands that make up a β-sheet and the distance between β-sheets respectively (Ecroyd and Carver, 2008b).

It was assumed that the proteinaceous aggregates most toxic to cells were likely to be the mature amyloid fibrils, the form of aggregates that have been commonly detected in pathological deposits, e.g. fibrillar plaques in the brains of victims of Alzheimer’s disease (Stefani and Dobson, 2003). However, an increasing quantity of experimental data suggest that, in many cases, at least the species that are most highly toxic to cells are the pre-fibrillar aggregates of dimers, trimers or other such oligomers, rather than the mature fibrils into which they often develop. In particular, a number of reports concerning Aβ peptides and α-synuclein, responsible for Alzheimer’s and Parkinson’s disease respectively, indicate that their early aggregates are the most toxic species (Chiti and Dobson, 2006, Conway et al., 2000). Others have concluded that the mature fibril can also be toxic (Novitskaya et al., 2006, Ward et al., 2000). In fact, the cytotoxic species may vary depending on the fibril-forming protein (Ecroyd and Carver, 2008b).

A similar level of debate exists surrounding the exact mechanism behind the toxicity of amyloid fibril-forming proteins. A number of hypotheses have been proposed, including the ‘channel hypothesis’. The idea stems from various pieces of evidence leading to the proposal that unchaperoned, positively charged and misfolded proteins, or early aggregates of such species, can interact with lipid membranes and cell membranes (Kourie and Shorthouse, 2000, Kourie and Henry, 2002) that is reminiscent of the action of eukaryotic pore-forming proteins such as peptides found in venoms and antimicrobial secretions and bacterial toxins (Kourie and Shorthouse, 2000). The charged residues of misfolded proteins are thought to interact with the polar lipid head groups, which are then followed by the insertion of hydrophobic regions into the membrane hydrophobic interior (Kourie and Henry, 2002). The
hydrophobic regions of the misfolded species interact with the plasma membrane and other cell membranes and via the formation of non-specific ion channels (Figure 1.7). These channels, or pores, have been described for a number of peptides and proteins associated with amyloid disease including Aβ peptides (Lin et al., 2001), their fragments (Mattson et al., 1997) and α-synuclein (Volles and Lansbury, 2002). Others have suggested that the toxicity of pre-fibrillar amyloid species is due to the production of reactive oxygen species (e.g. hydrogen peroxide) by the aggregating target protein itself, which are generated as a consequence of the fibril-forming process (Tabner et al., 2005).

Diseases associated with amyloid fibril formation, such as Alzheimer’s, Parkinson’s and Type II diabetes represent some of the world’s most debilitating conditions, particularly in developed countries (Stefani and Dobson, 2003). These diseases, of which many are associated with old age, will become more prevalent over the coming decades as the population ages (Ecroyd and Carver, 2008b). As such, a greater understanding is required of the mechanism by which functional proteins are converted in vivo into amyloid fibrils and strategies to prevent their formation (Ecroyd and Carver, 2008b).

1.6 Casein as Fibrils

There is a pathology associated with every gland or organ involved in calcium transport, and the mammary gland is no exception (De Kruif and Holt, 2003). In this case, the disease manifests itself in an accumulation of calcium stones in the ducts and cisterns of the gland, known as corpora amylacea (CA) granules because of a superficial likeness to starch particles (De Kruif and Holt, 2003). These round or oval amyloid bodies have been observed in humans and other species (Niewold et al., 1999, Brooker, 1978, Beems et al., 1978, Taniyama et al., 2000), however, the most detailed information is available on bovine CA in mammary tissue which were first described by Ottolenghi in 1901 (Ottolenghi, 1901). Their prevalence,
physio-pathological features, histochemistry and electron microscopy have since been reported (Reid, 1972, Arnold and Weber, 1977, Brooker, 1978, Nickerson et al., 1985, Nickerson and Sordillo, 1985).

Histologically, the majority (90%) of CA are located within the alveolar lumen (Nickerson et al., 1985), where milk is stored, and subsequently secreted into the ducts through ductules. Amyloid-like deposits have also been identified in the stroma (7%) and within the cytoplasm of mammary epithelial cells (3%), which are involved in the synthesis and secretion of caseins and other milk constituents (Reid, 1972, Nickerson et al., 1985) (Figure 1.8A). Corpora amylacea is observed throughout lactation with a progressive increase in the prevalence of these structures from early to later stages of lactation, resulting in a prevalence peak at early involution of the udder (Brooker, 1978, Nickerson and Sordillo, 1985). CA decreases in number towards the next lactation, probably by macrophage action (Nickerson and Sordillo, 1985, Nickerson and Sordillo, 1987). As mentioned previously, deposition of amyloid is often related to degenerative pathologies such as type II diabetes, Alzheimer’s and Parkinson’s disease (Dobson, 2003, Horwich, 2002, Kelly, 1998), its accumulation has also been noted in cases of invasive breast carcinoma (Santini et al., 1992). In spite of this, CA does not result in major clinical abnormalities, however, it does appear to be linked to mammary infections (Nickerson et al., 1985). The occurrence of amyloid bodies appears to adversely affect milk production, as they fill large parts of the luminal areas, block ducts, and irreversibly damage the apical membrane of secretory cells, which leads to suppression of secretion (Brooker, 1978, Nickerson et al., 1985, Claudon et al., 1998).

Two morphological types of bovine CA were described: 70% represent strongly basophilic (calcified), dense, lamellated deposits, which sometimes show a fibrillar outer rim staining positively for amyloid (Brooker, 1978, Nickerson and Sordillo, 1987), and 30% are entirely
composed of tightly packed amyloid fibrils, 8-10 nm in diameter (Reid, 1972, Brooker, 1978, Nickerson et al., 1985) (panels B and C of Figure 1.8). While histochemically, bovine CA were described as staining for albumin, amyloid (Reid, 1972), hyalin-fibrin (Kuiken et al., 1956), glycoproteins and glycosaminoglycans (Claudon et al., 1998, Sordillo and Nickerson, 1988) and calcium (Brooker, 1978). In spite of all these data, the origin, genesis and exact biochemical composition of bovine CA amyloid fibrils has not been determined conclusively. Brooker, (1978) was perhaps the first to suggest that the CA originate from milk protein by aggregation, fusion and compaction of casein micelles. The increase in CA as lactation progresses through early involution and decrease during later stages of the dry period supports the idea that CA develops as a result of synthetic and secretory processes. The importance of active casein synthesis and secretion as prerequisites for the formation and growth of CA explains why they have not been observed in virgin and pregnant heifers (Brooker, 1978, Reid, 1972, Kuiken et al., 1956) and is also supported by the observation that the presence of deposits are closely correlated with fully developed alveolar epithelium (Brooker, 1978, Nickerson et al., 1985). Moreover, immunoblotting and amino acid sequence analysis of bovine CA gave evidence of the occurrence of various caseins (αs2-, β-, and κ-), β-lactoglobulin and α-lactalbumin in CA (Claudon et al., 1998, Niewold et al., 1999). While Niewold et al. (Niewold et al., 1999) isolated a group of peptide fragments derived from αs2-casein, designated AαS2C, 32 to 45 residues in length and all starting from Ala81, from amyloid deposits associated with mammary CA (Niewold et al., 1999).

1.7 Casein fibril formation

Of physiological relevance is the observation that two of the four caseins, αs2-casein and κ-casein, are able to form fibrils in vitro under physiological conditions (Farrell et al., 2003b, Thorn et al., 2008, Thorn et al., 2005). Unlike the other caseins, both αs2- and κ-casein, possess disulfide linkages, arising from the two cysteine residues in each molecule. The two-
cysteiny1 residues in αS2-casein form both intramolecular and intermolecular disulphide bonds (Rasmussen et al., 1992a, Rasmussen et al., 1994). Thus, the protein occurs as a monomer with a disulphide linkage between residues 36 and 40 and as dimers with either parallel or antiparallel disulphide linkages. In κ-casein the cysteine residues are randomly crosslinked by intermolecular disulphide bonds resulting in the formation of polymers, from monomer to multimeric structures larger than decamers (Rasmussen et al., 1992b). Interestingly, while both native αS2-casein and κ-casein have the propensity to form fibrils, the conditions under which fibril formation is favoured are different. Reduction and subsequent carboxymethylation of native κ-casein, i.e. monomeric κ-casein, has an inherent susceptibility to form fibrils at physiological pH and temperature (Farrell et al., 2003b, Thorn et al., 2005). Native κ-casein also has the propensity to form fibrils under the same conditions, albeit at a slower rate (Thorn et al., 2005). Therefore, in native κ-casein, the monomers are tightly packed and stabilised by their intermolecular disulphide bonds and fibril formation is inhibited (Thorn et al., 2009). In contrast, αS2-casein fibril formation occurs most readily under non-reducing conditions (Thorn et al., 2008) and further investigation has shown that the disulfide-linked dimer, rather than the monomer, is the predominant amyloidogenic species (D. Thorn et al. unpublished results). While both αS2- and κ-casein have been shown to form fibrils in vitro, under physiological conditions in milk, this is kept in check by the action of other caseins (αS- and β-), which are able to inhibit their fibrillation (Thorn et al., 2005, Thorn, 2008 #32).

1.8 Molecular chaperones
Molecular chaperones, comprising several structurally unrelated protein families, assist in the stabilisation of partially folded target proteins, thereby preventing protein misfolding and aggregation. They also assist in other cellular tasks such as regulating the unfolding of proteins during their transport across intercompartmental membranes, oligomeric assembly,
protein folding and degradation of undesirable proteins (Ehrnsperger et al., 1997). The molecular chaperones are usually categorized into homologous families named heat shock proteins (Hsps) Hsp100, Hsp90, Hsp70, Hsp60, and the small heat shock proteins (sHsps) according to their molecular masses (Buchner, 1996). The chaperone behaviour of caseins has been said to be similar to that of sHsps (Morgan et al., 2005, Yong and Foegeding, 2010), therefore, these types of chaperones will be discussed below.

sHsps are a group of ubiquitous intracellular proteins that are found in all organisms and are the most strongly induced molecular chaperone family in response to stress, e.g. elevated temperature (Treweek et al., 2003). The chaperone function of sHsps involves direct association with, and sequestration of, partially folded target proteins (Jakob and Buchner, 1994). Binding of sHsps to target proteins is an ATP-independent process, which allows sHsps to sequester partially folded proteins even when cellular energy levels are low, as is generally the case when the cell is stressed (Ehrnsperger et al., 1997). Once bound, the sHsp oligomer simply maintains the solubility of the sHsp-target complex and does not assist in the repair of the partially folded complex (Lindner et al., 2000). However, since the target protein is complexed in a partially folded form, it can be refolded with the assistance of other molecular chaperones, such as Hsp70, when cellular levels are permissive (Treweek et al., 2003, Lee et al., 1997).

Under various stress conditions, such as elevated temperature (which weakens the strength of hydrogen bonds), changes in pH and salt concentration (which alters the electrostatic interactions between charged amino acids), and the addition of reducing agents (that break disulphide bonds between cysteines) proteins can unfold and denature. Such conditions cause the native, functional protein (N) to unfold and adopt partially folded, intermediate conformations (I₁, I₂, I₃...), which can progress to the unfolded state (U). These intermediate
conformations have varying degrees of structure, for example molten globule states which are highly dynamic containing elements of secondary structure but little or no tertiary structure (Treweek et al., 2003). As a result these partially unfolded proteins have their hydrophobic core, which is located in the interior of the native protein, more exposed to solution. If exposed for long enough, the intermediates can undergo mutual association via hydrophobic interactions, leading to aggregation and potentially precipitation (Bukau et al., 1999). When this occurs, the protein leaves the folding pathway and enters the slower, protein off-folding pathways (Figure 1.9). These comprise two possible routes: (i) the amorphous aggregation pathway in which interaction ultimately produces irreversibly precipitated, amorphously aggregated species, and (ii) the slower amyloid fibril-forming pathway which, via the formation of small, soluble protofibril species, leads to insoluble, highly ordered cross β-sheet fibril arrays (Carver et al., 2003). sHsps bind to the relatively long-lived, partially folded structural intermediates, primarily through hydrophobic interaction, to form stable, soluble complexes and preventing target protein aggregation (Ehrnsperger et al., 1997 , Ecroyd and Carver, 2008b, Ecroyd et al., 2007, Rekas et al., 2004) (Figure 1.9).

The first claim that caseins have molecular chaperone properties was by Bhattacharyya and Das, 1999. They recognised the ability of $\alpha_s$-casein (a combination of $\alpha_s$1- and $\alpha_s$2-casein) to stabilise heat-, light- and chemically-stressed proteins, preventing their aggregation and precipitation (Bhattacharyya and Das, 1999). Since then, numerous publications (including two reviews) have described the various chaperone-like properties of caseins. Work has been performed on sodium caseinate, whole casein, and its isolated components, $\alpha_s$- (comprising both $\alpha_s$1- and $\alpha_s$2-casein) $\beta$-casein and $\kappa$-casein, however, to date most of the work has focused on $\alpha_s$-, and $\beta$-casein (reviewed in July, 2009 see Yong and Foegeding, 2010).
1.9 Comparison of Caseins with Small Heat Shock Proteins

There are a number of properties that casein proteins share with sHsps (Carver et al., 2003). Though their monomeric weight is small 12-43kDa (Treweek et al., 2003), (1) they exist as heterogenous oligomers in which exchange takes place between subunits; (2) they are dynamic proteins, containing stretches of amino acids that have conformational flexibility and disordered structure; (3) and they are highly promiscuous in their chaperone interactions and prevent the stress-induced precipitation of a broad range of target proteins (Carver et al., 2003). As with sHps, casein micelles are dynamic rather than fixed structures. Changes in temperature, pH, and ionic strength lead to changes in size distribution and dissociation of the micelles (Swaisgood, 1993). In addition, each type of casein can undergo mutual association reactions to form homo-oligomeric micelles (Andrews et al., 1979, Swaisgood, 1993, Swaisgood, 1992). An important structural feature of caseins is their relatively high amount of proline residues, especially β-casein (αs1-, αs2-, β-, and κ-casein contain 9%, 5%, 17%, and 12% of their amino acid sequence as proline respectively (Fox and McSweeney, 2003)). The proline residues introduce kinks or bends into proteins causing distortion of α-helices and β-sheet formation (Swaisgood, 1993, Vanhoof et al., 1995), giving caseins their open structure. This flexibility and dynamism facilitate their interaction with a diversity of partially folded target proteins. NMR spectroscopy indicates that the heterogeneous mammalian sHsps have a highly flexible and unstructured region at their extreme C-terminus (Carver, 1999, Carver and Lindner, 1998). The flexible C-terminal extension is very important in chaperone action; its function seems to be as a solubilising region for the relatively hydrophobic sHsp and the complex it makes with the destabilised target protein during chaperone action (Carver et al., 1992, Carver and Lindner, 1998, Carver et al., 2002, Carver et al., 2003). NMR spectroscopy indicates that flexible polypeptide region(s) are also present in casein proteins (Rekas et al., 2007) and thus may also perform a similar, solubilising role, to the C-terminal extension in sHsps (Carver et al., 1992, Carver and Lindner, 1998, Carver et al., 2002, Carver et al., 2003).
In addition, as with other molecular chaperones, the caseins have distinct hydrophobic and hydrophilic domains (Swaisgood, 1992) to enhance solubility and to bind to lipophilic molecules (Bhattacharyya and Das, 1999).

1.10 Caseins as Molecular Chaperones

αs- and β-casein have been shown to possess effective chaperone-like activity in stabilising a range of unrelated target proteins from amorphous aggregation induced by heat-stress (Bhattacharyya and Das, 1999, Koudelka et al., 2009, Matsudomi et al., 2004, Morgan et al., 2005, O’Kennedy and Mounsey, 2006, Zhang et al., 2005, Guyomarc’h et al., 2009, Yong and Foegeding, 2008, Hassanisadi et al., 2008, Pulford et al., 2008), reduction-stress (Bhattacharyya and Das, 1999, Koudelka et al., 2009, Morgan et al., 2005, Zhang et al., 2005, Badraghi et al., 2009), and UV-induced aggregation (Bhattacharyya and Das, 1999). κ-Casein’s chaperone-like activity has also been investigated (Morgan et al., 2005, Guyomarc’h et al., 2009).

In preventing the heat-induced aggregation of catalase, β-casein has been shown to form a stable high molecular weight (HMW) complex with catalase (as assessed with size-exclusion chromatography) (Zhang et al., 2005). Similarly, αs-casein has also been shown to form soluble HMW aggregates with thermally stressed β-lactoglobulin and reduced apo α-lactalbumin to prevent their stress-induced aggregation (Morgan et al., 2005). In doing so, αs- and β-casein, act in a very similar manner to that of intracellular sHsps and extracellular clusterin (Carver et al., 2003, Lindner et al., 1997), whereby, they incorporate the partially folded target protein into a soluble high molecular weight complex to prevent it from precipitating. The sHsp oligomer is not able to refold the partially folded target protein (Lindner et al., 2000), however, it can be refolded with the assistance of other molecular chaperones, such as Hsp70 coupled with ATP hydrolysis, when cellular levels are permissive.
(Treweek et al., 2003, Lee et al., 1997). It is not known whether other chaperones with refolding ability exist in milk although clusterin (Kounnas et al., 1995) and another extracellular chaperone, haptoglobin (Eckersall et al., 2001), are present and may function in a synergistic chaperone manner with casein proteins to minimise protein aggregation (Morgan et al., 2005). Interestingly, when either α- or β-casein are added to reduced insulin that has been induced to aggregate amorphously, not only is further aggregation prevented, but already-aggregated insulin is also partially solubilised (Bhattacharyya and Das, 1999, Zhang et al., 2005). This finding warrants further attention, in particular with regards to the possibility of this property being present in the interaction of αS-casein with disease related, ordered target protein aggregation, i.e. fibril formation (Thorn et al., 2009).

The chaperone activity of the caseins changes with temperature. αs-Casein has demonstrated an enhanced chaperone activity at lower temperatures (from 37 to 25 °C) with reduced α-lactalbumin (Morgan et al., 2005) and insulin (Bhattacharyya and Das, 1999, Morgan et al., 2005) as target proteins. Both αs- and β-casein have been observed to have reduced chaperone activity at higher temperatures against horse alcohol dehydrogenase (Hassanisadi et al., 2008), β-lactoglobulin (Yong and Foegeding, 2008) and other target proteins (Pulford et al., 2008). This may be attributable to an indirect effect associated with the aggregation rate of the target protein, whereby increasing temperature leads to an increase in the rate of target protein aggregation. Indeed, a complex study by Hassanisadi et al. using multivariate curve resolution by alternating least squares (MCR-ALS) demonstrated that β-casein does not interact with either the native or aggregated species of alcohol dehydrogenase. Instead, β-casein forms a complex with aggregation-prone intermediates to prevent their accumulation and aggregation (Hassanisadi et al., 2008). This is in agreement with other molecular chaperones, e.g. α-crystallin, that bind only to the aggregation-prone conformers of the substrate protein and do not interact with the native protein or proteins, which are already aggregated (Bellotti et al.,
At lower temperatures, the native protein aggregates slowly, so β-casein immediately consumes aggregation-prone intermediates to form a complex (which is reversible) and a smaller amount of aggregate-prone intermediate will remain for aggregate formation. At higher temperatures, a faster conversion of the native species to aggregation-prone intermediates and a decomposition of the complex produce enough aggregate-prone intermediates for aggregation and precipitation, resulting in a decrease in chaperone activity (Hassanisadi et al., 2008). NMR studies have also shown that αs-casein interacts with and stabilises the partially folded, molten globule state of α-lactalbumin (Morgan et al., 2005), in a similar manner to that reported for α-crystallin (Carver et al., 2002, Lindner et al., 1997). Therefore, it is expected that the longer that these molten globule state(s) persist, i.e. the slower the aggregation, the better the ability of αS-casein to interact with them (Thorn et al., 2009).

The relative chaperone-like ability of the casein proteins remains controversial and may differ depending on the target protein as is observed for the sHsp, αB-crystallin (Ecroyd and Carver, 2008a, Ecroyd et al., 2007). For example αs-casein is significantly better at preventing the heat-induced aggregation of ovotransferrin (Matsudomi et al., 2004) than β-casein. However, β-casein is more effective at inhibiting the heat-induced aggregation of catalase (Zhang et al., 2005), β-lactoglobulin (Yong and Foegeding, 2008) and scroplasmic proteins (Pulford et al., 2008) than αs-casein. In addition, bovine β-casein is more effective at reducing the heat-induced aggregation of alcohol dehydrogenase than camel β-casein (Hassanisadi et al., 2008). In these cases, the higher effective hydrophobic surfaces of β-casein as compared to αs-casein and camel β-casein (Swaisgood, 1992) might have played an important role. Similar differences in chaperone activities are observed under reducing conditions with various substrate proteins. For example, αs-casein is more potent than β-casein at suppressing the aggregation of insulin-B chain (Morgan et al., 2005), while β-casein is more effective at
inhibiting the reduction-induced aggregation of lysozyme than αs-casein (Zhang et al., 2001). Apart from the stress-induced amorphous aggregation of target proteins, whole casein and β-casein also inhibit the aggregation of carbonic anhydrase that occurs when it is refolded from denaturant (Khodarahmi et al., 2008). Whole casein was found to be significantly better at inhibiting this form of aggregation than β-casein (Khodarahmi et al., 2008), which is most likely due to the presence of αs-casein in the whole casein fraction.

While most investigations focus on suppression of amorphous aggregation, several studies using αs-, β- and whole casein have investigated their ability to prevent amyloid fibril formation (Khodarahmi et al., 2008, Thorn et al., 2008, Thorn et al., 2005). Thorn et al., (2005) has shown that αs- and β-casein act in a chaperone-like manner to inhibit κ-casein fibril formation, with complete suppression achieved at a 3:1 molar ratio of αs-casein/ β-casein:κ-casein, concentrations similar to those pertaining in milk, i.e. 3g/L of κ-casein and 11.25 g/L of αs- and β-casein (see Table 1.1). Similarly αs2-casein fibril formation is inhibited by a two-fold molar excess of its natural binding partner αs1-casein (Thorn et al., 2008). Whole and β-casein are also able to inhibit heat-induced fibril formation by ovalbumin (Khodarahmi et al., 2008). The ability of αs- and β-casein to inhibit this ordered form of protein aggregation is not only of physiological relevance (limiting the amount of mammary CA) but is also significant due to the intimate association of fibril formation with a wide range of protein misfolding diseases, such as Alzheimer’s, Parkinson’s and Huntington’s diseases (Thorn et al., 2009). As such, casein chaperone ability warrants further study in order to determine the therapeutic potential of casein proteins or their peptide fragments in the treatment of such diseases.
1.11 Aims

The chaperone-like activity of the caseins is of particular commercial interest due to their ability to stabilise other proteins during food processing, e.g. the heat treatment of milk during pasteurisation and the production of milk-related products whereby the whey proteins (α-lactalbumin and β-lactoglobulin) along with κ-casein, are destabilised and aggregate under conditions of elevated temperature. In addition, whey and egg proteins are common food ingredients that are used in processed foods; therefore, understanding how caseins can improve the stability of these proteins under stressed conditions will provide information on how they can be used in various applications (Yong and Foegeding, 2010). Furthermore, understanding the αs- and β-casein chaperone mechanism and prevention of αs2- and κ-casein fibril formation is of great interest to the dairy industry and in the broader context of amyloid fibril diseases.

As such, in this thesis, the calcium-sensitive caseins, αs- and β-casein, were dephosphorylated to determine the effect of phosphate groups on the ability of caseins to act as molecular chaperones (Chapter 2). We also explored the binding site of β-casein with aggregating α-lactalbumin, a milk protein, using limited proteolysis and mass spectrometry to give insight into the mechanism of β-casein chaperone action with milk and other non-related proteins (Chapter 3). In addition, amyloid fibrils were formed from reduced and carboxymethylated κ-casein and dimeric αs2-casein, and the amyloidogenic regions of both these proteins were identified by limited proteolysis and mass spectrometry (Chapter 4). Lastly, considering milk exists in an oxidative extracellular environment the methionine residues of native κ-casein (Met-95 and Met-106) were oxidised selectively. The effect on methionine oxidation on κ-casein’s fibril-forming propensity, structure and cellular toxicity and its possible implication in vivo was examined (Chapter 5).
**Table 1.1:** Protein Composition of Milk (Karman and Van Boekel, 1986)

**Table 1.2:** Bovine mole fractions and definition of phosphate centres (from (De Kruif and Holt, 2003))

<table>
<thead>
<tr>
<th>Casein</th>
<th>Mole fraction</th>
<th>Phosphoamino acids (max)</th>
<th>Phosphate centres(^1)</th>
<th>No. of centres</th>
</tr>
</thead>
<tbody>
<tr>
<td>(\kappa)</td>
<td>0.13</td>
<td>1 (3)</td>
<td>None</td>
<td>0</td>
</tr>
<tr>
<td>(\beta)</td>
<td>0.41</td>
<td>5</td>
<td>f14-21</td>
<td>1</td>
</tr>
<tr>
<td>(\alpha_{s1})</td>
<td>0.37</td>
<td>8 (9)</td>
<td>f41-51</td>
<td>2</td>
</tr>
<tr>
<td>(\alpha_{s2})</td>
<td>0.09</td>
<td>10 (13)</td>
<td>f8-16, f56-63, f126-133</td>
<td>3</td>
</tr>
</tbody>
</table>

\(^1\)The number of phosphoamino acid residues in a phosphate centre is 4 with the exception of \(\alpha_{s1}\)-casein 8P (f41-51) and \(\alpha_{s2}\)-casein (f126-133), which have only 2 and \(\alpha_{s1}\)-casein 9P (f41-51), which has 3.

"NOTE: This table is included on page 24 of the print copy of the thesis held in the University of Adelaide Library."
**Table 1.3:** Secondary structural predictions for caseins (adapted from Horne, 2002)

<table>
<thead>
<tr>
<th>Reference</th>
<th>Method</th>
<th>α-helix</th>
<th>β-sheet</th>
<th>Turns</th>
<th>Irregular</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-casein (Byler et al., 1988)</td>
<td>Raman</td>
<td>13</td>
<td>22</td>
<td>35</td>
<td>30</td>
</tr>
<tr>
<td>β-casein (Caessens et al., 1999)</td>
<td>CD</td>
<td>13</td>
<td>15</td>
<td>-</td>
<td>72</td>
</tr>
<tr>
<td>β-casein (Farrell et al., 2001)</td>
<td>CD</td>
<td>20</td>
<td>32</td>
<td>28</td>
<td>22</td>
</tr>
<tr>
<td>β-casein (Farrell et al., 2001)</td>
<td>FTIR</td>
<td>29</td>
<td>34</td>
<td>32</td>
<td>4</td>
</tr>
<tr>
<td>α_{s1}-casein (Byler et al., 1988)</td>
<td>Raman</td>
<td>13</td>
<td>20</td>
<td>34</td>
<td>33</td>
</tr>
<tr>
<td>κ-casein (Farrell et al., 1996)</td>
<td>FTIR</td>
<td>17</td>
<td>35</td>
<td>25</td>
<td>23</td>
</tr>
<tr>
<td>κ-casein (Farrell et al., 2002)</td>
<td>CD</td>
<td>9</td>
<td>40</td>
<td>26</td>
<td>24</td>
</tr>
<tr>
<td>α_{s2} casein (Hoagland et al., 2001)</td>
<td>CD</td>
<td>24</td>
<td>30</td>
<td>24</td>
<td>22</td>
</tr>
<tr>
<td>α_{s2} casein (Hoagland et al., 2001)</td>
<td>FTIR Amide I</td>
<td>32</td>
<td>27</td>
<td>31</td>
<td>9</td>
</tr>
<tr>
<td>α_{s2} casein (Hoagland et al., 2001)</td>
<td>FTIR Amide II</td>
<td>29</td>
<td>37</td>
<td>34</td>
<td>34</td>
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</tbody>
</table>
Figure 1.1: Waugh's proposed model for the casein micelle; (A) Monomer model for $\alpha_{s1}$- or $\beta$-caseins with charged loop, (B) a tetramer of $\alpha_{s1}$-casein monomers, (C) planar model for a core polymer of $\alpha_{s1}$- and $\beta$-caseins (from Wong, 1988).

Figure 1.2: The structure of casein micelle in the sub-micelles model showing the protruding C-terminal parts of $\kappa$-casein as proposed by Walstra (from Walstra and Jenness 1984)
Figure 1.3: Model of the casein micelle (adapted from Holt, 1994). It shows a more or less spherical, highly hydrated, and fairly open particle. Polypeptide chains in the core are partly cross-linked by nanometer sized clusters of Ca phosphate; the internal structure gives rise to an external region of lower segment density known as the hairy layer, which confers steric and charge stability to native casein particles. Electron microscopy shows that casein micelles are generally spherical in shape and have an average diameter of ~150nm (Fox and McSweeney, 2003).
Figure 1.4: Conformations of (A) $\alpha_s$-casein with a train-loop-train structure and (B) $\beta$-casein with a tail-train structure adsorbed at hydrophobic interfaces. The Polymeric structures of (C) $\alpha_s$-caseins and (D) $\beta$-caseins (adapted from Horne (1998)).

Figure 1.5: The dual binding model of casein micelle structure, with $\alpha_s$-, $\beta$-and $\kappa$-casein portrayed as indicated. Bonding appears between the hydrophobic regions, shown as rectangular bars, and by linkage of hydrophilic regions containing phosphoserine clusters to colloidal calciumphosphate clusters. Molecules of $\kappa$-casein (K) limit further growth of the structure. (from Horne, 1998).
Figure 1.6: Transmission electron microscopy of amyloid fibrils assembled from human lysozyme, showing the typical long, unbranched, rope-like structure of amyloid fibrils (A). Scale bar represents 400 nm (from Chamberlain et al., 2000). Model of the structural organisation of insulin fibrils (from Jimenez et al., 2002) (B). The image shows a fibril with four protofilaments wound around each other. In this model the core structure of each protofilament is a row of β-strands where each insulin molecule occupies two layers connected by the interchain disulfide bonds and each β-strand runs perpendicular to the fibril axis. X-ray fiber diffraction of amyloid fibrils showing the diagnostic meridional and equatorial reflections at 4.7 Å and ~ 9–11 Å respectively, which form the “cross β-sheet” pattern (C) (from Ecroyd and Carver, 2008b).
**Figure 1.7:** Some amyloid-related peptides/proteins form early aggregates of globular appearance that further organise into beaded chains, globular annular ‘doughnut’ shaped assemblies and eventually form mature protofilaments and fibrils (from Stefani and Dobson, 2003). Pre-fibrillar aggregates may interact with reconstituted phospholipid membranes and with cell membranes where they form aspecific channels (pores) disrupting cellular homeostasis. The latter possible mechanism of toxicity is similar to that displayed by antimicrobial peptides, pore-forming eukaryotic proteins and bacterial toxins (Kourie and Shorthouse, 2000). The electron micrographs of the rings of the α-synuclein A53T (upper row), A30P (middle row) mutants and of the Alzheimer precursor protein (lower row) are from Lashuel *et al.*, 2002.
**Figure 1.8:** Overview of the mammary alveolus showing the aveolus lumen with subtending ducts, blood supply, adipocyte stroma, and myoepithelial cells. Milk constituents are secreted from the epithelial cells into the aveolus lumen, while removal of milk from the alveoli is accomplished by contraction of the myoepithelial cells surrounding the alveoli and ducts (A). Figure showing calcified CA in the alveolar lumen (B) (stained with hematoxylin and eosin, original magnification 100x (from Niewold et al., 1999). Non-calcified amyloid bodies within the alveolar lumen (C) (stained with hematoxylin and eosin, original magnification 100x (from Niewold et al., 1999).
Figure 1.9: The protein folding/unfolding and off-folding pathways and the interaction of sHsps to prevent protein aggregation (adapted from Carver et al., 2003). A native protein (N) unfolds via a variety of intermediate states (I₁, I₂, …) to the unfolded state (U). This folding/unfolding pathway is fast and reversible. However, if the intermediate states linger for too long (e.g. during times of cellular stress), they can self-associate via exposed hydrophobic regions. When this self-association occurs, the partially folded intermediates enter the off-folding pathways, which are slow and irreversible. The off-folding pathways comprise either the disordered, amorphous aggregation pathway resulting in amorphous protein precipitates, or the ordered, amyloid fibril pathway which, via the process of β-sheet stacking, leads to highly ordered, cross β-sheet fibrils being formed. sHsps exist as large, dynamic aggregates which are in constant exchange with dissociated dimeric species. The dissociated form is thought to be the active species which interacts with the partially folded intermediates (Iₐ), which are subsequently sequestered into a high-mass complex containing both proteins. Refolding of the protein to the native state (N) can then occur via the action of another chaperone such as Hsp70 using ATP (Treweek et al., 2003).
STATEMENT OF AUTHORSHIP

Dephosphorylation of $\alpha_{s}$- and $\beta$-Casein and its Effect on Chaperone Activity: a Structural and Functional Investigation


Koudelka, T. (Candidate)
Performed experimental work (unless stated otherwise, see below), interpreted data and prepared manuscript.
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Chapter 2

Dephosphorylation of \( \alpha_s \)- and \( \beta \)-Casein and its Effect on Chaperone Activity: a Structural and Functional Investigation

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Chapter 3

During chaperone interaction, the C-terminal domain of β-casein is protected from proteolysis by bound α-lactalbumin.

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3.1 Abstract

β-Casein, a major milk protein, functions as a molecular chaperone by binding to and solubilising stressed, partially unfolding target proteins. β-Casein has a mass of 24 kDa and is predicted to be composed of two domains roughly encompassing residues 1-50 and 51-209 which are hydrophilic and hydrophobic respectively in character (Berry and Creamer, 1975). Under chaperone conditions, size-exclusion chromatography was used to purify the complex between β-casein and reduced, partially unfolded α-lactalbumin. A combination of limited proteolysis and mass spectrometry was performed to identify the regions of β-casein directly involved in binding to reduced α-lactalbumin. In the presence of endoproteinase Glu-C, β-casein bound to α-lactalbumin showed markedly reduced proteolysis at residues A91, A101 and E121 compared with β-casein in the absence of α-lactalbumin, indicating that the bound target protein restricted access of Glu-C to the C-terminal region of β-casein. Chymotrypsin also cleaved within the C-terminal domain of β-casein, with reduced proteolysis at residues between G94-W143 when α-lactalbumin was bound to β-casein. Trypsin cleaved both the N- and C-terminal domains of β-casein. β-Casein with reduced α-lactalbumin bound showed no reduction in proteolysis at R25 and K28 compared with a control, indicating that the highly charged N-terminal domain is not involved in binding to the target protein. It is concluded that a portion of the hydrophobic C-terminal domain of β-casein, from A91 to W143, encompasses its chaperone-binding site.
3.2 Introduction

Approximately 95% of casein in milk exists as micelle-like particles (Fox and McSweeney, 2003). Casein is the principal component of these micelles and is a heterogeneous mix of phosphoproteins comprising the unrelated \( \alpha_{S1} \), \( \alpha_{S2} \), \( \beta \), and \( \kappa \)-caseins (Swaisgood, 1992). Casein proteins have been classified as „natively unfolded“ (Uversky et al., 2000) and under physiological conditions, have poorly ordered secondary and tertiary structures in comparison with typical globular proteins (Swaisgood, 1993). The flexible nature of the caseins renders them susceptible to proteolysis, which facilitates their physiological function, as a source of amino acids (Swaisgood, 1992). Although the caseins are relatively small proteins, ranging in mass between 19 and 25 kDa, they are usually associated with calcium and other ions in milk to form micelles that have an average molecular mass of \( 10^8 \) Da and a diameter of ~150nm (Fox and McSweeney, 2003). The micelles have colloidal dimensions and scatter light; the white colour of milk is due largely to the light scattering of casein micelles (Fox and McSweeney, 2003).

\( \beta \)-Casein, as with the other unrelated caseins (i.e. \( \alpha_{S1} \), \( \alpha_{S2} \) and \( \kappa \)) prevents a variety of proteins, including whey proteins, against thermal-, chemical-, and UV-light-induced aggregation (Bhattacharyya and Das, 1999; Morgan et al., 2005; Zhang et al., 2005; O'Kennedy and Mounsey, 2006), and as such behaves as a molecular chaperone in a very similar manner to intracellular small heat-shock proteins (sHsps) and the extracellular protein, clusterin (Carver et al., 2003). Although no common amino acid sequence has been identified among these chaperone proteins, they share common structural features, for example, they have distinct hydrophilic and hydrophobic domains that enable them to bind to and solubilise stressed, partially unfolded target proteins (Carver and Lindner, 1998; Carver, 1999). \( \beta \)-Casein has a distinct polar N-terminal domain and a hydrophobic C-terminal domain; a net charge of -13 at pH 7.0 resides on the first fifty N-terminal amino acids, while the remainder of the protein
contains few hydrophilic residues (Berry and Creamer, 1975). In addition to casein proteins, other chaperones also exhibit micelle-like structures, e.g. GroEL exits as well-structured 14-mer (Braig et al., 1994), while α-crystallin and clusterin are both found as heterogeneous aggregates, with the former possessing a large central cavity (Carver et al., 1994; Haley et al., 2000; Carver et al., 2003).

Limited proteolysis is a powerful tool for probing the higher order structure of proteins. Amino acids buried in the protein core and in regular secondary structure are less accessible to an enzyme’s active site and are therefore not cleaved as quickly (Hubbard, 1998). Hence, it would be expected that proteolytic cleavages occur at broadly “surface” sites that are exposed to the surrounding solvent (Hubbard, 1998). Limited proteolysis can be used to determine the regions of amino acids involved in chaperone-target protein interactions. If peptide bonds are readily cleaved in the absence of a target protein, yet exhibit restricted cleavage in the presence of substrate, it can be assumed that these bonds are involved in interaction with the target protein (Aquilina and Watt, 2007). Limited proteolysis studies have been used to probe the structure of α-crystallin, e.g. to investigate the relationship between chaperone activity and oligomeric size of the protein (Saha and Das, 2004), while Aquilina et al. (2007) used these techniques coupled with mass spectrometry to determine the regions of αB-crystallin directly involved in binding a target protein, α-lactalbumin, which partially unfolds, aggregates and precipitates upon reduction of its disulfide bonds (Carver et al., 2002). In this study, limited proteolysis and mass spectrometry was performed using β-casein and reduced α-lactalbumin, a physiologically relevant whey protein. It is concluded that the hydrophobic C-terminal domain of β-casein, residues A91-W143, are responsible for the interaction of β-casein with unfolding target proteins.
3.3 Materials and methods

3.3.1 Materials

Bovine β-casein and α-lactalbumin (calcium reduced >85%) were purchased from Sigma Aldrich (St Louis, Missouri). Trypsin, chymotrypsin, (from bovine pancreas, protein sequencing grade), endoproteinase Glu-C (from Staphylococcus aureus V8, sequencing grade) and the reducing agent 1,4-dithiotheritol (DTT) were also purchased from Sigma Aldrich. All other chemicals were of reagent grade, and unless otherwise stated, all solutions were prepared in MilliQ water.

3.3.2 Monitoring the aggregation of reduced α-lactalbumin.

α-Lactalbumin (1.5 mg/ml) was dissolved in 100mM ammonium acetate, 10mM EDTA, pH 7.0 with various amounts of β-casein (i.e. 0:1, 0.5:1, 1:1 or 2:1 mole ratio of β-casein: α-lactalbumin). Aggregation was induced by the addition of DTT to a final concentration of 20 mM. The aggregation was monitored as light scattering over two hours at either 25°C or 37°C using a Fluostar Optima plate reader (BMG Labtechnologies) in a 96-well Falcon 3072 plate at a wavelength of 340 nm.

3.3.3 Size-exclusion chromatography

Size-exclusion chromatography was performed on samples following the α-lactalbumin reduction experiments in the presence and absence of β-casein. The various samples were spun down for 5 mins at 14,000 rpm using a Sigma 3-18K centrifuge (Sigma, Germany) at 4°C and 100 µl of sample was loaded on to a Superdex 200 10/30 column (Amersham Bioscience, Little Chalfont) at a flow rate of 0.5 ml/min in the presence of 100mM ammonium acetate column buffer at pH 7.0. Samples were collected in 1 ml fractions and stored at 4°C. The column was calibrated using gel filtration markers (Bio-Rad, Hemel Hampstead).
3.3.4 SDS PAGE

The peaks eluting from the Superdex 200 column were analysed using a 15% SDS-PAGE gel to determine their composition (Laemmli, 1970). Samples were mixed with equal volumes of reducing gel sample buffer and then heated (90 °C, 10 mins) before being loaded onto the gels. ImageQuant was the software program used to quantitatively analyse the amount of β-casein in the SDS-PAGE gels. The software compares the relative intensity of different bands on the scanned image of a gel. Each pixel was assigned a numerical value corresponding to the optical density of the gel at that point, which represents the amount of the sample at that point. Known concentrations of β-casein were loaded onto a 15% SDS-PAGE gel alongside the eluting fraction containing the β-casein: α-lactalbumin complex. Depth analysis in ImageQuant was used to obtain a standard curve of β-casein from known concentrations and consequently the unknown concentration of β-casein in the β-casein-target protein complex was determined.

3.3.5 Limited proteolysis

For limited proteolysis experiments, 100 µl of the complex peak in 100 mM ammonium acetate (pH 7.0) was left at ambient temperature for 15 mins. Digestion was then initiated by the addition of either 1:25 (w:w) of trypsin or 1:10 (w:w) of either chymotrypsin or V8 (Endoproteinase Glu-C) and heated at 37°C. Control samples in 100 mM ammonium acetate (pH 7.0) consisted of β-casein at the same concentrations found in the complex. Digestion was quenched at various time points (5, 15, 30 and 60 mins) by taking 10 µl of digestion solution and adding it to 10 µl of stop solution: Glu-Fib at the same concentration as β-casein (0.02-0.03 mg/ml) in 90% acetonitrile and 1% (v/v) trifluoroacetic acid. The solution was spun down using a SpeedVac vacuum concentrator and resuspended in 50% acetonitrile, 0.1% trifluoroacetic acid and kept frozen prior to analysis by a MALDI-TOF mass spectrometer.
3.3.6 Mass spectrometry

All samples were prepared for mass spectrometry by spotting 1 µl of analyte solution to 1 µl of 5 g/l 2,5 dihydroxybenzoic in 50% acetonitrile, 0.1 % trifluoroacetic acid on a MTP Anchor Chip Target 600/384 (Bruker Daltonics) and leaving to dry. All mass spectra were acquired on an Ultraflex III MALDI-TOF/TOF mass spectrometer (Bruker Daltonics) in reflector mode using Flex Control (Bruker Daltonics). The identity of peptides was confirmed by matching their observed masses to the expected masses of the in silico tryptic digest of the known β-casein primary sequence using Biotools 3.0 software (Bruker Daltonics). All spectra were processed using FlexAnalysis software (Bruker Daltonics) with minimal smoothing applied to the raw data.

3.4 Results

3.4.1 Light scattering of reduced α-lactalbumin.

The aggregation of α-lactalbumin at 25 °C was initiated by the addition of DTT to reduce its four disulfide bonds (Carver et al., 2002) and the resultant light scattering was measured over time in the presence and absence of β-casein (Figure 3.1A). In the absence of β-casein, an increase in light scattering was observed 10 minutes after addition of DTT which is indicative of α-lactalbumin aggregation. The addition of β-casein at a molar ratio of 0.5:1 β-casein: α-lactalbumin resulted in a significant decrease in protein aggregation, while a 1:1 molar ratio resulted in complete inhibition of α-lactalbumin aggregation. The aggregation assay of α-lactalbumin in the presence of β-casein was also performed at 37 °C. However, β-casein was significantly less able to suppress the aggregation and subsequent precipitation of α-lactalbumin at this temperature, which is consistent with β-casein being a better chaperone at lower temperature (Morgan et al., 2005). A 2:1 molar ratio of β-casein: α-lactalbumin at 37 °C resulted in only 70% protection against α-lactalbumin aggregation (Figure 3.1B). As a
consequence the aggregation of α-lactalbumin and β-casein at 25 °C was studied in all further experiments.

3.4.2 Size-exclusion chromatography

Under stress conditions, particularly those that lead to amorphous aggregates of target proteins, sHsps often form stable complexes with their partially folded target proteins (Ehrnsperger et al., 1997; Lee et al., 1997; Carver and Lindner, 1998). To determine whether such complexes were formed with β-casein and reduced α-lactalbumin, size-exclusion chromatography (SEC) was employed. As shown in the chromatograph presented in Figure 3.2A, non-covalent complexes between β-casein and reduced α-lactalbumin were detected as a peak (7 mL) with a significantly lower elution volume than peaks associated with β-casein (13 mL) and reduced α-lactalbumin (16 mL) alone. The complex peak eluted at the same volume as blue-dextran, consistent with a large soluble aggregate being formed between β-casein and reduced α-lactalbumin. Reduced α-lactalbumin in the absence of chaperone aggregated significantly forming large insoluble complexes which precipitated and were removed by centrifugation. As a consequence an elution profile corresponding to monomeric α-lactalbumin is presented in Figure 3.2A. SDS-PAGE analysis was performed on the eluting fractions from the size-exclusion column (Figure 3.2B). Lanes 2 and 3 correspond to the elution of native β-casein and reduced α-lactalbumin respectively, while lanes 4 and 5 correspond to the first peak eluted off the chromatograph presented in Figure 3.2A for 0.5:1 and 1:1 molar ratios of β-casein: α-lactalbumin respectively. Both β-casein and α-lactalbumin are observed in lanes 4 and 5, demonstrating the presence of soluble chaperone-target protein complex.

While a stable complex between β-casein and α-lactalbumin was formed and separated at a molar ratio 0.5:1 and 1:1 β-casein: α-lactalbumin, it is evident from the chromatogram that
not all the β-casein was incorporated into the chaperone-target protein complex as a peak corresponding to that of native β-casein is also observed (Figure 3.2A). To determine the concentration of β-casein within the β-casein: α-lactalbumin complex a second gel was performed prior to limited proteolysis experiments (Figure 3.2C). Known concentrations of β-casein were placed in the lanes alongside that from the eluting fraction containing the β-casein: α-lactalbumin complex. Depth analysis was employed in ImageQuant to estimate the concentration of β-casein in the complex as described in the methods section. By this analysis, it was determined that 0.02-0.03 mg/ml of β-casein was present in the complex peak. Depth analysis was also performed on α-lactalbumin, typically concentrations between 0.04 and 0.065 were determined.

3.4.3 Limited proteolysis and mass spectrometry

The β-casein: α-lactalbumin complex as purified from size-exclusion chromatography was anticipated to contain species in which the chaperone binding site(s) of β-casein were occupied by α-lactalbumin and was used for the subsequent proteolysis experiments. Limited proteolysis experiments were performed on the β-casein: α-lactalbumin complex and native β-casein, under the same conditions, with endoproteinase Glu-C, trypsin or chymotrypsin as the protease.

Glu-C from *Staphylococcus aureus* strain V8 cleaves at the carboxyl side of glutamyl and aspartyl residues unless they are followed by a proline residue (Drapeau, 1976). The specificity of Glu-C is also dependent upon buffer and pH conditions. In ammonium acetate or ammonium bicarbonate (pH 7.8), the enzyme preferentially cleaves glutamyl bonds whereas in phosphate buffer (pH 7.8), Glu-C will cleave at glutamyl and aspartyl residues (Drapeau, 1976). The possible cleavage sites accessible to Glu-C in β-casein are indicated in Figure 3.3A. Digestion was performed over 60 mins at 37 °C in ammonium acetate buffer
(pH 7.8) with samples removed at intervals and quenched with Glufib solution in 99 % acetonitrile and 1% trifluoroacetic acid. This enabled monitoring of cleavage sites and the extent of proteolysis over time. Glutamic acids are not distributed uniformly throughout the sequence of β-casein; almost all of them are situated on the highly polar N-terminal domain in close proximity to each other (Figure 3.3A). These peptides may not be observed as cleavage at these N-terminal domains yield peptides of small mass (<700 Da) which are too small to be detected by MALDI-TOF under normal conditions used in these experiments. Figure 3.3B shows mass spectra of the limited proteolysis of native β-casein and α-lactalbumin (left panel) and the β-casein: α-lactalbumin complex obtained under reduction stress conditions (right panel). In the mass spectrum, the large peak at 1570.6 Da was observed in all spectra and belongs to Glu1-fibrinopeptide B (Glu-fib) that was added to the digestion mixture upon quenching. A standard amount of Glu-fib was added to the digestion mixture as an internal standard to enable the peak intensities of the cleaved peptides from β-casein to be normalised and quantified. The large peak at 2474.2 Da corresponds to the β-casein peptide fragment, A101-E121 (Figure 3.1, Appendix 7.3 A). The intensity of this peak was significantly greater at all time points for native β-casein and α-lactalbumin (left panel) compared to the β-casein: reduced α-lactalbumin complex (right panel). The small peak at 3431.7 Da corresponds to the peptide fragment V92-E121 (Figure 3.2) and was present for native β-casein but was not observed for the β-casein: reduced α-lactalbumin complex. The peak at 2107.2 Da, which was present in all spectra, corresponds to L191-V209 from β-casein but is not due to a Glu-C cleavage and possibly arises from laser-induced dissociation of a larger peptide fragment (Appendix 7.3 B). The peak at 1381.7 corresponds to α-lactalbumin peptide (Appendix 7.3 C). In order to more accurately quantify the relative abundance of the cleavage products, the peak intensities of the cleaved peptides were normalized against the signal arising from Glu-fib (Figure 3.3C). A significant difference in the signal intensity was observed for native β-casein compared to β-casein:reduced α-lactalbumin complex for
peptides V92-E121 and A101-E121, suggesting that the target protein is bound to β-casein in this region which hinders the enzyme from gaining access to this region.

Trypsin cleaves at the carboxyl side of lysine and arginine unless they are followed by a proline residue in which case no cleavage occurs (Figure 3.4A). β-Casein has relatively few basic amino acids and as a result the sequence coverage upon digestion with trypsin is not extensive. However, many of the expected tryptic peptide fragments were observed in the mass spectra (Figure 3.4B) and their peak intensities were normalised against the signal arising from the internal standard, Glu-fib (Figure 3.4C). The peaks at approximately 3122.3 and 3477.6 Da correspond to proteolysis at R25 and K28 resulting in the peptide fragments R1-R25 and R1-K28 respectively, a missed cleavage of β-casein, in the highly polar N-terminal domain. The peaks are observed in native β-casein and the β-casein: reduced α-lactalbumin complex with very similar intensities suggesting that the highly polar region is not involved in binding unfolding target proteins. The other peptide fragments arose from the highly hydrophobic C-terminal end of β-casein. The peaks at approximately 780.5 and 830.4 Da are associated with β-casein peptide fragments V170-K176 and A177-R183 respectively (Appendix 7.3 D), with both having similar peak intensities for native β-casein and the β-casein: reduced α-lactalbumin complex (Figure 3.4C). A slight reduction in peak intensity between the native and complex bound β-casein was observed for the peaks at 1591.9 and 2909.6 Da corresponding to the peptide fragment V170-R183 and D184-V209 respectively (Appendix 7.3 E and F). However, the results were not statistically significant and therefore we were unable to draw any definitive conclusions.

Proteolysis was also performed using α-chymotrypsin which hydrolyses peptide bonds on the C-terminal sides of the hydrophobic amino acids tyrosine, phenylalanine, tryptophan and leucine with secondary hydrolysis occurring on the C-terminal side of methionine, isoleucine,
serine, threonine, valine, histidine, glycine, and alanine (Burrell, 1993). A significant number of peptide fragments were observed for β-casein, all of which arose from the hydrophobic C-terminal region of β-casein, i.e. T78 to V209. The peptide fragments that were observed had their peak intensities normalised against the signal arising from the standard, Glu-fib (Figure 3.5). A significant difference in the normalised signal intensities was observed for the peaks arising from native β-casein compared to those from the β-casein: reduced α-lactalbumin complex for peptides T126-I139 and T126-W143, suggesting the reduced target protein may be bound to β-casein which hinders the enzyme’s access to this region. A statistical difference was also observed between native β-casein compared to β-casein bound to reduced α-lactalbumin for peptides G94-F119 and G94-I125 while peptide fragments from M144-V209 and T78-M93 had similar signal intensities for β-casein and β-casein bound to reduced α-lactalbumin. A slight reduction in peak intensity was observed for the peptide fragments M144-L163, with a smaller peak intensity observed when β-casein was bound to reduced α-lactalbumin. However, the results were not statistically different and no conclusion could be drawn. Therefore, the region of β-casein protected from chymotrypsin cleavage was determined to be between G94-W143, consistent with the region of amino acids protected from Glu-C cleavage, i.e. V92-E121. The β-casein peptide fragments observed for all protease digestions in the presence and absence of reduced α-lactalbumin have been summarized in Figure 3.6. The figure indicates the regions of β-casein that are cleaved by each enzyme and those that are significantly protected from proteolysis as a result of bound α-lactalbumin. The C-terminal peptide fragments that are not involved in binding to the reduced α-lactalbumin, i.e. M144-V209 and V170-V209 from chymotrypsin and trypsin proteolysis respectively, are also in agreement.
Figure 3.1: The reduction of α-lactalbumin (1.5 mg/mL) induced by the addition of 20 mM DTT in 100 mM ammonium acetate buffer (pH 7.0, 10 mM EDTA) and monitored by light scattering at 340 nm over 120 min at (A) 25 °C and (B) 37 °C in the presence of increasing amounts of β-casein.
**Figure 3.2:** SEC after the incubation of either α-lactalbumin (0:1), β-casein (1:0) or α-lactalbumin with β-casein (0.5:1 and 1:1) in the presence of 20 mM DTT at 25 °C. Proteins were loaded onto a Superdex 200 10/30 column at a flow rate of 0.5 ml/min in the presence of 100mM ammonium acetate column buffer at pH 7.0. Calibration of the column was performed using (from left to right ▼) blue dextran, 2000 kDa; thyroglobulin, 670 kDa; beta amylase, 210 kDa; ovotransferrin, 76 kDa; carbonic anhydrase, 29 kDa; myoglobin, 17 kDa; insulin 11 kDa (A). After SEC, eluted fractions were resolved by SDS-PAGE (1) SDS size-markers; (2) β-casein, 1:0; (3) α-lactalbumin, 0:1; (4) β-casein and α-lactalbumin, 0.5:1; (5) β-casein and α-lactalbumin, 1:1 (B). Known concentrations of β-casein were loaded onto a SDS-PAGE alongside the eluting fraction containing the β-casein: α-lactalbumin complex. Lane (1) SDS size-markers; (2) β-casein and α-lactalbumin (1:1, mole to mole ratio); (3) β-casein, 0.015 mg/ml; (4) β-casein, 0.03 mg/ml; (5) β-casein, 0.045 mg/ml; (6) β-casein, 0.06 mg/ml (C). The concentration of β-casein in the complex peak was determined from the bands of the known concentrations via densitometry using ImageQuant as described in the Methods.
**Figure 3.3:** The sequence of β-casein (Ribadeau Dumas *et al.*, 1972) showing the predicted cleavage sites of the enzyme Glu-C. Only sites indicated by downward arrows were observed (A). The raw mass spectrum from the limited proteolysis of native β-casein (left panel) and
the β-casein: α-lactalbumin complex (right panel) with enzyme Glu-C after 5, 15, 30, or 60 mins of digestion. The peaks at approximately 2474.2 and 3431.7 Da correspond to the β-casein peptide fragments, A101-E121 and V92-E121 respectively. Signals arising from both peptides are clearly more abundant in the native β-casein spectra. Arb. U. Arbitrary Units (B). Relative abundances of peptides released from β-casein over time as a result of limited proteolysis. Peak intensities were normalized against the signal arising from the standard of Glu-fib. Error bars represent standard error of at least three independent experiments (C).
Figure 3.4: The sequence of β-casein (Ribadeau Dumas et al., 1972) showing the predicted cleavage sites of the enzyme trypsin. Only the sites indicated by downward arrows were observed (A). The raw mass spectra from the limited proteolysis of native β-casein (left panel) and the β-casein: α-lactalbumin complex (right panel) with trypsin after 5 and 60 mins of digestion. Peak assignments corresponding to the tryptic peptides of β-casein are indicated. (Arb. U.) Arbitrary Units (B). Relative abundances of peptides released from β-casein over time as a result of limited proteolysis. Peak intensities were normalized against the signal arising from the standard amount of Glu-fib (C). Error bars represent standard error of at least three independent experiments.
**Figure 3.5:** Relative abundance of peptides released from β-casein over time as a result of limited proteolysis with chymotrypsin. Limited digestion of native β-casein or β-casein: α-lactalbumin complex at 5, 15, 30 or 60 mins with chymotrypsin. The identity of the β-casein peptides resulting from chymotrypsin digestion were confirmed with MS/MS (Appendix 7.3 G – M) Peak intensities were normalized against the signal arising from the standard of Glu-fib. Error bars represent standard error of at least three independent experiments.
Figure 3.6: Summary of limited proteolysis experiments. The figure indicates the β-casein peptide fragments that were generated by Glu-C, trypsin or chymotrypsin cleavage and were observed by mass spectrometry. Shaded regions indicate a section of the polypeptide sequence whose access to the enzyme’s active site was restricted in the presence of bound α-lactalbumin.

Table 3.1: The composition of β-casein based on its primary sequence

<table>
<thead>
<tr>
<th>Residues considered</th>
<th>Net charge$^2$</th>
<th>Charge frequency$^3$</th>
<th>Average hydrophobicity$^3$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 → 45</td>
<td>-18</td>
<td>0.51</td>
<td>821</td>
</tr>
<tr>
<td>46 → 95</td>
<td>-0.5</td>
<td>0.08</td>
<td>1511</td>
</tr>
<tr>
<td>96 → 145</td>
<td>0.5</td>
<td>0.28</td>
<td>1310</td>
</tr>
<tr>
<td>146 → 177</td>
<td>2.5</td>
<td>0.09</td>
<td>1417</td>
</tr>
<tr>
<td>178 → 209</td>
<td>0</td>
<td>0.13</td>
<td>1738</td>
</tr>
<tr>
<td>1 → 209</td>
<td>-15.5</td>
<td>0.23</td>
<td>1335</td>
</tr>
</tbody>
</table>

$^1$The primary structure of β-casein A$^2$-5P (Ribadeau Dumas et al., 1972)

$^2$Serine phosphate = -2, histidine = 0.5, other charges (+1 or -1) per residue

$^3$Calculated as described by Bigelow (1967) and expressed as calories per residue
3.5 Discussion

Various studies have shown that β-casein has chaperone-like activities in inhibiting the aggregation of a wide range of target proteins under a variety of stress conditions (Bhattacharyya and Das, 1999; Matsudomi et al., 2004; Morgan et al., 2005; Zhang et al., 2005). In this paper, α-lactalbumin, a bovine whey protein, was reduced and its aggregation and precipitation was inhibited by the addition of β-casein (Figure 3.1). β-Casein was significantly more effective at suppressing the aggregation of α-lactalbumin at lower temperature as we observed previously (Morgan et al., 2005). By contrast, sHsps (e.g. α-crystallin) have enhanced chaperone action at higher temperature (Das and Surewicz, 1995). β-Casein forms stable complexes with heat-stressed catalase (Zhang et al., 2005), and this study has shown that β-casein also forms a stable complex with reduced α-lactalbumin (Figure 3.2A). Following the aggregation assay of α-lactalbumin and β-casein, SEC was performed to separate the complex from non-aggregated α-lactalbumin and native β-casein (Figure 3.2A). Limited proteolysis was performed on the stable β-casein: reduced α-lactalbumin complex and the digestion pattern, as assessed by MS, was compared to that of native β-casein to identify the region(s) of β-casein involved in binding to the unfolding target protein, reduced α-lactalbumin.

The β-casein peptide regions encompassing G94-W143 and A91-E121 were significantly protected from proteolysis by chymotrypsin and Glu-C when β-casein was complexed to reduced α-lactalbumin (Figure 3.4C & 3.5). Both peptides reside in the hydrophobic domain of β-casein. Perhaps this result is not surprising as hydrophobic interactions are critical in protein–protein chaperone interactions, with a correlation between exposed hydrophobic sites and the chaperone-like activity of many chaperone proteins being observed (Lee et al., 1997; Bhattacharyya and Das, 1999; Manna et al., 2001). Molecular modelling of β-casein (Kumosinski et al., 1993) has proposed that the protein contains long hydrophilic arms, one of
those comprising proline-based turns between residues V84-Y119 which are solvent accessible. Experimental evidence is consistent with this proposal as plasmin in solution readily hydrolyses the bonds between residues K105-H106 and K107-E108 in β-casein (Eigel \textit{et al.}, 1984). Therefore, while residues G94-W143 are not exceptionally hydrophobic (\textbf{Table 3.1}) they are sufficiently exposed in solution to bind to unfolding reduced α-lactalbumin. In the tryptic digest of β-casein, the phosphopeptide fragments R1-R25 and R1-K28 in the highly polar N-terminal domain (\textbf{Figure 3.4B}) were present in the mass spectra of both native β-casein as well as the β-casein: reduced α-lactalbumin complex. Both spectra had these peaks with very similar intensities suggesting that the highly polar region is not responsible for binding to unfolding target proteins. While the anionic phosphopeptide R1-R25 has been shown to be important in β-casein’s chaperone ability (Koudelka \textit{et al.}, 2009; Yousefi \textit{et al.}, 2009), it does not seem to be involved in chaperone binding which is consistent with experiments performed by Matsudomi \textit{et al.}, (2004) whereby the phosphopeptide R1-R25 (derived from β-casein) showed no measureable level of suppression against ovotransferrin aggregation (Matsudomi \textit{et al.}, 2004). The peptide fragments V170-V209 (present in the tryptic digest (\textbf{Figure 3.4C})) and M144-V209 (from the chymotrypsin digest (\textbf{Figure 3.5})) did not show any statistical differences in their intensities between native β-casein and β-casein: reduced α-lactalbumin complex. The Q146-V209 region contains many apolar residues and few charged residues and as a result, the most hydrophobic portion of β-casein resides in the last C-terminal third of the protein (\textbf{Table 3.1}). It is interesting that the residues M144-V209 are not implicated in binding to unfolding α-lactalbumin considering hydrophobic interactions are important in assisting target protein interactions by molecular chaperones (Lee \textit{et al.}, 1997; Manna \textit{et al.}, 2001; Zhang \textit{et al.}, 2005), and in preventing the irreversible misfolding and aggregation of proteins by providing a hydrophobic surface to unfolding proteins (Bhattacharyya and Das, 1999). The important role that the C-terminal
region of β-casein plays in the aggregation of β-casein provides a rationale for its lack of interaction with reduced α-lactalbumin.

It is well established that β-casein undergoes self-association that is highly temperature dependent. At 0-4 °C, only monomers are observed, while as the temperature is increased β-casein undergoes a highly cooperative, reversible, rapidly equilibrating self-association, yielding large polymers with a narrow size distribution, similar to the formation of detergent micelles (Waugh et al., 1970; Andrews et al., 1979; Takase et al., 1980; Tai and Kegeles, 1984). Like detergent micelle formation, there is a critical micelle concentration above which micelles are formed, ranging from less than 0.5 mg/ml to 2 mg/ml depending on the temperature, ionic strength and pH (Evans et al., 1979). The hydrophobic domains of β-casein are proposed to associate to form the core of the polymer micelle (Swaisgood, 1992). Pearce (1975) demonstrated a blue shift in fluorescence when β-casein self associates at higher temperature, implying that Trp-143 is exposed in the monomer and is subsequently buried upon polymerization. It has been shown by NMR that upon association there is loss of flexibility in the hydrophobic C-terminal region which is packed toward the centre of the aggregate (Andrews et al., 1979). Furthermore, removal of the three C-terminal hydrophobic residues, Ile-Ile-Val, greatly reduces the association of β-casein (Thompson et al., 1967) while the removal of the last 20 amino acids, destroys the ability of the protein to associate and eliminates the binding of the hydrophobic surface probe, 8-anilino-1-naphthalene sulphonate (Berry and Creamer, 1975).

In this paper, complete suppression of reduced α-lactalbumin (1.5 mg/ml) at 25 °C was observed when incubated with 2.6 mg/ml of β-casein, corresponding to a 1:1 mole:mole ratio of α-lactalbumin:β-casein (Figure 3.1A). At this concentration, β-casein is above its critical micelle concentration. The β-casein regions between M144-V209 and V170-V209 observed
in the mass spectra from the addition of chymotrypsin and trypsin respectively to β-casein, were not involved in binding to unfolding reduced α-lactalbumin. Instead, this hydrophobic C-terminal region of β-casein is likely to be buried in the centre of the aggregate and therefore not available to bind to unfolding α-lactalbumin. However, once the β-casein complex was isolated by SEC the concentration of β-casein, approximately 0.03 mg/ml, would have favoured the monomer. β-Casein bound to reduced α-lactalbumin, and similarly native β-casein, would have its C-terminal region exposed to solution. As a consequence the β-casein peptides M144-V209 and V170-V209 following the addition of chymotrypsin and trypsin respectively to β-casein were observed in the mass spectra for both native β-casein and the β-casein: reduced α-lactalbumin complex. In addition, it has been suggested that the monomer may undergo a small conformational change to a more open structure (Pearce, 1975; Evans et al., 1979) and possibly further exposing the C-terminal region of β-casein to proteolysis by chymotrypsin and trypsin.

In summary, via a combination of limited proteolysis and mass spectrometry, the regions of β-casein directly involved in binding to an unfolding target protein, reduced α-lactalbumin, have been identified. The region of β-casein protected from chymotrypsin cleavage of the complex between the two proteins was determined to be G94-W143 consistent with the region protected from the enzyme Glu-C, i.e. A91-E121. The hydrophobic residues between M144-V209 were not involved in binding to destabilised and reduced α-lactalbumin. Instead, they are most likely to be involved in the hydrophobically driven self-association of β-casein polymers.
Chapter 4

The structural core within $\alpha_{s2}$- and $\kappa$-casein amyloid fibrils as identified by limited proteolysis and mass spectrometry.
4.1 Introduction

Amyloid and amyloid-related diseases result in the formation of insoluble fibrils that deposit, usually with high specificity, in different locations in the human body (Pepys, 2006). The various peptides and proteins associated with amyloid diseases have no obvious similarities in size, amino acid composition, sequence or structure. Nevertheless, the fibrils generated all contain a common cross-β structure, in which the core of the macromolecular structure is comprised of β-strands oriented perpendicular to the long axis of the fibril (Sunde and Blake, 1997). This structure gives rise to a characteristic cross formed by the meridional and equatorial reflections in X-ray fibre diffraction studies (Sunde and Blake, 1997). Many amyloid also have the ability to bind hydrophobic dyes such as thioflavin T or Congo red, as well as antibodies that bind to a common epitope (LeVine, 1999; Myers et al., 2006). However, amyloid fibril formation is not confined to disease-related proteins. Studies have shown that non-disease related proteins, regardless of their initial secondary and tertiary structure, can also be induced to form amyloid-like fibrils in vitro by judicious choice of the solution conditions (Guijarro et al., 1998; Chiti et al., 1999; Chiti et al., 2001a). This has led to the proposal that the amyloid state is a generic conformation accessible to all polypeptide chains under appropriate conditions (Dobson, 1999). Although it may be true that all proteins are capable of forming fibrils given the right conditions, naturally unfolded or intrinsically disordered proteins can do so under non-denaturing conditions at physiological pH and temperature (Thorn et al., 2009). Moreover, the tendency of a polypeptide chain to aggregate rather than fold correctly depends on a number of intrinsic factors, such as hydrophobicity, its propensity to form β-strand and its overall net charge (Taddei et al., 2001; Chiti et al., 2002).

Amyloid fibrils formed in vitro by αS2- and κ-casein have all the hallmarks of those associated with disease, for instance, they possess a β-sheet core structure and the ability to bind amyloidophilic dyes such as Thioflavin T (Thorn et al., 2005; Thorn et al., 2008). Unlike αs1-
and β-casein, which do not have the propensity to form fibrils, both αS2- and κ-casein possess disulfide linkages arising from the two cysteine residues in each molecule. The two-cysteiny l residues in αS2-casein form either a monomer or dimer (as a result of intramolecular and intermolecular disulphide bonds respectively), which are present in milk in equal proportions (Rasmussen et al., 1992a; Rasmussen et al., 1994). In κ-casein the cysteine residues are randomly cross-linked by intermolecular disulphide bonds resulting in the formation of polymers, from monomers to multimeric structures larger than decamers (Rasmussen et al., 1992b). Reduction and subsequent carboxymethylation of native κ-casein, i.e. leading to monomeric κ-casein, has an inherent susceptibility to form fibrils at physiological pH and temperature (Farrell et al., 2003; Thorn et al., 2005). Native κ-casein also has the propensity to form fibrils under physiological conditions, albeit at a slower rate (Thorn et al., 2005), which is not surprising, since reduction and carboxymethylation of the protein does not significantly alter its secondary or tertiary structure (Farrell et al., 2003). While both αS2- and κ-casein have an inherent susceptibility to fibril formation at physiological conditions in milk, this is kept in check by the chaperone action of the other caseins (αs1- and β-casein), which are able to inhibit their fibrillation (Thorn et al., 2005; Thorn et al., 2008).

Of physiological relevance, amyloid-like plaques displaying many of the hallmarks of amyloid are, on occasion, observed extracellularly within the proteinaceous deposits or inclusions of mammary corpora amylacea (CA) (Reid, 1972; Beems et al., 1978; Taniyama et al., 2000). Histologically, the majority (90%) of CA is located within the alveolar lumen (Nickerson et al., 1985), where milk is stored, and subsequently secreted into the ducts through ductules. Amyloid-like deposits have also been identified in the stroma and within the cytoplasm of mammary epithelial cells surrounding the CA, which are involved in the synthesis and secretion of caseins and other milk constituents (Reid, 1972; Nickerson et al., 1985). Although the identity of the proteins forming these fibrillar structures is not explicitly
known, the prevalence of CA within the epithelial cells (where milk is synthesised) and the alveolar lumen (where milk is stored) points to casein proteins as the likely candidates (Brooker, 1978; Nickerson et al., 1985). Immunoblotting and amino acid sequence analysis obtained from mammary CA have suggested that several milk proteins, including caseins are present (Claudon et al., 1998; Niewold et al., 1999). In addition, Niewold et al., (1999) isolated a group of peptide fragments derived from αS2-casein, from amyloid deposits associated with mammary CA (Niewold et al., 1999). The isolated peptide fragments, designated as AαS2C, were 32 to 45 residues in length and starting from Ala81. Therefore, the fibrils associated with CA may result from the incapacity of other casein proteins to prevent αs2- and κ-casein fibril formation. Moreover, because of the link between casein amyloid fibril formation, corpora amylacea, and the intracellular fibril-like bundles identified in cells surrounding these deposits a more detailed understanding of the mechanism by which αs2- and κ-casein forms fibrils is required.

Understanding the mechanism of aggregation into amyloid fibrils requires knowledge of the structure of the amyloid precursor, the nature and identity of any assembly intermediates, and the structure of the fully formed amyloid fibrils themselves (Myers et al., 2006). In the absence of a complete understanding of the general formation of amyloid structure, i.e. how peptides and proteins with dissimilar native folds are capable of accessing a common amyloid folding motif, local structural information on protein and disease-specific amyloid fibrils remains critical (Wilson et al., 2007). Over the last few years, the nature of the amyloid precursor conformation for a number of amyloidogenic proteins has been elucidated using approaches spanning bioinformatics, peptide libraries, and NMR spectroscopy (Chiti et al., 2001b; Kelly, 2002; Jahn and Radford, 2005; Platt et al., 2005). Limited proteolysis in conjunction with mass spectrometry has also been used on fibrillar apolipoprotein C-II, α-synuclein, and β2-microglobulin to identify their β-sheet core regions (Myers et al., 2006; Qin
et al., 2007; Wilson et al., 2007). Subsequent synthesis/expression of these β-sheet peptide regions indicates that they readily form amyloid fibrils (Qin et al., 2007; Wilson et al., 2007). Therefore, these peptides have been postulated to drive fibril formation in their corresponding full-length proteins (Qin et al., 2007; Wilson et al., 2007). Herein we also use proteolysis and mass spectrometry to identify the protease-resistant core regions of αs2- and κ-casein fibrils. Using trypsin and proteinase K we determined that residues from Tyr25 to Lys86 were incorporated into the β-sheet core of κ-casein fibrils. Digestion of αs2-casein fibrils with trypsin indicated a protease-resistant core between Ala81 to Lys181. These protease-resistant core regions within αs2- and κ-casein may underlie the propensity of αs2- and κ-casein and their peptide derivatives to accumulate in amyloid deposits in vivo.

4.2 Materials and methods.
Bovine αs-casein and κ-casein (catalogue no. C6780 and C0406, respectively) were purchased from Sigma Aldrich (St Louis, Missouri). Snakeskin pleated dialysis tubing with 3.5 kDa cut-off was obtained from Pierce Chemical Company (Rockford, Illinois). The thioflavin T (ThT), and 1,4-dithiotheritiol (DTT) were purchased from Sigma Aldrich. All other chemicals were of reagent grade, and unless otherwise stated, all solutions were prepared with MilliQ water.

4.2.1 In situ THT assay
Reduced and carboxymethylated (RCM) κ-casein was prepared by adapting the experimental procedure used by Schechter et al., (1973). The formation of amyloid fibrils by RCM κ-casein was monitored using an in situ ThT binding assay method adopted from Nielsen et al., (2001). ThT binds selectively to anti-parallel β-pleated sheets, the major structural element of amyloid fibrils, and fibril formation is therefore accompanied by an increase in ThT fluorescence emission at approximately 490 nm (LeVine, 1999). RCM κ-casein (1 mg/mL)
was incubated at 37 °C for 20 h in 50 mM ammonium bicarbonate buffer (pH 7.4). Samples were prepared in duplicate and were incubated with a final concentration of 10 µM ThT in a 96-microwell plate. The plates were sealed to prevent evaporation, and the fluorescence was measured with a Fluostar Optima plate reader (BMG Labtechnologies) with a 440 nm excitation/490 nm emission filter set.

4.2.2 TEM (Transmission Electron Microscopy)
Samples for TEM were prepared by adding 5 µL of protein solution to Formvar and carbon-coated nickel grids (SPI Supplies, West Chester, PA). The grids were then washed 3 times with 10 µL of water and negatively stained with 10 µL of uranyl acetate (2% (w/v); Agar Scientific, U.K.). The grids were dried with Whatman filter paper between each step. The samples were viewed under 25 000-64 000 magnifications at 80 kV excitation voltages using a Philips CM100 transmission electron microscope (Philips, The Netherlands). Error bars were inserted using SIS Image Analysis software.

4.2.3 Proteolytic Cleavage of Amyloid Fibrils Formed by RCM κ-CN
Prior to proteolysis, RCM κ-CN fibrils were formed by incubating in 50 mM ammonium bicarbonate buffer, pH 7.4, overnight at 37 °C, with the presence of fibrils confirmed by TEM. Limited proteolysis experiments were carried out by incubating RCM κ-casein and fibrillar RCM κ-casein with either trypsin or proteinase K at 37 °C using an enzyme-to-substrate ratio of 1:100 and 1:1000 (w/w) for trypsin and proteinase K respectively. The presence of amyloid fibrils following proteolysis was confirmed by TEM. At the end of the assay, an aliquot of each sample was taken for SDS-PAGE analysis, and the remainder was used for MALDI-TOF MS analysis. For SDS-PAGE, aliquots were removed at various time intervals, and the reaction was stopped by mixing with an equal volume of reducing sample buffer. These samples were then heated to 95 °C for 5 min before being loaded onto gels.
Proteolysis products were analysed using 15 % acrylamide gels (v/v) and standard techniques (Laemmli, 1970).

4.2.4 Mass Spectrometry
An Ultraflex III MALDI-TOF/TOF mass spectrometer (Bruker Daltonics, Bremen, Germany) was used for the analysis of intact protein and proteolytic cleaved peptides. The instrument was used in linear mode for peptides greater than 6000 Da and in reflectron mode for smaller peptides. Linear mode was calibrated using protein calibration standard I (Bruker Daltonics). The protein mixture enables calibrations and testing in a mass range of m/z 3000 to 25,000 with an error of approximately 200 ppm. Reflector mode was calibrated using peptide calibration standard II (Bruker Daltonics), which enables calibrations and testing in a mass range of 500 to 4000 Da to a mass accuracy greater than 50 ppm. Aliquots taken for analysis by mass spectrometry were first mixed with urea to a final concentration of 6 M and left overnight at room temperature in order to disaggregate the fibrils. The sample was then mixed with trifluoroacetic (TFA) acid to a final concentration of 0.1% (v/v), acidified with HCl (final pH < 4), before being taken up in a reverse phase column ZipTipC-18 (Millipore, Billerica, MA). After washing with acetonitrile (5%, v/v), containing 0.1% (v/v) trifluoroacetic acid (TFA) multiple times to remove any salt and urea present, the sample was then stepwise eluted with increasing concentrations of acetonitrile (20, 30, 40, 50, and 60% (v/v) in 0.1% (v/v) TFA). The proteinase-resistant peptide of interest was eluted with 50 and 60% (v/v) acetonitrile. Samples were analysed by MALDI-TOF MS in linear mode using 2,5-dihydroxybenzoic acid as the matrix (5 g/L) in 50% (v/v) acetonitrile with 0.1% (v/v) TFA. To confirm that this peptide matched the protease-resistant band identified by SDS-PAGE, a tryptic in-gel digest was performed according to the method of Shevchenko et al., (1996) with the following modifications. The Coomassie Blue-stained bands from the gel were cut with a sterile scalpel blade into small blocks and destained using 50mM ammonium bicarbonate in
30% (v/v) acetonitrile. The blocks were rinsed and incubated overnight at 37 °C with 10 ng/µl trypsin in 5 mM ammonium bicarbonate. The tryptic fragments were extracted from the gel pieces by sonicating the samples for 15 min with 50% (v/v) acetonitrile with 0.1% (v/v) trifluoroacetic acid. The extracted peptide solution was then dried down to a final volume of ~5 µl using a vacuum centrifuge. The tryptic peptides were analysed directly by MALDI-TOF MS in reflector mode using α-cyano-4-hydroxycinnamic acid (Bruker Daltonics) as the matrix. The identity of these peptides was confirmed by matching their observed masses to the expected masses of the in silico tryptic digest of the known κ-casein sequence using Biotools 3.0 software (Bruker Daltonics).

4.2.5 Purification of αs2-casein monomer and dimer
Whole αs-casein obtained from Sigma was separated into three major components by cation-exchange chromatography using a Mono S 5/50 GL column (Amersham Biosciences, UK) according to a method described by Rasmussen et al., (1994), with minor modifications. The column was equilibrated with 50 mM ammonium acetate-8 M urea (pH 7.0) and eluted with a linear gradient of 1 M ammonium acetate-8 M urea (pH 7.0). The eluted proteins were dialysed at 4°C against 20 mM ammonium bicarbonate for six days, replacing the dialysis buffer twice daily. The dialysate was then lyophilised.

4.2.6 Conventional ThT assays
The low binding of ThT to αs2-casein fibrils at pH 6.8, i.e the physiological pH of milk (Fox and McSweeney, 2003) and the optimal pH for αs2-casein fibril formation (Thorn et al., 2008), precluded the use of in situ assays. As an alternative, 10 µL aliquots were periodically withdrawn from stock protein solutions that were incubated without shaking at 37 °C and then snap frozen at -20 °C. Freezing had no affect on fibril morphology as noticed previously (Thorn et al., 2008). At the completion of the time-course, each sample was thawed and then
mixed with 1.6 mL of 10 µM ThT in 50 mM glycine buffer, raised to pH 9.0 with NaOH. The fluorescence was then measured using a glass cuvette (10 mm light path) and a Cary Eclipse spectrofluorimeter (Varian) with the excitation and emission wavelengths set at 442 and 485 nm, respectively.

4.2.7 Proteolysis experiments

Prior to proteolysis, dimeric αs2-casein fibrils were formed by incubating in 50 mM phosphate buffer, pH 6.8, for a week at 37 °C, with the presence of fibrils confirmed by TEM. Limited proteolysis experiments were carried out by incubating native and fibrillar dimeric αs2-casein with trypsin at 37 °C using an enzyme-to-substrate ratio of 1:100 (w/w). At various time intervals, an aliquot of the digest was taken for SDS-PAGE analysis and mixed with an equal volume of non-reducing sample buffer and then snap frozen at -20 °C. At completion of the experiment these samples were then heated to 95 °C for 5 min before being loaded onto 15 % gels. A tryptic in-gel digest was performed on protease-resistant bands identified by SDS-PAGE and the peptides analysed using an Ultraflex III MALDI-TOF mass spectrometer in reflector mode as described above.

The MALDI TOF mass spectrometer was also used in linear mode for the analysis of intact proteins. Aliquots of native and fibrillar αs2-casein were taken following digestion with trypsin and an equal volume of 99.8 % acetonitrile/ 0.2% TFA was added to quench the trypsin. The samples were then sonicated for 5 mins to disaggregate remaining fibrils. Samples were further diluted 1/10 with 0.1% TFA before being analysed by MALDI-TOF MS in linear mode using the dried droplet in sinapinic acid (10 g/L) in 90% (v/v) acetonitrile with 0.1% (v/v) TFA as described by Megan et al., (2009).
4.3 Results

4.3.1 Formation of RCM κ-casein fibrils

RCM κ-casein (1 mg/mL) was incubated at 37 °C overnight in 50 mM ammonium bicarbonate (pH 7.4) and 10 µM ThT. A sigmoidal increase in fluorescence at 490 nm was observed which reached a plateau after 16 hours (Figure 4.1A). The presence of fibrils in the sample was confirmed by TEM (Figure 4.1B).

4.3.2 Identification of the protease-resistant core region of fibril formed from RCM κ-casein

In order to identify the core region of these fibrils, proteolysis experiments were performed on native and fibrillar forms of the protein. In the absence of trypsin, RCM κ-CN derived from fibrils gave rise to a band in the SDS-PAGE gel arising from the full-length protein (19 kDa) (Figure 4.2A). The addition of trypsin to native RCM κ-casein (at a 1:100 (w/w) ratio of trypsin: RCM κ-casein) resulted in its rapid proteolysis after two hours, generating small peptide fragments of less than 4 kDa in mass (Figure 4.2A). In contrast, fibrillar RCM κ-casein was much more resistant to degradation, such that even following four hours of incubation with trypsin, a protease-resistant peptide of approximately 6 kDa was evident by SDS-PAGE. Following proteolysis with trypsin, fibrils with a similar morphology to those present before digestion were observed by TEM (panels B and C of Figure 4.2). Similar results were obtained when proteinase K, at an enzyme-to-substrate ratio of 1:1000 (w/w), was used (Figure 4.3A). Thus, native RCM κ-casein was degraded by proteinase K into small peptide fragments, whereas the fibrillar form was resistant to proteolytic degradation. After 2 h of incubation of fibrillar RCM κ-CN with proteinase K, four peptide bands were observed by SDS-PAGE at 18, 15, 10, and 8 kDa. Following four hours of digestion with proteinase K, amyloid fibrils were still present, as monitored by TEM (panels B and C of Figure 4.3).
We identified the protease-resistant core region of these amyloid fibrils by mass spectrometry. Since digestion with trypsin produced a small, single protease-resistant peptide fragment compared with digestion with proteinase K, we used this treatment in order to identify the protease-resistant core. The trypsin-treated fibrils were disaggregated with 6M urea overnight. The urea and salt was removed using a C-18 reverse phase ZipTip column and the peptide fragment(s) were separated using increasing concentrations of acetonitrile as described in the methods. The resultant peptide was subjected to MALDI-TOF MS in linear mode and gave rise to a [M+H]⁺ ion at approximately 7207.6 ± 1.4Da in the spectrum (Figure 4.4A); thus the mass approximated that of the peptide identified by SDS-PAGE (Figure 4.2A). A number of smaller tryptic fragments were also identified that matched fragments generated when the native form of the protein was treated with trypsin (data not shown). Based on its mass, the identity of this tryptic fragment could not be unambiguously assigned; it could correspond to the κ-casein fragment (25-86) or (35-97) (Table 4.1). We confirmed the identity of the peptide by performing in-gel tryptic digestion of the band identified by SDS-PAGE and analysing the fragments by MALDI-MS and MS/MS. Three peaks with an approximate m/z of 4009.9, 1980.0, and 1251.7 (Figure 4.4B) were identified and were matched to the κ-casein tryptic fragments Tyr³⁵-Arg⁶⁸, Ser⁶⁹-Lys⁸⁶, and Tyr²⁵-Arg³⁴ respectively (Table 4.1), with the sequence identity of these in-gel tryptic fragments confirmed by tandem mass spectrometry (supplementary data, Appendix 7.4A-C). Thus, the intact tryptic fragment corresponded to the κ-casein peptide Tyr²⁵-Lys⁸⁶.

We also confirmed that the same region of the protein was resistant to proteolytic degradation by proteinase K by performing an in-gel digest of the bands identified by SDS-PAGE (Figure 4.3A) and analysing the fragments by MALDI-MS (panels A and B of Figure 4.5). Five peaks and four peaks were identified for the 10 kDa and 8 kDa bands, respectively. Peaks at m/z 4009.9, 3515.7 2872.4, 2188.2, 1980.0, and 1251.7 (panels A and B of Figure 4.5) were
matched to the tryptic fragments Tyr$^{35}$-Arg$^{68}$, Tyr-Lys, Ser$^{87}$-Lys$^{111}$, Phe$^{17}$-Arg$^{34}$, Ser$^{69}$-Lys$^{86}$, and Tyr$^{25}$-Arg$^{34}$ respectively (Table 4.1, Appendix 7.4A-D). Therefore, the 10 and 8 kDa fragments correspond to the κ-casein peptides Phe$^{17}$-Lys$^{111}$ and Phe$^{17}$-Lys$^{86}$, respectively. The region identified by trypsin proteolysis of the fibrils (Tyr$^{25}$-Lys$^{86}$) is encompassed within these two peptides. Similar tryptic fragments were identified from the 18 and 15 kDa bands in Figure 4.3A; therefore, these higher molecular mass bands incorporate the same protease-resistant region of κ-casein and represent larger peptides that were not fully digested by the proteinase K treatment. Figure 4.6 highlights the protease-resistant core as well as key regions of κ-casein with regard to its amyloid fibril-forming ability (see discussion).

Acknowledgement must be given to Heath Ecroyd who performed the majority of proteolysis and SDS-PAGE experiments that were subsequently used for mass spectrometry experiments. SDS-PAGE gel used in Figure 4.3A was performed by Heath Ecroyd and taken from (Ecroyd et al., 2008) with permission.

### 4.3.3 Purification of α$_{s2}$-casein monomer and dimer

Whole α$_{s}$-casein was separated into three major components, α$_{s1}$-casein, monomeric and dimeric α$_{s2}$-casein (due to intra- and interchain disulfide linkages respectively, which exist in equal proportions (Rasmussen et al., 1992a; Rasmussen et al., 1994)) by cation-exchange chromatography (Figure 4.7A). A broad peak corresponding to the predominant α$_{s1}$-casein component eluted at 50 mM ammonium acetate-8M urea, as observed previously (Rasmussen et al., 1994; Thorn et al., 2008). Once the α$_{s1}$-casein peak trailed off, a linear gradient up to 35% eluent (1 M ammonium acetate-8 M urea) allowed the elution of monomeric and dimeric α$_{s2}$-casein. A peak containing peptide fragments and possibly other contaminants (e.g., β-casein) was also observed before the elution of α$_{s1}$-casein (elution volume of 2 mL). Analysis of each fraction and taking into consideration the relative extinction coefficients of α$_{s1}$ and α$_{s2}$ at 280 nm resulted in a molar ratio of 4:1 respectively, consistent with the proportion
observed in bovine milk (Karman and Van Boekel, 1986). SDS-PAGE analysis of the αs2-casein fractions showed that monomeric and dimeric αs2-casein was successfully separated by the purification process (Figure 4.7B). On the basis of their electrophoretic mobility, the dimeric form of the protein migrated predominantly as a single band of 50 kDa, while the monomer migrated with a mass of 25 kDa.

4.3.4 Formation of dimeric αs2-casein fibrils

While whole αs2-casein forms fibrils (Thorn et al., 2008), the purified dimer was used instead of whole αs2-casein for subsequent experiments as (1) to reduce the complexity of proteolysis products and (2) preliminary results suggest that the disulphide-linked dimer, rather than the monomer, is the predominant amyloidogenic species (D. Thorn, unpublished results). In support of this, under conditions that reduce disulphide bonds, fibril formation of whole αs2-casein is inhibited (Thorn et al., 2008). Dimeric αs2-casein was incubated at 37 °C and monitored using a conventional ThT assay (Figure 4.8A). Fibril formation started to plateau after approximately one week (170 hrs) with fibril formation confirmed using TEM (Figure 4.8B). The fibrils had a tangled, twisted-ribbon morphology that had a tendency to circularise and form closed loop structures very similar to those of apolipoprotein C-II fibrils (Hatters et al., 2000).

4.3.5 Identification of the protease-resistant core region of dimeric αs2-casein fibrils

In order to identify the core region of these fibrils, proteolysis experiments were performed on native and fibrillar forms of the protein. In the absence of trypsin, dimeric αs2-casein derived from fibrils was identical to native (non-fibrillar) dimeric αs2-casein and both gave an identical migration pattern on SDS-PAGE, with several protein bands observed between 54 and 41 kDa (Figure 4.9A). The addition of trypsin to native dimeric αs2-casein (1:100 (w/w) ratio of trypsin: αs2-casein) resulted in its rapid proteolysis after two hours, generating small
peptide fragments less than 4 kDa in mass (Figure 4.9A). In contrast, fibrillar $\alpha_{s2}$-casein was much more resistant to degradation such that, even following four hours of incubation with trypsin, two protease-resistant peptides, a predominant band at 9 kDa and another at 5 kDa, were still evident by SDS-PAGE (denoted with an asterisk). Unlike $\kappa$-casein, the morphology of $\alpha_{s2}$-casein fibrils changed significantly over the duration of digestion (as observed by TEM, panels B-F of Figure 4.9). While a long, twisted-ribbon morphology was observed for $\alpha_{s2}$-casein fibrils after 30 mins of incubation with trypsin, only amorphous aggregates and very short, straight fibrils, 50-100 nm in length, were observed after four hours of incubation with trypsin (panels B-F of Figure 4.9).

The identity of the two peptides giving rise to the major bands in the SDS-PAGE gel were confirmed by performing an in-gel tryptic digestion and analysing the fragments by MALDI-TOF MS and MS/MS. The high mass fragment (~9 kDa) observed in the gel gave rise to five peaks at 2725.5, 1762.0, 1633.9, 1367.7 and 979.5 Da (Figure 4.10A, Appendix 7.4E-I). These peptides were matched to the $\alpha_{s2}$-casein tryptic fragments Phe$^{92}$-Lys$^{113}$, Leu$^{153}$-Lys$^{166}$, Leu$^{153}$-Lys$^{165}$, Ala$^{81}$-Lys$^{91}$, and Phe$^{174}$-Lys$^{181}$ respectively, with the sequence identity of these in-gel tryptic fragments confirmed by tandem mass spectrometry (Table 4.2). Thus, the high mass fragment (~9 kDa) corresponds to the tryptic fragment Ala$^{81}$-Lys$^{181}$. The lower mass band (~5 kDa) gave rise to five peaks at $m/z$ 1863.096, 1762.032, 1610.757, 1247.663 and 979.574 (Figure 4.10B). These peaks were identified and matched to the $\alpha_{s2}$-casein tryptic fragments Thr$^{151}$-Lys$^{165}$, Leu$^{153}$-Lys$^{166}$, Thr$^{138}$-Lys$^{150}$, Thr$^{151}$-Arg$^{160}$, and Phe$^{174}$-Lys$^{181}$ respectively, with the sequence identity of these in-gel tryptic fragments confirmed by tandem mass spectrometry (Table 4.2, Appendix 7.4E-J). Thus, the lower fragment corresponds to tryptic fragment Thr$^{138}$-Lys$^{181}$. Therefore, the 9 and 5 kDa fragments correspond to $\alpha_{s2}$-casein peptides Ala$^{81}$-Lys$^{181}$ and Thr$^{138}$-Lys$^{181}$. 
In addition, we also tried to identify the protease-resistant core region of αs2-casein fibrils, by
disaggregating the fibrils following four hours of proteolysis with trypsin (similar to experiments performed on RCM κ-casein). RCM κ-casein fibrils were disaggregated with 6M urea and purified using a C-18 reverse phase ZipTip. The resultant peptide was subjected to MALDI-TOF MS in linear mode and gave rise to a [M+H]+ ion at 7207.7 Da in the spectrum (Figure 4.4A); thus the mass matched that of the peptide identified by SDS-PAGE (Figure 4.2A). The same experiments were performed on αs2-casein fibrils following proteolysis (disaggregation with urea, etc.) however, a resultant peptide was not observed. Instead, after four hours of proteolysis of αs2-casein fibrils (and native, non-fibrillar αs2-casein) with trypsin, samples were quenched and disaggregated by adding an equal volume of 99.8% ACN/0.2 % TFA and sonicating for 5 mins. Samples were further diluted with 0.1 % TFA and the resultant peptides were analysed by MALDI-TOF MS in linear mode using a sinapinic acid/dried droplet method as described in the methods. Unfortunately, the spectra did not give rise to peaks that matched the mass fragments observed by SDS-PAGE (~9 and 5 kDa in mass, Figure 4.9A). Instead, the spectra of both native and fibrillar αs2-casein gave rise to various peaks at approximate m/z values of 4216.8, 4237.2, 4295.1, 4362.1 and 4843.9 (Figure 4.11). The identity of these peptides was confirmed by matching their observed masses to the expected masses of the in silico tryptic digest of the known αs2-casein sequence using Biotools 3.0 software (Bruker Daltonics). The peaks were matched to the αs2-casein tryptic fragments Ala81-Lys113, Ala81-Arg114, Asn46-Lys80, Asn115-Lys150, Glu126-Arg160, Leu153-Lys190, respectively (Table 4.2). However, a difference between the two spectra was observed. For example, a peak at m/z 4059.9, corresponding to residues from Ala81 to Lys113, was observed in the fibrillar αs2-casein spectrum but was absent in the spectrum of native αs2-casein (Figure 4.11). In addition, different peak intensities were observed for native and fibrillar αs2-casein with the same m/z ratio. For example, the peak at m/z ratio of 4216.8, corresponding to Ala81-Lys114, which was the most intense peak in fibrillar αs2-casein, was not
very intense in native αs2-casein. In contrast, the peak at \( m/z \ 4237.2 \), corresponding to residues Asn\textsuperscript{46}-Lys\textsuperscript{80}, was the most intense peak in native αs2-casein, and not very intense in fibrillar αs2-casein (Figure 4.11). The difference in peak intensities between native and fibrillar αs2-casein suggests that at least Ala\textsuperscript{81}-Lys\textsuperscript{113}, and Ala\textsuperscript{81}-Lys\textsuperscript{114} are somewhat protected from proteolysis by trypsin in fibrillar αs2-casein, compared to native αs2-casein. Unfortunately, peaks corresponding to peptide fragments observed by SDS-PAGE (~9 and 5 kDa, Figure 4.9A) were not observed in the spectrum of fibrillar αs2-casein (Figure 4.11). This may be a result of the poor ionisation of these large phosphorylated peptides and/or the conditions used to disaggregate the fibrils, i.e. urea, and ACN/TFA conditions, were not sufficient. Acknowledgement must be given to David Thorn for performing the majority of proteolysis experiments and all the SDS-PAGE experiments that were subsequently used for mass spectrometry experiments. The SDS-PAGE gel used in Figure 4.9A was performed by David Thorn.
Figure 4.1: ThT fluorescence analysis of RCM κ-casein (1 mg/ml) fibril formation in 50 mM ammonium bicarbonate buffer (pH 7.4) and incubated at 37 °C. A.U., arbitrary units (A). Electron micrograph of RCM κ-casein following 20 h of incubation (B). Scale bar represents 200 nm.
Figure 4.2: SDS-PAGE analysis of native (N) and fibrillar (F) RCM κ-casein following treatment with trypsin for 0, 2 and 4 h (A). The position of molecular weight markers (M) is shown. Transmission electron micrographs of fibrillar RCM κ-casein before (B) and after (C) incubation with trypsin for 4 h. Scale bars represent 200 nm.
**Figure 4.3:** SDS-PAGE analysis of native (N) and fibrillar (F) RCM κ-casein following treatment with proteinase K for 0, 0.5, 1 and 2 h (A). The position of molecular weight markers is shown on the left. The 10 and 8 kDa bands identified by mass spectrometry (shown in **Figure 4.5**) are denoted by an asterisk. Transmission electron micrographs of fibrillar RCM κ-casein before (B) and after (C) incubation with proteinase K for 2 h. Scale bars represent 500 nm.
Figure 4.4: MALDI-TOF MS spectrum of peptide fragments present following treatment of RCM κ-CN fibrils with trypsin and their subsequent disaggregation and elution with 60% ACN/ 0.1% TFA using ZipTip clean-up (A). MALDI-TOF MS spectrum of the in-gel digest obtained from the Coomassie Blue-stained SDS-PAGE band (Figure 4.2A) that was identified following treatment of RCM κ-CN fibrils with trypsin (B). A.U., arbitrary units.
Figure 4.5: MALDI-TOF MS spectrum of the in-gel digest obtained from the 10 kDa (A) and 8 kDa (B) Coomassie Blue-stained SDS-PAGE bands (in Figure 4.3A, denoted with an asterisk) that were identified following treatment of RCM κ-CN fibrils with proteinase K. Arb.U., arbitrary units.
Table 4.1: Identification of peptide fragments of RCM κ-casein by time of flight mass spectrometry (adapted from (Ecroyd et al., 2008))

NOTE:
This table is included on page 101 of the print copy of the thesis held in the University of Adelaide Library.
Figure 4.6: A summary of the key regions of κ-casein with regard to its amyloid fibril-forming ability (taken from (Ecroyd et al., 2008) with permission). The diagram indicates the regions of the protein predicted to form β-sheet by three-dimensional modeling (Kumosinski et al., 1993), the protease-resistant fibrillar core fragments generated by digestion with proteinase K and trypsin and identified by mass spectrometry, the region of the protein predicted to be ordered by the algorithms PONDR (Romero et al., 2001) and FoldIndex (Uversky et al., 2000; Prilusky et al., 2005), the most amyloidogenic regions of the protein as predicted by TANGO (Fernandez-Escamilla et al., 2004) and a modified version of Zyggregator (Pawar et al., 2005).
**Figure 4.7:** Purification of monomeric and dimeric $\alpha_{s2}$-casein. Elution profile of $\alpha_s$-casein separated by cation-exchange chromatography. Proteins were eluted using a linear gradient (dotted line) of 1 M ammonium acetate-8 M urea (pH 7.0) on a Mono-S column (A). SDS-PAGE analysis of monomeric (middle lane) and dimeric $\alpha_{s2}$-casein (left lane) fractions obtained from cation-exchange chromatography (B). Molecular weight standards are shown on the right.
Figure 4.8: ThT fluorescence analysis of dimeric α_{s2}-casein (3 mg/mL) fibril formation in 50 mM phosphate buffer (pH 6.8) incubated at 37 °C. A.U., arbitrary units (A). Fibril formation was confirmed using TEM (B). Scale bar represents 200 nm.
**Figure 4.9:** SDS-PAGE analysis of native (N) and fibrillar (F) dimeric κ_{s2}-casein following treatment with trypsin for 0, 0.5, 1, 2 and 4 h (A). The two protease resistant bands observed after four h of incubation with trypsin, which were subsequently analysed using mass spectrometry (Figure 4.10) are denoted with an asterisk. Molecular weight markers (M) are also shown. Transmission electron micrographs (B-F) represent fibrillar κ_{s2}-casein after 0, 30, 60, 120 and 240 mins of incubation with trypsin, respectively. Scale bars represent 500 nm. Arrows point towards short fibrils.
Figure 4.10: MALDI-TOF MS spectrum of the in-gel digest obtained from the top (A) and bottom (B) Coomassie Blue-stained SDS-PAGE bands (denoted with an asterisk in Figure 4.9A) that were identified following treatment of α-s2-casein fibrils with trypsin for 4 h.
Peptides confirmed by tandem mass spectrometry are denoted by an *asterisk* (Appendix 4.3). Peptides in blue contain a post-translational modification. Arb.U., arbitrary units.

**Figure 4.11**: MALDI-TOF MS spectra of peptide fragments present following treatment of native (top) and fibrillar (bottom) αs2-casein with trypsin for 4 hrs and disaggregation with equal volume of 99.8% ACN/ 0.2% TFA and sonication as described in the methods. Only peaks that were matched to an *in silico* tryptic digest of αs2-casein using Biotools 3.0 are shown. Arb.U., arbitrary units.
Table 4.2: Identification of peptide fragments of dimeric α_{s2}-casein by time of flight mass spectrometry

<table>
<thead>
<tr>
<th>Peptide source and matching tryptic fragment</th>
<th>Expected mass [M+H]^+</th>
<th>Observed mass [M+H]^+</th>
<th>Intensity (%)</th>
<th>Deviation (ppm)</th>
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<td>Trypsin-treated disaggregated fibrils(^a)</td>
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<tr>
<td>Leu(^{153})-Lys(^{190})</td>
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<td>4843.9</td>
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<td>In-gel digest of top band SDS-PAGE band(^b)</td>
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<tr>
<td>Ala(^{81})-Lys(^{91})</td>
<td>1367.69</td>
<td>1367.7(^c)</td>
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<td>2725.5(^c)</td>
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<td>1762.0(^c)</td>
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<td>Phe(^{174})-Lys(^{181})</td>
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<td>979.5(^c)</td>
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<tr>
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<td>979.5(^c)</td>
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\(^a\)Data obtained in linear mode and represents average masses of the [M+H]^+ ions in Da.
\(^b\)Data obtained in reflector mode and represents monoisotopic masses of the [M+H]^+ ions in Da.
\(^c\)The sequence identity of these peptides were confirmed using tandem mass spectrometry (Appendix 4.3).
\(^d\)Includes either/both oxidation of methionine to sulfoxide or/and phosphorylation of oxygen on serine, with a subsequent mass change of 16 and 80 Da, respectively.
4.4 Discussion

4.4.1 RCM κ-casein’s protease-resistant core

Fibrillar RCM κ-casein was much more resistant to proteolysis than native RCM κ-casein. Incubation of fibrillar RCM κ-casein with trypsin led to a protease-resistant fragment between residues Tyr\(^{25}\)-Lys\(^{86}\) (Table 4.1), while incubation with proteinase K led to protease-resistant fragments of various sizes (Figure 4.3A), the two smaller ones comprising residues Phe\(^{17}\)-Lys\(^{86}\) and Phe\(^{17}\)-Lys\(^{111}\) (Table 4.1), which encompass the same region identified by trypsin proteolysis. Interestingly, treatment with trypsin, a relatively specific protease, with cleavage on the C-terminal side of lysine and arginine residues, cleaved closer to the core of the amyloid fibrils than proteinase K, a relatively nonspecific protease, with cleavage at the carboxyl group of aliphatic and aromatic amino acids. This may reflect differences in the residue accessibility of the two proteases (due to packing of the fibrils) or due to variations in their size (proteinase K is a larger protease), mode of action or proteolysis conditions (proteinase K was ten times more dilute (w/w ratio)).

Proteolysis experiments indicate that the region of RCM κ-casein from Tyr\(^{25}\) to Lys\(^{86}\) represents the protease-resistant β-sheet core of amyloid fibrils formed by κ-casein. Thus, a significant portion of the full-length protein is protected from proteolysis and is of similar length to what has been reported for other amyloid fibril forming proteins. For example α-synuclein (residues from 32-102 were found to be resistant to degradation by trypsin and endoproteinase GluC (Qin et al., 2007)) and β\(_2\)-microglobulin (residues 10–99 were found to be resistant to degradation by pepsin (Myers et al., 2006)). Since the protease-resistant region of RCM κ-casein is relatively long and continuous, it is highly likely that a single κ-casein monomer may contribute more than one β-strand to the fibril structure, in a similar manner to that shown by α-synuclein (Qin et al., 2007), amyloid Aβ-peptide (1-40) (Petkova et al., 2002) and the C-terminal fragment of the HET-s protein (Ritter et al., 2005).
κ-Casein, as with all the caseins, has been classified as an intrinsically disordered protein (Dunker et al., 2002; Syme et al., 2002) and is envisaged as being extremely dynamic, essentially unfolded with some regular secondary structure, with interactions between remote parts of the sequence expected to be minimal and many of the side chains to have conformational flexibility (Syme et al., 2002). Various spectroscopic techniques (i.e. circular dichroism, Raman and Fourier transform infra-red) suggest that the overall secondary structure of κ-casein includes considerable β-sheet (35%-40%, (Farrell et al., 1996; Farrell et al., 2002)) with much of it predicted to reside between residues 22 and 79 (Creamer et al., 1998). Disorder-predicting algorithms (VL-XT algorithm using PONDR, (Romero et al., 2001)) indicate that, although almost half of the residues in the protein are predicted to be disordered (44%), the region Lys^{24}–Pro^{64} is the most likely to adopt an ordered conformation (Ecroyd et al., 2008) (Figure 4.6). A similar region of the protein (Tyr^{30}–Gln^{89}) was predicted to fold into a defined conformation using the FoldIndex algorithm (Uversky et al., 2000; Prilusky et al., 2005) (Figure 4.6).

Theoretical three-dimensional modelling of κ-casein has predicted the residues from Lys^{21} to Phe^{55} adopt an antiparallel β-sheet formation in which the β-strands are separated by proline turns (Kumosinski et al., 1993). Rather than compacting itself within the cavity of the folded polypeptide, this hydrophobic sheet-turn-sheet motif is predicted to be highly exposed and free to interact intermolecularly in solution, making an ideal site for sheet-sheet interactions (Kumosinski et al., 1993). Under physiological conditions, such interactions would presumably occur with hydrophobic domains of other caseins, facilitating its incorporation into oligomeric species, such as milk micelles. This region of the protein would also favour β-sheet stacking with other κ-casein subunits and therefore facilitate amyloid fibril assembly. While κ-casein exhibits a substantial degree of conformational pliability, the pervasiveness of
this β-sheet structure is illustrated by the propensity of non-fibrillar κ-casein to bind Thioflavin T (Farrell et al., 2003; Thorn et al., 2005), an “amyloid” dye, which is thought to interact with anti-parallel β-sheets (LeVine, 1999). Algorithms to predict aggregation-prone regions of proteins and peptides have also been used on κ-casein (Ecroyd et al., 2008). The algorithm TANGO (Fernandez-Escamilla et al., 2004) predicts the residues Ile^{28}–Leu^{32} and Ile^{73}–Leu^{79} to be the most aggregation prone, while a modified version of the Zyggregator algorithm (Pawar et al., 2005), also predicts regions of significant aggregation propensity between Tyr^{25} and Gln^{29} and between Leu^{74} and Ser^{80} (Ecroyd et al., 2008) (Figure 4.6). Therefore, residues Tyr^{25} to Lys^{86}, which represent the protease-resistant β-sheet core of amyloid fibrils formed by κ-casein correlate very well with previous modelling work (Kumosinski et al., 1993), protein disorder algorithms and are aggregation-prone (Figure 4.6).

Fibril formation is an ordered process that typically involves the unfolding of a protein to partially folded states that subsequently interact and aggregate through a nucleation-dependent mechanism (Ecroyd et al., 2008). According to the mechanism the rate-limiting step is the formation of a stable nucleus (nucleation), which is limited by the protein concentration (Harper and Lansbury, 1997). The time required for the formation of this stable nucleus corresponds to the lag phase of polymerisation. Increasing the protein concentration increases the amount of intermediates that can associate to form a nucleus, decreasing the lag phase. The lag phase can also be shortened by the addition of seeds (preformed fibrils), which overcomes the time required for the formation of a stable nuclei (Harper and Lansbury, 1997). Once nuclei are formed, the aggregates grow rapidly (elongation phase) until a thermodynamic equilibrium between aggregate and monomer is reached (Wood et al., 1999). In recent studies investigating the inherent propensity of RCM κ-casein to form amyloid fibrils, we have demonstrated that the lag phase of RCM κ-casein fibril formation is
independent of protein concentration, and the rate of fibril formation does not increase upon the addition of seeds (preformed fibrils) (Ecroyd et al., 2008). Therefore, the mechanism of RCM κ-casein is not governed by the simple nucleation-dependent model that has been proposed for most other amyloid fibril-forming systems studied to date (i.e. the rate-limiting step is not the formation of a stable nucleus). Instead, the rate-limiting step is the dissociation of the monomer from this oligomeric state (Ecroyd et al., 2008). The dissociated monomer is highly amyloidogenic and is readily incorporated into nuclei or growing fibrils when it becomes available. Since dissociation of the monomer from its aggregate is independent of protein concentration, the lag phase of fibril-formation by RCM κ-casein is concentration-independent. In addition, no free pool of this amyloidogenic species is available to be incorporated into exogenous seeds as occurs in the simple nucleation-dependent model (Ecroyd et al., 2008). To help explain the amyloidogenic nature of RCM κ-casein monomer, the protease-resistant core region of RCM κ-casein fibril was investigated. By digesting RCM κ-casein fibrils with trypsin or proteinase K and using mass spectrometry for identification, we determined that the region from Tyr$^{25}$ to Lys$^{86}$ is incorporated into the core of the fibrils. Theoretical three-dimensional modelling of κ-casein monomer predicts that the region from Lys$^{21}$ to Phe$^{55}$ adopts an anti-parallel β-sheet (Kumosinski et al., 1993). Residues from Tyr$^{25}$ to Lys$^{86}$ are also predicted to fold into a well defined conformation by protein disorder algorithms and are aggregation prone (Ecroyd et al., 2008) (see Figure 4.6). Therefore, we have proposed that the region from Tyr$^{25}$ to Lys$^{86}$ accounts for the amyloidogenic nature of κ-casein (Ecroyd et al., 2008).

### 4.4.2 Dimeric $\alpha_{s2}$-casein’s protease-resistant core

Fibrillar $\alpha_{s2}$-casein was much more resistant to trypsin proteolysis than native $\alpha_{s2}$-casein, such that after two hours of digestion, only small peptide fragments (<4 kDa) were observed for native $\alpha_{s2}$-casein, while two protease-resistant peptides, 9 and 5 kDa were observed for
fibrillar $\alpha_{s2}$-casein even after four hours of digestion (Figure 4.8). The identity of 9 and 5 kDa protease-resistant bands observed from the SDS-PAGE gel were confirmed by performing an in-gel tryptic digestion analysing the fragments by MALDI-MS and MS/MS and corresponded to the $\alpha_{s2}$-casein peptides Ala$^{81}$-Lys$^{181}$ and Thr$^{138}$-Lys$^{181}$, respectively (Table 4.2, Appendix 4.3). Disaggregation of fibrillar $\alpha_{s2}$-casein following trypsin proteolysis and subsequent analysis with MALDI-MS suggested that residues from Ala$^{81}$ to Arg$^{114}$ were also resistant to proteolysis, when compared with native $\alpha_{s2}$-casein under the same conditions (Figure 4.10).

Circular dichroism (CD) and FTIR experiments indicate that $\alpha_{s2}$-casein is the most "structured" casein whereby it possesses about 24–32% $\alpha$-helix, 30–37% $\beta$-sheet and 24–31% turns (Hoagland et al., 2001). This high content of secondary structure is thought to be attributed to the low content of proline residues in $\alpha_{s2}$-casein, by comparison to the other caseins (Tauzin et al., 2003). From secondary structure predictions (Garnier et al., 1978), the most likely candidates for $\beta$-structure are predicted to encompass the hydrophobic residues between Phe$^{89}$ and Pro$^{120}$ and those found at the C-terminus, following Pro$^{177}$ (Hoagland et al., 2001). In addition, the residues from Ile$^{85}$-Val$^{112}$ possess 43% sequence similarity to residues Ile$^{28}$ to Phe$^{55}$ in $\kappa$-casein (Thompson et al., 1994; Thorn et al., 2008), the region corresponding to the sheet-turn-sheet motif proposed to initiate $\kappa$-casein fibril formation (Farrell et al., 2003). The pervasiveness of the $\beta$-sheet structure is also illustrated by the propensity of reduced and non-reduced $\alpha_{s2}$-casein to bind ThT (Thorn et al., 2008), a dye which has been shown to bind to anti-parallel $\beta$-sheet (LeVine, 1999). In contrast, $\alpha_{s1}$-casein, a protein devoid of disulfide linkages, shows no capacity to bind ThT or form fibrils under reducing or non-reducing conditions (Thorn et al., 2008). Molecular modelling on $\alpha_{s2}$-casein indicates that the central hydrophobic portion of $\alpha_{s2}$-casein, residues from His$^{77}$ to Ile$^{119}$, protrudes into solution and provides a good site for potential protein-protein interactions.
(Farrell et al., 2009). Therefore, the protease-resistant core determined for αs2-casein, i.e. Ala81 to Lys181 and Ala81 to Lys114 would incorporate the highly hydrophobic residues Ile85-Val112, which are predicted to adopt extended β-sheet structure (Hoagland et al., 2001). More importantly, it also incorporates AαS2-C, i.e. the peptide fragments, 32 to 45 residues in length and all starting from Ala81, which have been isolated from amyloid deposits associated with mammary CA (Niewold et al., 1999).

It is somewhat surprising that residues from Thr138 to Lys181 were significantly protease resistant. These residues have very low average hydrophobicity and a high charge frequency (Farrell et al., 2009). Theoretical secondary structure prediction on native αs2-casein also suggests that most of these residues, Thr144-Leu176, form an α-helix, rather than β-sheet, which are stabilised by favourable salt bridges (E145; K149, E155; K158 and E156; K160) (Hoagland et al., 2001; Tauzin et al., 2003). In addition, Kizawa et al. (1996) found that residues 164 to 179 of αs2-casein inhibited calmodulin activation of phosphodiesterase; such calmodulin-binding proteins are thought to be helical when bound to calmodulin (Kizawa et al., 1996). If this region, rather than forming anti-parallel β-sheet necessary for fibril formation, has a propensity to adopt an α-helical structure, why is it so resistant to proteolysis? A possible explanation may come from the experiments by Tauzin et al., (2003) who performed trypsin digestion of native αs2-casein and observed that residues from Asn115 to Lys150 were the slowest to be hydrolysed. He proposed that intermolecular ionic interaction could occur through the area Glu126–Lys158, which is composed of five alternatively charged clusters (5 negative, 2 positive, 4 negative, 3 positive, 3 negative residues). Consequently, the central part of the protein (Asn115 to Lys150) would be locked within αs2-casein aggregates and relatively protected from trypsin (Tauzin et al., 2003). Similarly, the C-terminal extremity of αs2-casein is proposed to be implicated in intra- and intermolecular bonding (Snoeren et al., 1980). The fragment Thr3-Glu18; that contains up to 9 successive negatively charged residues,
appears to be the most probable part of the protein to exchange ionic interactions with the fragment Lys$^{158}$-Lys$^{173}$ (6 positive charges) (Tauzin et al., 2003). Therefore, the protease-resistant band Thr$^{138}$-Lys$^{181}$, which is highly charged and proposed to exist as α-helical structures (Thr$^{144}$-Leu$^{176}$, (Hoagland et al., 2001; Tauzin et al., 2003)) may also be somewhat protected from trypsin proteolysis as a result of intra- and intermolecular interactions in α$\text{a}_\text{2}$-casein fibrils.

Qin et al., (2007) using limited proteolysis and MS determined that residues ~32-102 comprise the β-sheet core of α-synuclein fibrils. These trypsin-treated fibrils, however, only formed long protofilaments while the full-length protein consists of four intertwined protofilaments to make the mature fibrils. Using three truncated α-synucleins: Syn30-140, Syn1-103, and Syn30-103, Qin et al., (2007) determined that the C-terminal region, while not incorporated into the β-sheet core of α-synuclein fibrils, plays a key role in the interaction between protofilaments and is necessary for full length mature α-synuclein fibril formation.

The TEM of α$\text{a}_\text{2}$-casein fibrils during various stages of proteolysis also indicates a change in the morphology of α$\text{a}_\text{2}$-casein fibrils: from long, twisted-ribbon morphology to short, straight fibrils (panels B-F of Figure 4.8). While peptides Ala$^{81}$-Lys$^{181}$, Ala$^{81}$-Lys$^{114}$ and Thr$^{138}$-Lys$^{181}$ were determined to be resistant to proteolysis, it may be that the longer peptide fragment, Ala$^{81}$-Lys$^{181}$, or residues outside this region are also necessary for the formation of stable, mature α$\text{a}_\text{2}$-casein fibrils. Cleavage of these “other residues” may begin to unravel the mature α$\text{a}_\text{2}$-casein fibrils, further exposing them to proteases.

4.3 Conclusion

By digesting RCM κ-casein fibrils with trypsin/proteinase K and using mass spectrometry for identification, we have determined that the region from Tyr$^{25}$ to Lys$^{86}$ is incorporated into the core of the fibrils. We predict that this region accounts for the amyloidogenic nature of κ-
casein (Ecroyd et al., 2008). Digestion of $\alpha_{s2}$-casein fibrils with trypsin indicated a protease-resistant core between Ala$^{81}$ to Lys$^{181}$, which incorporates peptide fragments that have been isolated from amyloid deposits associated with mammary CA. Isolated $\alpha_{s2}$- and $\kappa$-casein both form amyloid fibrils at physiological pH and temperature. However, caseins do not exist as isolated entities in their native state but instead have a strong tendency to associate with themselves and each other through hydrophobic and electrostatic interactions (Swaisgood, 1992). It is this tendency to associate, along with calcium and other minerals present in milk, that ultimately leads to the formation of casein micelles (Fox and McSweeney, 2003). Therefore, the fibril-forming tendency of $\alpha_{s2}$- and $\kappa$-casein is kept in check via their interactions with other caseins in milk. Nonetheless, the proteinaceous deposits found within CA of mammary tissue in various species have been shown to be amyloid in nature (Reid, 1972; Beems et al., 1978; Taniyama et al., 2000), and peptides corresponding to casein fragments (e.g. $\alpha_{s2}$-casein) have been isolated from within these amyloid-like deposits (Niewold et al., 1999). Electron microscopy has also identified numerous fibril-like structures within mammary epithelial cells (Beems et al., 1978; Nickerson et al., 1985), which is the site of casein synthesis and secretion. That amyloidoses associated with $\alpha_{s2}$- and $\kappa$-casein fibril formation are not more prevalent in vivo is probably a testament to the interaction between the casein proteins, which prevents, through their chaperone-like activity, the release of these amyloidogenic caseins, thereby inhibiting their large-scale fibril formation (Thorn et al., 2005; Thorn et al., 2008).
STATEMENT OF AUTHORSHIP

Selective methionine oxidation of κ-casein and its effect on κ-casein’s amyloid fibril-forming propensity, chaperone ability, cellular toxicity and structure.

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Chapter 5

Selective methionine oxidation of κ-casein and its effect on κ-casein’s amyloid fibril-forming propensity, chaperone ability, cellular toxicity and structure.

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5.1 Abstract

Methionine residues are linked to the pathogenicity of several amyloid diseases, including Alzheimer’s disease. These diseases are characterised, in vivo, by the accumulation of insoluble proteinaceous plaques, of which the major constituents are amyloid fibrils. Amyloid bodies are also found in mammary secretory tissue known as corpora amylacea (CA) of humans and various other species. The identity of the protein(s) involved in formation of amyloid plaques has not been determined conclusively, however, immunoblotting and sequence analysis of peptides obtained from mammary CA suggest that several milk proteins are present including κ-casein, which has been shown to form amyloid fibrils under reducing and non-reducing conditions, in vitro.

As milk exists in an extracellular oxidising environment, we selectively oxidised the two methionine residues in κ-casein (Met-95 and Met-106) to determine whether oxidation affects κ-casein’s fibril-forming propensity, cellular toxicity, chaperone ability, and structure. Oxidation of κ-casein’s methionine residues resulted in an increase in the rate of fibril-formation (ThT fluorescence) and a greater level of cellular toxicity. In addition, β-casein, which is able to inhibit κ-casein fibril-formation, in vitro, was less effective at suppressing fibril-formation of κ-casein upon methionine oxidation. No significant changes to structure or chaperone ability were observed for κ-casein upon oxidation.
5.2 Introduction

Casein is arguably the best characterised milk protein and constitutes over 70-80% of total bovine milk protein (Swaisgood, 1992). In its native state in milk, caseins exist as large micelle-like particles that comprise four unrelated casein subunits (α\textsubscript{S1}, α\textsubscript{S2}, β-, and κ-caseins) and calcium phosphate with an average mass of approximately 10\textsuperscript{8} Da and an average diameter of around 200 nm (Fox and McSweeney, 2003). Owing to their high content of phosphate groups, which occur in clusters, α\textsubscript{S1}, α\textsubscript{S2} and β-caseins have a strong tendency to bind metal ions, which in milk are mainly calcium. The calcium-sensitive proteins (α\textsubscript{S1}, α\textsubscript{S2} and β-caseins), which represent approximately 85% of total casein, are insoluble at calcium concentrations greater than ~6 mM. Since bovine milk contains a calcium concentration of approximately 30 mM, one would expect that the caseins would precipitate under the conditions prevailing in milk (Fox and McSweeney, 2003). However, κ-casein which contains one organic phosphate group (Ser-149) binds calcium very weakly and, when mixed with the calcium-sensitive caseins, stabilises them by forming the large colloidal casein micelles. Like other caseins, particularly β-casein, κ-casein is extremely amphiphatic and possesses a very hydrophobic N-terminal domain encompassing roughly residues Glu\textsuperscript{1}-Phe\textsuperscript{105} (para- κ-casein) and a polar C-terminal domain (Met\textsuperscript{106}-Val\textsuperscript{169}) (Swaisgood, 1992). The flexible hydrophilic C-terminal region of κ-casein is thought to reside on the surface of the casein micelle (Schmidt, 1980; Swaisgood, 1985) which stabilises the micelle through electrostatic and steric interactions. Separation of the hydrophobic and polar domains by chymosin-catalysed hydrolysis of the Phe\textsuperscript{105}-Met\textsuperscript{106} peptide bond increases the surface hydrophobicity of casein micelles allowing them to associate and coagulate, which occurs during cheese manufacture (Fox and McSweeney, 2003).

Amyloid fibrils are ordered aggregates of normally soluble peptides or proteins that are characterised by a highly structured array of cross β-sheet arranged into fibrils (Ecroyd and
Carver, 2008). The assembly of partially folded proteins into amyloid fibrils is associated with several devastating human diseases, including Alzheimer’s and Parkinson’s diseases (Markesbery and Carney, 1999; Duda et al., 2000). Amyloid-like deposits have also been identified in bovine, rat, and canine mammary glands within calcified stones known as corpora amylacea (CA) (Reid, 1972; Beems et al., 1978; Taniyama et al., 2000). The majority of CA is located within the alveolar lumen (Nickerson et al., 1985), where milk is stored, and subsequently secreted into the ducts through ductules. In addition, amyloid-like fibrils have been identified within the cytoplasm of mammary epithelial cells, which are involved in the synthesis and secretion of caseins and other milk constituents (Reid, 1972; Nickerson et al., 1985). It has been postulated that CA can cause complications during late lactation by engorging luminal spaces and clogging small ducts, leading to milk stasis and involution (Nickerson et al., 1985). Although the identity of the protein(s) involved in plaque formation has not been determined conclusively, analysis of peptides obtained from CA has suggested that several milk proteins, in particular caseins are present (Claudon et al., 1998). It is thought that κ-casein may play a significant role in the formation of CA as κ-casein forms fibrils under reducing and non-reducing conditions in vitro at physiological conditions (Thorn et al., 2005). However, large scale fibril-formation of κ-casein in vivo is prevented by the chaperone ability of the other casein proteins present in milk (Thorn et al., 2005).

The formation of reactive oxygen species is a normal by-product of metabolism in cells (Hokenson et al., 2004). Under normal conditions, cells are able to eliminate reactive oxygen species (ROS) by antioxidants, metal chelators or enzymatic reduction (Uversky et al., 2002b). However, when the level of ROS exceeds its defence mechanisms, the cell may suffer from oxidative stress. Proteins that are modified due to oxidation often accumulate during normal aging and are implicated in the pathogenesis of a wide range of disorders including Alzheimer’s disease (AD) (Markesbery and Carney, 1999), Parkinson’s disease (Duda et al.,
2000), Huntington’s disease (Browne et al., 1999) and cataract (Fu et al., 1998). Post-mortem analysis of AD brains has shown high levels of protein oxidation, lipid peroxidation, and oxidative damage to mitochondria, indicating that oxidative stress is a characteristic of AD (Markesbery and Carney, 1999). The oxidative state of the Aβ peptide is proposed to be critical to its neurotoxic properties in vivo where the single methionine at position 35 is proposed to be involved in the generation of ROS (Barnham et al., 2003).

Protein oxidation in milk can be initiated either enzymatically, e.g. by the lactoperoxidase system (Ostdal et al., 2000), by photo-oxidation (Dalsgaard et al., 2007), or by transition metal ions, which can induce protein oxidation in the presence of ascorbic acid by a Fenton reaction (Leclère et al., 2002). Protein oxidation leads to different types of modifications, including cross-linkages (Ostdal et al., 2000), fragmentation of covalent bonds, and changes in amino acids, e.g. methionine (Kim and Morr, 1996), histidine (Zittle, 1965), tryptophan (Zittle, 1965), and tyrosine (Ostdal et al., 2000).

Because of the prevalence of oxidative injury in disease along with methionine being one of the most readily oxidised amino acids (Vogt, 1995), we examined the effect of methionine oxidation on κ-casein’s structure and function. Bovine κ-casein has 2 methionine residues at positions 95 and 106 which were selectively oxidised with hydrogen peroxide under mild conditions. Interestingly, while methionine oxidation in other fibril-forming proteins (e.g α-synuclein, amyloid β, prion protein, transthyretin, and apolipoprotein C-II) inhibits fibril formation by the protein (Hou et al., 2002; Uversky et al., 2002b; Breydo et al., 2005; Maleknia et al., 2006; Binger et al., 2008), the oxidation of methionine residues in κ-casein resulted in increased fibril formation and enhanced cell toxicity. However, the presence of β-casein incubated with oxidised κ-casein resulted in complete inhibition of κ-casein fibril
formation, underlying the importance of the other caseins (α_{S1}-, α_{S2}-, and β-casein) in preventing large-scale aggregation of κ-casein in vivo.

5.3 Materials and Methods

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

5.3.2 Oxidation of native κ-casein

50 mg of native κ-casein was dissolved in 5 mls of 50 mM phosphate buffer (pH 7.4). 0.1% (v/v) Hydrogen peroxide was added, the reaction mixture was stirred overnight at 4 °C and then desalted using a HiPrep™ 26/10 Desalting column (GE Healthcare), with 50 mM ammonium bicarbonate (pH 7.8) to remove the hydrogen peroxide. The sample was then lyophilised and stored at -20 °C. Prior to lyophilisation, a 10µl aliquot was taken for SDS-PAGE analysis and mixed with an equal volume of reducing sample buffer before being heated (95 °C, 5 mins) and then analysed using 15% acrylamide gels (v/v) and standard techniques (Laemmli, 1970).

5.3.3 Mass Spectrometry

To confirm the oxidation of κ-casein’s methionine residues a tryptic in-gel digest was performed on the hydrogen peroxide treated κ-casein according to the method of Shevchenko et al. (1996) with the following modifications. The Coomassie Blue-stained bands from an
SDS PAGE were cut into 1 mm² blocks, rinsed in 100 mM NH₄HCO₃ and destained using 50 mM NH₄HCO₃ with 30% acetonitrile (v/v) and then incubated overnight with 100 ng of trypsin in 5 mM NH₄HCO₃ at 37°C. The tryptic fragments were extracted from the gel by sonicating the samples for 15 mins with 50% acetonitrile (v/v) in 0.1% trifluoroacetic acid (v/v) and then in 100% acetonitrile. All samples were prepared for mass spectrometry by spotting 1 µl of analyte solution to 1 µl of 2,5-dihydroxybenzoic (5 g/l) in 50% acetonitrile (v/v), 0.1 % trifluoroacetic acid (v/v) on a microtitre plate (MTP) Anchor Chip Target 600/384 (Bruker Daltonics) and left to dry. All mass spectra were acquired on an Ultraflex III MALDI-TOF/TOF mass spectrometer (Bruker Daltonics) in reflector mode using Flex Control (Bruker Daltonics). The identity of peptides was confirmed by matching their observed masses to the expected masses of the in silico tryptic digest of the known κ-casein primary sequence using Biotools 3.0 software (Bruker Daltonics). All spectra were processed using FlexAnalysis software (Bruker Daltonics) with the following parameters: a maximum of 200 peaks were selected, with a signal to noise threshold of 5, and a quality factor of 75. A Gauss smoothing algorithm (0.02 m/z, 2 cycles), and a TopHat baseline subtraction was also performed. Electrospray MS experiments were also performed on native and oxidized κ-casein with a Q-Tof II spectrometer (Micromass UK) in positive mode. Protein solutions were made up to 1 mg/mL in acetonitrile and 0.1% formic acid (1:1 ratio) and diluted to a desired concentration using an acetonitrile and 0.1% formic acid solution (1:1 ratio). Spectra were smoothed (2 channels, 1 cycle) and base line subtracted (cubic, 5% peak area) and deconvoluted using MassLynx 4.1 software (Waters, Milford, Massachusetts)

### 5.3.4 Thioflavin T Assays

The effect of oxidation on the fibril formation of native and reduced and carboxymethylated (RCM) κ-casein was monitored using an in situ thioflavin T (ThT) binding assay (Nielsen et al., 2001). Native and oxidised κ-casein was incubated at 37 °C in 50 mM phosphate buffer,
pH 7.4, with and without 1,4-dithiotheritiol (DTT) to a final concentration of 20 mM. Samples were prepared in duplicate and incubated with 10 µM ThT in black µClear 96-microwell plates (Greiner Bio-One). The plates were sealed to prevent evaporation, and the fluorescence levels were measured with a FLUOstar Optima microplate reader (BMG Labtechnologies) with a 440/490-nm excitation/emission filter set. To monitor the effect of β-casein on κ-casein fibril formation, native and oxidised κ-casein (3.45 mg/ml) in 50 mM phosphate buffer, pH 7.4, was incubated with 20 mM DTT and increasing concentrations of β-casein up to a 1:1 (mole: mole) ratio. Samples were prepared in duplicate and incubated with 10 µM ThT in black µClear 96-microwell plates. The plates were sealed to prevent evaporation, and the fluorescence levels were measured with a FLUOstar Optima microplate reader.

5.3.5 Chaperone Activity

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

\[ \% \text{ Protection} = \left( \frac{k_{\text{target}} - k_{\text{chaperone}}}{k_{\text{target}}} \right) \times 100 \]
where $k_{\text{target}}$ is the rate of aggregation of the target protein and $k_{\text{chaperone}}$ is the rate of aggregation of the target protein with the chaperone (κ-casein) present. The rates were determined from the maximum slope of the plots.

5.3.6 TEM (Transmission Electron Microscopy)

Samples for TEM were prepared by adding 2 µL of protein solution to Formvar and carbon-coated nickel grids (SPI Supplies, West Chester). The grids were then washed three times with 10 µL of water and negatively stained with 10µL of uranyl acetate (2% (w/v); Agar Scientific, U.K.). The grids were dried with Whatman filter paper between each step. The samples were viewed under 25 000-64 000 magnifications at 80 kV excitation voltages using a Philips CM100 transmission electron microscope (Philips, The Netherlands). Particle size measurements were performed using SIS Image Analysis software.

5.3.7 Pheochromocytoma-12 cell culture and MTT assay

The effect of methionine oxidation on κ-casein’s fibrillar toxicity was investigated by treating pheochromocytoma-12 (PC-12) cells with native and oxidised κ-casein (200 µM), in 50 mM phosphate buffer (pH 7.4), pre-incubated at 37 °C for 20 h under reducing conditions (2 mM DTT). PC12 cells were grown in RPMI 1640 medium supplemented with 10% v/v horse serum, 5% v/v foetal bovine serum, 10 U/mL of penicillin and 10 µg/mL of streptomycin. Cells were cultured in uncoated 75-cm$^2$ plastic flasks in an incubator with 95% air and 5% CO$_2$ at 37 °C. The medium was refreshed every 2–3 days. For treatment, cells from culture were seeded in 96-well plates at a density of $2\times10^4$ cells per well in 100-µL full-serum medium and then incubated for 24 h. The plated cells were then treated (six replicates per treatment) with 10 µL of either native or oxidised κ-casein fibrils, which were pre-incubated for 20 h with 2 mM DTT, to give a final κ-casein concentration of 0.05, 0.5 or 2 µM. Control wells were treated with 10 µL of 2 mM DTT (in 50 mM phosphate buffer, pH 7.4) to ensure

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that the buffer was non toxic to cells. Reduced and carboxymethylated κ-casein has been shown to induce cell death in a concentration-dependent manner (Dehle et al., submitted for publication; Hudson et al., 2009). Similar concentrations to those used by Hudson et al., (2009) are used herein. After incubation of the treated cells for 48 h, the treatment mixture was removed and 100 μL of serum-free medium containing MTT (0.6 mM) was added to each well. The cells were then incubated for an additional 2 h, and the MTT-containing medium was replaced with 100 μL of DMSO. Formazan absorption was then measured at 560 nm using a BMG Polarstar microplate reader (BMG Labtechnologies, Offenburg, Germany). Mean absorption readings from sextuplet wells were taken, and the percentage cell viability was calculated using the following equation: A(treated)/A(untreated)×100, where A(treated) is the mean formazan absorption reading for each treatment and A(untreated) is the mean formazan absorption reading for cells treated with phosphate buffer only.

5.3.8 Intrinsic and extrinsic fluorescence spectroscopy

Native and oxidised κ-casein were dissolved in 50 mM phosphate buffer (pH 7.4) at 0.5 mg/mL (25 μM). Intrinsic tryptophan fluorescence spectra were recorded at ambient temperature using a Cary Eclipse fluorescence spectrophotometer (Varian) equipped with temperature control. The excitation wavelength was set at 295 nm and emission was monitored between 300 and 450 nm. The excitation and emission slit widths were 5 nm. The fluorescence emission of buffer alone was subtracted from that obtained in the presence of protein. For extrinsic fluorescence measurements, 2-4 μL aliquots of a 10 mM stock solution of ANS, a hydrophobic probe, in 50 mM phosphate buffer (pH 7.4) were added to 25 μM native and oxidised κ-casein. The ANS fluorescence of each sample was measured using a Cary Eclipse fluorescence spectrophotometer (Varian), with the excitation wavelength set at 387 nm, and emission spectra measured between 400 and 550 nm. The excitation and emission bandwidths were both 5 nm. The fluorescence intensity was plotted versus
wavelength for each sample after the sequential addition of 2-4 μL aliquots of 10 mM ANS until the maximum fluorescence intensity was observed. The fluorescence emission of ANS in buffer alone was deducted from that obtained in the presence of protein.

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

5.3.10 1H NMR Spectroscopy
Native and oxidised κ-casein were dissolved at 2 mg/ml in 50 mM deuterated phosphate buffer (pH 7.4 in D₂O). 1H 2-D TOCSY NMR spectra were acquired at 25 °C at 600 MHz using a Varian Inova NMR spectrometer, while cross peaks were measured using the Sparky 3.106 software package. The spin lock mixing time in the TOCSY experiment was 60 ms. The residual HDO resonance was removed by presaturation during the delay between scans. Deuterated phosphate buffer (50 mM) was prepared by dissolving appropriate amounts of mono- and di-sodium salts in 5 mL of D₂O and equilibrated with acid/base to pH 7.4. To remove residual water, the resulting buffer was lypholised and redissolved in D₂O. No correction was made to the buffer as a consequence of the deuterium effect.
5.4 Results

5.4.1 Oxidation of native κ-casein

The oxidation of native κ-casein was performed by incubating κ-casein with 0.1% (v/v) hydrogen peroxide overnight at 4 °C. Excess hydrogen peroxide was removed with a HiPrep™ 26/10 desalting column. Following purification, an in-gel tryptic digest of native (control) and oxidised κ-casein was performed. The subsequent solutions were analysed by MALDI-TOF mass spectrometry to verify that oxidation of κ-casein had occurred. A section of the raw mass spectra of native and oxidised κ-casein with tryptic peptide fragments of interest are highlighted in Figure 5.1. The two unmodified methionine residues in κ-casein, located at position 95 and 106, are indicated by peaks with a m/z of approximately 1193 and 1608 and correspond to the tryptic fragments Ser^{87}-Arg^{97} and His^{98}-Lys^{111} respectively. The identity of the more intense peak (m/z of 1608.779) was also confirmed using MS/MS (data not shown). The oxidation of methionine is characterised by an addition of 16 Da. Thus, the two peaks observed in the mass spectra of oxidised κ-casein with a m/z of approximately 1209 and 1624 correspond to the oxidation of the Met^{95} and Met^{106} on the tryptic fragments Ser^{87}-Arg^{97} and His^{98}-Lys^{111} respectively (Figure 5.1). Other κ-casein tryptic fragments observed in the mass spectrum are also indicated. Native and oxidised κ-casein were also analysed by electrospray ionization mass spectrometry (Appendix 7.5A-C). The poor MS spectra observed is due to the extreme heterogeneity of the protein. In κ-casein the cysteine residues are randomly crosslinked by intermolecular disulphide bonds resulting in the formation of polymers, from monomer to multimeric structures larger than decamers (Rasmussen et al., 1992b). κ-Casein also contains one to three phosphorylated residues (Whitney, 1988) and is variously glycosylated on serine and threonine residues resulting in additional heterogeneity (a total of 19 variants make-up this protein family) (Swaisgood, 1993). Nevertheless, the deconvoluted MS spectrum of native κ-casein gave rise to several peaks at 19004, 19036, 19050 and 19080 Da. The peak at 19004 Da corresponding to monomeric κ-casein which is
consistent with the literature (19005 Da, (Swaisgood, 1993)), while the peak at 19036 is a sodium adduct of monomeric κ-casein (the addition of 22 Da, Appendix 7.C). Deconvolution of the MS spectra of oxidised κ-casein resulted in various peaks at 19034, 19050, 19068, 19086 and 19112 Da (Appendix 7.C). The oxidation of two methionine residues is characterised by an addition of 32 Da. Therefore, the major peaks at 19034, 19068 and 19112 Da in the MS spectrum of oxidised κ-casein, correspond to the peaks at 19004, 19036 and 19080 Da of native κ-casein which have had their methionine residues oxidised. Thus, both MALDI and ES ionization mass spectrometry results show that no other modifications by hydrogen peroxide have been introduced.

5.4.2 Fibril forming propensity of native and oxidised κ-casein

Previous in vitro studies have shown that κ-casein under reducing conditions forms long, rod-like aggregates at neutral pH and 37 °C that have the characteristics of amyloid fibrils (Farrell et al., 2003; Ecroyd et al., 2008). Native κ-casein also has a propensity to form fibrils, although at a significantly reduced level compared to the reduced protein (Thorn et al., 2005). Various concentrations (1.0-2.5 mg/ml) of native and oxidised κ-casein were incubated in 50 mM phosphate buffer (pH 7.4, 10 µM) at 37°C under reducing (20 mM DTT) and non-reducing conditions to determine the effect of oxidation on fibril formation. A concentration-dependent increase in ThT fluorescence is observed for both native and oxidised κ-casein under non-reducing and reducing conditions (panels A to D of Figure 5.2) with oxidised κ-casein having a significantly greater initial rate of increase in ThT fluorescence compared to native κ-casein under both non-reducing (Figure 5.2E) and reducing conditions (Figure 5.2F) at the same concentration. For example, under non-reducing conditions, at 2.5 mg/mL, the ln(initial rate constant) was 4.96 and 5.56 for native and oxidised κ-casein respectively, while under reducing condition the ln(initial rate constant) was 5.64 and 6.63 for native and oxidised κ-casein respectively. Since the rate of increase in ThT fluorescence was observed
to be dependent on the protein concentration (panels A to D of Figure 5.2), we sought to determine the reaction rate for the formation of fibrils by native and oxidised κ-casein under reducing and non-reducing conditions. The reaction order can be determined according to the equation $\ln V = A + n \ln C$, where $V$ represents the initial reaction rate, $C$ is the protein concentration, and $n$ is the apparent reaction order (Ecroyd et al., 2008). The plot of $\ln$(initial rate constant) against $\ln$(protein concentration) gave a slope ($n$) = 0.82 +/- 0.14 and 0.86 +/- 0.07 for native and oxidised κ-casein under reducing conditions respectively (Figure 5.2F). Thus for both native and oxidised κ-casein under reducing conditions the rate of increase in ThT fluorescence, in relation to the κ-casein protein concentration, approaches a first-order kinetic mechanism. This result is slightly lower than what was found for RCM κ-casein, whereby a first-order mechanism was also found for RCM, with a reaction order of 1.13 ± 0.15 (Ecroyd et al., 2008).

TEM was used to verify that the increase in ThT fluorescence of native and oxidised κ-casein under reducing and non-reducing conditions was the result of fibril formation. TEM is only a qualitative method for evaluating fibril formation, nevertheless smaller and shorter fibrils were clearly observed for native and oxidised κ-casein under non-reducing conditions (panels A and B of Figure 5.3), when compared to native and oxidised κ-casein in the presence of reducing agent (panels C and D of Figure 5.3), consistent with previous results (Thorn et al., 2005). No significant difference in morphology was observed between native and oxidised κ-casein fibrils under the same conditions. Interestingly, even though the rate of increase in ThT fluorescence was lower, the maximum change in ThT fluorescence was observed to be greater for native and oxidised κ-casein under non-reducing conditions than under reducing conditions (panels A to D of Figure 5.2). These results did not correlate to the TEM data whereby significantly more fibrils were observed under reducing conditions than under non-reducing conditions (Figure 5.3). ThT binds selectively to antiparallel β-pleated sheets, the
major structural element of amyloid fibrils, therefore, fibril formation is accompanied by an increase in the ThT fluorescence (LeVine, 1999). However, it has been shown that ThT fluorescence is not always proportional to the amount of amyloid fibrils present and can change depending on the morphology of the fibrils (Kozhukh et al., 2002; Chiba et al., 2003). Kozhukh et al., (2002) observed that fresh β2-microglobulin amyloid fibrils without lateral aggregation show a high fluorescence intensity, however, further incubation decreases their ThT fluorescence, probably due to the lateral aggregation expelling ThT bound between fibrils (Kozhukh et al., 2002). Although the exact mechanism is not clear, it is thought that a change in the high order structure of amyloid fibrils may account for such a decrease in ThT fluorescence (Kozhukh et al., 2002; Chiba et al., 2003). Similarly, while significantly more κ-casein fibrils are observed by TEM under reducing conditions than non-reducing conditions (Figure 5.3), the lateral elongation and clumping of the fibrils may be expelling ThT bound between fibrils and reducing the maximum fluorescence intensity at 490 nm.

In vivo, the fibrillar aggregation propensity of κ-casein is kept in check by the chaperone ability of the other casein proteins in milk (i.e., αs- and β-caseins) (Thorn et al., 2005). As such, β-casein was incubated with native and oxidised κ-casein (3.45 mg/ml) under reducing and non-reducing conditions to determine whether it could also prevent oxidised κ-casein from forming fibrils under non-reducing and reducing conditions (panels A to D of Figure 5.4). Under non-reducing conditions, β-casein is able to suppress the fibril formation of both native and oxidised as effectively (panels A and B of Figure 5.4). β-Casein provides approximately 70% protection against fibril formation of both native and oxidised κ-casein at a 0.25:1.0 molar ratio of β-casein: κ-casein, while at a 1:1 molar ratio, greater than 90% protection is observed for both κ-casein species (panels A and B of Figure 5.4). In contrast, under reducing conditions, β-casein is significantly less able to suppress the fibril formation of oxidised κ-casein at lower concentration (panels C and B of Figure 5.4). Thus, at a
0.25:1.0 molar ratio of β-casein:κ-casein, 35% protection is observed for κ-casein, while only a 5% protection is observed for oxidised κ-casein (Figure 5.4F). TEM was also performed on samples immediately following the ThT assay. Both native and oxidised κ-casein formed fibrils under reducing conditions in the absence of β-casein (panels A and E of Figure 5.5). Fibrils are also observed for both native and oxidised κ-casein at a 0.25:1 molar ratio of β-casein: κ-casein under reducing conditions (panels B and F of Figure 5.5). No fibrils were observed when κ-casein was incubated at a 0.5:1 molar ratio of β-casein: κ-casein under reducing conditions, instead, amorphous aggregates were present (Figure 5.5C). Therefore, at a 0.5:1 molar ratio of β-casein: κ-casein, β-casein was able to suppress κ-casein fibril formation. In contrast, when oxidised κ-casein was incubated at a 0.5:1 molar ratio of β-casein: κ-casein, fibrils were still observed by TEM (Figure 5.5G). No fibrils were observed for both native and oxidised κ-casein under reducing conditions in the presence of a molar equivalent of β-casein (panels D and H of Figure 5.5). Therefore, the TEM results are consistent with the ThT data with β-casein being less able to suppress fibril formation of oxidised κ-casein under reducing conditions. However, β-casein is still able to suppress oxidised κ-casein fibril formation at higher concentrations.

5.4.3 Chaperone activity of native and oxidised κ-casein

Native κ-casein has previously been shown to inhibit the amorphous aggregation and subsequent precipitation of a variety of target proteins (Morgan et al., 2005; Guyomarc'h, 2009 #219). Native and oxidised κ-casein were incubated with various target proteins under heat stress to determine whether oxidation of methionine residues to the more polar methionine sulfoxide had an effect on κ-casein chaperone activity. When alcohol dehydrogenase (ADH, 1 mg/mL) was incubated at 42 °C in 50 mM phosphate buffer (pH 7.4) with 2 mM EDTA, an increase in light scattering was observed at 30 mins with aggregation reaching a plateau following 180 min of incubation (panels A and B of Figure 5.6). The
presence of increasing amounts of native κ-casein led to a decrease in aggregation, such that, at a 2:1 molar ratio of κ-casein:ADH, the aggregation of ADH was almost completely inhibited (Figure 5.6A). Oxidised κ-casein was also effective at suppressing the aggregation and subsequent precipitation of ADH (Figure 5.6B). Under more severe heating conditions, i.e. when catalase (1 mg/ml) was incubated at 55°C in 50 mM phosphate buffer (pH 7.4), an increase in light scattering was observed after 25 mins with aggregation continuing over 120 min of incubation. The presence of increasing concentration of native and oxidised κ-casein led to a decrease in the aggregation of catalase, such that, at a 2:1 molar ration of catalase: κ-casein, the aggregation of catalase was completely suppressed (panels C and D of Figure 5.6). The rate of aggregation of ADH and catalase with and without chaperone present was quantified and expressed as a percentage of protection (panels E and F of Figure 5.6). Oxidised κ-casein offered slightly better protection against the aggregation of both catalase and ADH, however, their chaperone activity was not statistically different (p-values > 0.05, for all t-tests performed (one tailed, unequal variance)).

5.4.4 Cellular toxicity of fibrillar native and oxidised κ-casein

To assess the toxicity of oxidised κ-casein fibrils, pheochromocytoma-12 (PC12) cells were treated with native and oxidised κ-casein (4 mg/ml) pre-incubated at 37 °C for 20 h with 2 mM DTT in 50 mM phosphate buffer, pH 7.4. Samples were prepared in duplicates, with and without 10 µM ThT, in order to monitor fibril formation without exposing the cells to residual ThT (0.1 µM), which has been shown to be toxic at certain concentrations (Francis, unpublished results). The cells were incubated with a final κ-casein concentration of 0.05 µM to 2 µM. Following incubation of the treated cells for 48 h, cellular survival was measured by methylthiazolyldiphenyl-tetrazolium bromide (MTT) reduction (Figure 5.7). A significant difference in the amyloid-associated toxicity was observed between native and oxidised κ-
casein fibrils, which correlated with ThT data, whereby a greater degree of fibril formation corresponded to an increase in amyloid-fibril associated toxicity.

5.4.5 Structural studies

Extrinsic fluorescence (ANS binding), intrinsic fluorescence and CD spectroscopy were used to determine changes to the structure of κ-casein upon oxidation. Aliquots of a stock solution of ANS were added to solutions of native and oxidised κ-casein (0.5 mg/ml) at neutral pH. The fluorescence intensity was plotted versus wavelength for each sample after sequential addition of ANS until the maximum fluorescence intensity was observed. The maximum κ-casein fluorescence intensity was 565 arb.u. which increased to 650 upon oxidation (Figure 5.8), suggesting an increase in exposed and clustered hydrophobicity of κ-casein upon oxidation. Intrinsic tryptophan fluorescence was also performed at neutral pH on native and oxidised κ-casein (0.5 mg/ml). The magnitude and wavelength of fluorescence gives an indication of the environment of the fluorophore (Freifelder, 1976). Native κ-casein has a maximum fluorescence intensity of 345 arb.u. which decreased to 285 upon oxidation (Figure 5.9). However, the quenching of fluorescence did not accompany a red shift in fluorescence suggesting that the protein is not unfolding around the tryptophan (Freifelder, 1976), but instead, it is being quenched. The circular dichroism spectra were used to examine alteration in overall secondary structure upon oxidation of κ-casein. Figure 5.10 shows that the CD spectra of native and oxidised κ-casein are very similar. Both spectra exhibit a maximum negative mean residue ellipticity at 200 nm and a shoulder at 213 nm and 230 nm, which are indicative of a predominantly random coil structure, and are very similar with previously published CD spectra of κ-casein (Creamer et al., 1998) and RCM κ-casein (Ecroyd et al., 2008; Hudson et al., 2009).
One- and two-dimensional NMR spectroscopy was undertaken on native and oxidised κ-casein at 25°C in 50 mM deuterated phosphate buffer (pH 7.4). Native κ-casein in aqueous solution forms large spherical polymers, with an average molecular mass of 1.18 MDa (Groves et al., 1998). The large micelles are assembled from smaller multimeric subunits (monomers to decamers) that result from intermolecular disulfide bonding (Fox and McSweeney, 2003). An aggregate of this size would have a long correlation time, and thus, very broad, overlapping and uninformative resonances would be expected to be observed giving rise to a poorly-resolved $^1$H NMR spectrum (Carver and Lindner, 1998). Instead, a series of comparatively sharp resonances are observed in the 1D NMR spectra of native and oxidised κ-casein in D$_2$O (Figure 5.11). The spectra are very similar to those of the κ-casein macropeptide (M$^{106}$-V$^{169}$) (Griffin and Roberts, 1985), the region of the polypeptide chain that extends into solution from the casein micelle (Griffin and Roberts, 1985). These relatively sharp resonances arise from protons in the molecule that have independent flexibility (i.e. a much shorter correlation time) compared to the bulk of the protein (Carver and Lindner, 1998). The 1D $^1$H NMR spectra of native and oxidised κ-casein are very similar, however, an additional resonance at 2.75 ppm in the $^1$H NMR spectra of oxidised κ-casein is observed (Figure 5.11). The resonance corresponds to the ε-methyl protons of oxidised methionine and is approximately 0.6 ppm downfield, due to the addition of the electronegative oxygen on the sulphur moiety, consistent with the literature (Concetti and Gariboldi, 1990; Wishart et al., 1995).

Two-dimensional TOtal Correlation SpectroscopY (TOCSY) experiments of native and oxidised κ-casein were also performed (Figure 5.12). No cross-peaks from methionine residues (Met-95 and Met-106) were observed in the TOCSY spectra of native κ-casein, presumably as they reside within the hydrophobic portion of para-κ-casein, encompassing residues from Glu$^{1}$ to Phe$^{105}$ (Swaisgood, 1992)). Therefore, the methionine residues are
buried within with the κ-casein micelle and are not observed as a result of resonance broadening due to slow protein tumbling. In contrast, cross-peaks were observed for methionine sulfoxide residues in the TOCSY spectra of oxidised κ-casein, resulting from an increase in conformational flexibility of the methionine residue upon oxidation. The methionine sulfoxide spin-spin systems: $H^\alpha$ (4.47) - $2H^\gamma$ (2.90, 2.91) - $2H^\beta$ (2.22, 2.10) (Figure 5.12) were consistent with those of other methionine sulfoxides in the literature (Rashid et al., 2000). The γ-methylene protons are significantly more downfield than those expected of native methionine (Wishart et al., 1995) as a result of the electronegative oxygen on the sulphur moiety, consistent with previously published NMR data (Rashid et al., 2000). The α- and β-methylene protons are less affected by the addition of the electronegative oxygen as they are situated further away and as such, exhibit chemical shifts similar to that of native methionine (Wishart et al., 1995; Rashid et al., 2000). No cross-peaks were observed from N-H protons as they exchange with D$_2$O in solution. Arginine cross-peaks (Wishart et al., 1995) were also observed in the TOCSY spectra of oxidised κ-casein which were absent in the TOCSY spectra of native κ-casein (Figure 5.12). The five arginine residues present in κ-casein (at positions 10, 16, 34, 68, and 97) are located within para κ-casein (Glu$^1$-Phe$^{105}$) and therefore are buried within the κ-casein micelle and not observed in the native TOCSY spectra due to resonance broadening. The presence of arginine cross-peaks in oxidised κ-casein, possibly due to Arg-97, i.e. adjacent to Met-96, provides further evidence of localised unfolding of κ-casein upon oxidation. However, no significant structural changes to κ-casein upon oxidation were observed by $^1$H NMR spectroscopy, consistent with CD and fluorescence spectroscopy data.
Figure 5.1: MALDI-TOF mass spectra of native and oxidised κ-casein following tryptic in-gel digest. Peak assignments corresponding to the tryptic κ-casein peptide fragments are indicated. An addition of 16 Da corresponds to the oxidation of a methionine residue. Arb.U., arbitrary units.
Figure 5.2: Fibril formation of native and oxidised κ-casein (1.0-2.5 mg/mL) monitored by ThT fluorescence over 18 hrs in the absence (A and B) or presence of 20 mM DTT (C and D). Samples were incubated in 50 mM phosphate buffer, pH 7.4 at 37 °C. The reaction order of native and oxidised κ-casein fibril formation was determined by plotting, ln(rate) against ln[κ-
casein], in the absence (E) or presence of DTT (F). The reaction rate was determined from the initial rate of increase in ThT fluorescence from data, shown in A-D. A.U., arbitrary units.

**Figure 5.3**: Transmission electron micrographs of native and oxidised κ-casein. Protein samples, at 1.5 mg/mL, were incubated in 50 mM phosphate buffer, pH 7.4 at 37 °C for 20 h. Native (A and C) and oxidised κ-casein (B and D) in the absence (A and B) or presence of DTT (C and D). Scale bars represent 500 nm and 200 nm respectively.
Figure 5.4: Fibril formation of native and oxidised κ-casein (3.4 mg/mL) incubated with increasing amounts of β-casein over 18 hrs in either the absence (A and B) or presence of 20 mM DTT (C and D). Samples were incubated in 50 mM phosphate buffer, pH 7.4 at 37 °C. The ability of β-casein to prevent fibril formation of native and oxidised κ-casein in absence (E) or presence of 20 mM DTT (F) was quantified as a percentage protection. Percentage protection was determined by comparing the maximum change in ThT fluorescence with and
without β-casein present. Molar ratios are indicated. Error bars represent the standard error of at least three independent experiments. * represent P value <0.05 (t-test, one-tailed, unequal variance). A.U., arbitrary units.

Figure 5.5: Transmission electron micrographs of native (A-D) and oxidised (E-H) κ-casein. Protein samples, at 3.4 mg/mL, were dissolved in 50 mM phosphate buffer, pH 7.4, at 37 °C under reducing conditions with 20 mM DTT and incubated for 20h with increasing
concentrations of β-casein (0:1, 0.25:1, 0.5:1 and 1:1 molar ratio of β-casein:κ-casein). Scale bars represent 500 nm.

**Figure 5.6:** The thermal aggregation at 42 °C of ADH (1 mg/mL) in 50 mM phosphate buffer, pH 7.4, 2 mM EDTA, in the presence of increasing concentration of native κ-casein (A) or oxidised κ-casein (B). The thermal aggregation at 55 °C of catalase (1 mg/mL) in 50 mM phosphate buffer, pH 7.4, in the presence of increasing concentration of native κ-casein

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(C) or oxidised κ-casein (D). The chaperone activity of native and oxidised κ-casein was quantified by comparing the initial rate of aggregation with and without chaperone present (E and F). Molar ratios are indicated. Error bars represent the standard error of at least three independent experiments.

**Figure 5.7:** Cell viability of PC-12 cells treated with native and oxidised κ-casein pre-incubated at 37 °C for 20 h in the presence of 2 mM DTT. Final concentration of κ-casein is shown. Error bars represent standard error of at least three independent experiments, * represent P value <0.05 (t-test, one-tailed, unequal variance).

**Figure 5.8:** Extrinsic ANS fluorescence spectra of native and oxidised κ-casein (0.5 mg/mL) in 50 mM phosphate buffer, pH 7.4. Figure illustrates the mean maximum fluorescence.
Figure 5.9: Intrinsic fluorescence spectra of native and oxidised κ-casein (0.5 mg/mL) in 50 mM phosphate buffer, pH 7.4. Arb.U., arbitrary units.

Figure 5.10: Far-UV circular dichroism spectra of native and oxidised κ-casein (0.2 mg/mL) in 10 mM phosphate buffer, pH 7.4 at 25 °C
**Figure 5.11:** One-dimensional $^1$H NMR spectra of native and oxidised κ-casein (100 µM) in deuterated phosphate buffer (50 mM, pH 7.4) in D$_2$O recorded at 25 °C. The ε-methyl protons of methionine are observed for oxidised κ-casein.
Figure 5.12: $^1$H 2-D TOCSY NMR spectra of native (red) and oxidised (blue) $\kappa$-casein in deuterated phosphate buffer (50 mM, pH 7.4) in D$_2$O at 25 °C. Cross-peaks for an oxidised methionine spin-spin system: $H^\alpha$ (4.47 ppm) - 2$H^\gamma$ (2.89 ppm, 2.91 ppm) - 2$H^\beta$ (2.22 ppm, 2.10 ppm) are indicated. No cross-peaks are observed for the singlet methionine methyl protons ($H^\delta$) in TOCSY experiments. Cross-peaks for an arginine are also observed after oxidation ($H^\alpha$ (4.26 ppm) - 2$H^\delta$ (3.14 ppm) - 2$H^\beta$ (1.82 ppm, 1.71 ppm) - 2$H^\gamma$ (1.57 ppm)).
5.5 Discussion

It is well known that side chain substitutions can have profound effects on protein stability and structure. The oxidation of methionine is regarded as a form of chemical ‘mutagenesis’ in which the methionine side chain is substituted with methionine sulfoxide, resulting in a larger and more polar side chain (Kim et al., 2001). It is therefore not surprising that oxidation of methionine can greatly perturb protein structure and affect its stability and hence activity (Volkin et al., 1997; DalleDonne et al., 1999). While the oxidation of proteins has been implicated as a causative or contributing factor in many diseases (Dean et al., 1997), the oxidation of methionine appears to be a common modulator of fibril formation whereby it inhibits the aggregation of Aβ (Hou et al., 2002), prion protein (PrP) (Breydo et al., 2005), transthyretin (Maleknia et al., 2006), α-synuclein (Uversky et al., 2002b) and apolipoprotein C-II (Binger et al., 2008). It is therefore interesting that oxidation of κ-casein did not restrict its ability to form fibrils in the presence or absence of DTT (Figure 5.2). In fact, a significantly greater rate of increase in ThT fluorescence was observed upon oxidation of κ-casein’s methionine residues, which was a result of fibril formation, as confirmed by TEM (Figure 5.3). Furthermore, β-casein, which has been proposed to prevent large-scale fibril formation of κ-casein in vivo (Thorn et al., 2005), was less efficient at suppressing amyloid fibril formation by oxidised κ-casein (Figure 5.4 & 5.5).

Several amyloidogenic proteins whose fibril formation is inhibited by methionine oxidation have a key methionine residue located within their fibril core region. For example, Aβ has a methionine at residue 35, which is positioned within the core fibril region (Luhrs et al., 2005), and oxidation of this residue inhibits fibril formation (Hou et al., 2002). Fibril formation of apolipoprotein C-II is particularly sensitive to oxidation or substitution of Met-60 (Binger et al., 2008), which lies within one of its fibril-forming core regions (Wilson et al., 2007). Likewise in the peptide, ccb-Met (Steinmetz et al., 2008), a number of site-specific
hydrophobic interactions are responsible for the formation of the highly stable amyloid structure (Steinmetz et al., 2008). Perturbation of one of these residues by the insertion of a single oxygen moiety has drastic effect on the kinetics and dynamics of fibril assembly (Steinmetz et al., 2008). Therefore, in general, the particular environment and/or position of a methionine residue in a protein play an important role in its ability to regulate fibrillogenesis. κ-Casein contains two methionine residues; Met-95 is situated in the very hydrophobic N-terminal domain roughly encompassing resides Glu\textsuperscript{1}-Phe\textsuperscript{105} (para-κ-casein), while Met-106, is located in the polar C-terminal domain (Swaisgood, 1992). We have shown that the fibril-forming core of κ-casein encompasses residues Tyr\textsuperscript{25}-Lys\textsuperscript{86} (Ecroyd et al., 2008), and therefore, it is not surprising that oxidation of Met-95 and Met-106 to the more polar methionine sulfoxide has no inhibitory effect on fibril formation. It may also help to explain why oxidation of native κ-casein makes it a more effective chaperone against the thermally aggregating target proteins, catalase and ADH (Figure 5.6). The larger more polar methionine residues may help solubilise κ-casein and the complex it forms with unfolding target proteins in an analogous manner to the phosphate groups which solubilise α\textsubscript{s} and β-caseins complexes with partially folded target proteins during chaperone action (Matsudomi et al., 2004; Koudelka et al., 2009). The flexible, unstructured and polar C-terminal extensions in small heat-shock proteins, e.g. α-crystallin, perform a similar role for this class of chaperone proteins during interaction with destabilised target proteins (Carver and Lindner, 1998; Carver, 1999; Carver et al., 2003).

In contrast, α-synuclein has four methionines (Met1, Met5, Met116 and Met 127), outside its fibril forming core, i.e. E22-E105 (Heise et al., 2005), and its fibril formation is completely inhibited upon methionine oxidation (Uversky et al., 2002b). Similarly, selective oxidation of Met-9 of apolipoprotein C-II, which is not in its fibril core, also significantly inhibits fibril formation, but not to the same extent as oxidation of Met-60 (Binger et al., 2008). The
difference in fibril-forming sensitivity to oxidation between κ-casein and α-synuclein may be
attributed in their different modes of fibril aggregation. Fibril-formation of α-synuclein occurs
via a nucleation-dependant polymerisation mechanism (Wood et al., 1999), with a critical
structural transformation from the unfolded conformation of α-synuclein to a partially folded
species (Uversky et al., 2001). Fibril-formation of RCM-κ-casein occurs by a novel
mechanism, whereby the rate of fibril formation is limited by the dissociation of the
amyloidogenic precursor from an oligomeric state (Ecroyd et al., 2008). Molecular modelling
of the dissociated κ-casein monomer indicates that, although predominately unfolded, it can
adopt a structural conformation containing a large hydrophobic and aromatic rich sheet-turn-
sheet motif (Kumosinski et al., 1993). It is the anti parallel β-sheet structure of RCM-κ-casein
monomers that make it an ideal fibril precursor that is readily incorporated into nuclei or
growing fibrils, without the need for further unfolding or folding (Ecroyd et al., 2008).
Because folding/unfolding is not required for κ-casein’s fibril formation, and that methionine
residues are absent in its fibril-forming core region, it is conceivable that oxidation of
methionine residues has no inhibitory effect on κ-casein fibril formation. This conclusion is
supported by the observation that acidification of α-synuclein results in an acceleration of
fibril formation (Uversky et al., 2001; Uversky et al., 2002a). The acceleration of α-synuclein
fibril formation is attributed to the pH-induced stabilisation of a partially folded intermediate,
that has significant β-structure, (Uversky et al., 2001) which is not affected by methionine
oxidation (Uversky et al., 2002b; Hokenson et al., 2004). Similarly, because κ-casein exists as
a paritally folded, amyloidogenic species that readily undergoes fibrillation and little, if any,
folding is required for its fibrillation, methionine oxidation has no inhibitory effect.

As discussed above, in vitro, the rate of fibril formation upon κ-casein oxidation was
significantly higher than in the native state. To determine the possible implication of
increased fibril formation in a cellular context, PC12 cells were used to assess the toxicity of
κ-casein upon oxidation. κ-Casein fibrils were formed by incubating κ-casein with 2 mM DTT for 20 hrs at physiological temperature. DTT has been shown to protect against amyloid toxicity, e.g. in β-amyloid cytotoxicity to neurons in culture is prevented with 2 mM DTT (Brera et al., 2000). However, the presence of residual DTT (20 μM) did not have a protective effect on cell survival. Moreover, by first allowing for the formation of amyloid fibrils in a controlled environment in vitro, we avoided interference by protective extracellular molecular chaperones, such as clusterin and BSA, which are present in cell media (Marini et al., 2005; Yerbury et al., 2007). Oxidised κ-casein was significantly more toxic to cells with reduced cell survival when compared to native κ-casein fibrils formed under the same conditions (Figure 5.7). It has been shown that mature and protofibrillar RCM-κ-casein fibrils are more toxic than their pre-fibrillar counterparts (Hudson et al., 2009). Therefore, the increase rate of ThT fluorescence observed for oxidised κ-casein under reducing and non-reducing conditions (Figure 5.2) may correlate to more mature and protofibrillar species present after 20 hrs of incubation, increasing cellular toxicity.

To determine the effect of methionine oxidation on κ-casein’s secondary and tertiary structure CD, intrinsic and extrinsic fluorescence spectroscopic studies were undertaken. Somewhat counterintuitively, the oxidation of methionine led to an increase in fluorescence of the hydrophobic probe ANS (panels A and B of Figure 5.8), presumably through greater unfolding of the protein leading to greater exposed, clustered regions of hydrophobicity at the protein surface. The intrinsic fluorescence of the single tryptophan residue in κ-casein (Trp-76), which resides in the fibril forming core of the protein i.e. Tyr25-Lys86 (Ecroyd et al., 2008), was affected marginally by methionine oxidation with a slight decrease in the maximum fluorescence intensity but with no change in the maximum wavelength of fluorescence (Figure 5.9). This implies that Trp-76 is quenched but not unfolded within the highly hydrophobic and structured area of the protein upon methionine oxidation (Freifelder,
The CD data are consistent with this conclusion since no significant change in gross secondary structure occurs in κ-casein upon oxidation (Figure 5.10). 1- and 2-D (TOCSY) NMR experiments resulted in the addition of weak cross-peaks corresponding to methionine and arginine upon oxidation which suggest that these residues have greater conformational flexibility and are more solvent exposed compared to the bulk of the protein (Figures 5.11 and 5.12). However, no significant structural changes occurred to the unstructured and flexible region(s) of κ-casein upon oxidation. The minor structural changes observed for κ-casein upon oxidation are similar to the situation observed for other amyloid forming proteins upon oxidation such as Aβ-(1-42) and α-synuclein. Both Aβ-(1-42) and α-synuclein do not exhibit significant change in their overall structure upon oxidation with both the native and oxidised forms of the proteins predominately random-coil in structure (Hou et al., 2002; Uversky et al., 2002b; Hokenson et al., 2004).

Unlike Aβ-(1-42), α-synuclein, prion protein (PrP) (Breydo et al., 2005), transthyretin (Maleknia et al., 2006), and apolipoprotein C-II (Binger et al., 2008), the oxidation of methionine on κ-casein does not inhibit its fibril formation, instead an increase in the rate of fibril formation was observed. A possible explanation is that (a) the methionine residues are outside of κ-casein’s fibril-forming region and (b) that κ-casein has a unique mechanism of fibril formation; whereby the rate-limiting step is the dissociation of an amyloidogenic precursor from an oligomeric state rather than the formation of stable nuclei (Ecroyd et al., 2008) and therefore, κ-casein fibril-formation is not inhibited by oxidation. A recent study of apolipoprotein A-1 (ApoA-1) has shown that while it does not form fibrils in its native state under various solution conditions that are known to induce fibril formation (e.g. extreme pH, temperature, presence of denaturants, etc.), ApoA-1 has a propensity to form fibrils upon methionine oxidation at 37 °C and pH 6.0 (Wong et al., 2010). Moreover, ApoA-1 is subject to an oxidative modification in vivo, and the oxidised form exists in normal plasma (Wong et
While κ-casein has been shown to form fibrils (Farrell et al., 2003; Thorn et al., 2005), we have shown that oxidation of methionine residues leads to an increase in fibril formation. This may have implications in vivo as casein micelles are secreted and stored as milk in the alveolar lumen (Nickerson et al., 1985), which is an extracellular oxidative environment. Furthermore, β-casein, which has been proposed to prevent large-scale fibril formation of κ-casein in vivo (Thorn et al., 2005), was less efficient at suppressing amyloid fibril formation by oxidised κ-casein.

5.6 Acknowledgements

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Chapter 6

Summary/Future Directions
6.1 Summary

Proteins constantly face stresses from their environments. Thus, stress conditions, e.g. elevated temperature, alteration in pH or oxidation can cause a native, functional protein to unfold and adopt partially folded, intermediate states. These intermediates have their hydrophobic core more exposed to solution and can mutually associate via hydrophobic interactions, leading to aggregation and potentially precipitation, with the aggregates adopting either highly ordered (amyloid fibril) or disordered (amorphous) forms. The deposition of amyloid fibrils in human tissue is implicated in the pathogenesis of some of modern society’s most debilitating diseases, including Alzheimer’s, Parkinson’s and Huntington’s diseases, and Type II diabetes (Kelly, 1998; Horwich, 2002; Dobson, 2003). From an applied point of view, the sensitivity of proteins to heat stress also creates challenges in developing protein-based beverages as thermal processing can cause undesirable levels of aggregation leading to excessive turbidity and possibly precipitation or gelation. However, when gelation and coagulation is desired (i.e. cheese production) this can be engineered through the addition of rennet, or acid treatment. In biological systems, molecular chaperone proteins inhibit undesirable protein aggregation, and assist in the stabilisation of partially folded target proteins, preventing protein misfolding and aggregation. Caseins, the major protein found in milk, exhibit chaperone activity as they stabilise heat-, light- and chemically-stressed proteins, inhibiting their aggregation and precipitation (Yong and Foegeding, 2010). The chaperone activity of caseins is of particular commercial interest due to their ability to stabilise other proteins during food processing, e.g. the heat treatment of milk during pasteurisation and the production of milk-related products whereby the whey proteins (e.g. α-lactalbumin and β-lactoglobulin) are destabilised and aggregate under conditions of elevated temperature.

While many recent papers have investigated the chaperone ability of caseins, in chapter 2 of this thesis, we examined the importance of casein phosphate groups, which are present in all
caseins to varying degrees. Both αs- and β-casein were dephosphorylated and their chaperone activity tested against two types of protein misfolding, i.e., amorphous aggregation and amyloid fibril-formation. It was conclusively shown that dephosphorylation of αs- and β-caseins resulted in a decrease in the chaperone efficiency against both heat- and reduction-induced amorphously aggregating target proteins. In contrast, both native and dephosphorylated αs- and β-casein were as effective at inhibiting amyloid fibril formation of target proteins. The decrease in casein chaperone ability against amorphously aggregating target proteins was attributed to the loss of charge and hydrophilicity of αs- and β-casein, decreasing their ability to solubilise unfolding target proteins upon binding. It was also shown that the large bulky phosphate groups contribute to maintaining the open, flexible nature of caseins, presumably as a result of charge-charge repulsion in the vicinity of the negatively charged phosphorylated serine residues. It was concluded that both the amphiphatic nature of αs- and β-casein and their flexible, dynamic, relatively unstructured nature are important for their chaperone action.

In chapter 3, we investigated the mechanism of β-casein chaperone action, by identifying particular residues in β-casein that are involved in binding to the unfolding milk-protein, reduced α-lactalbumin. Under chaperone conditions, size-exclusion chromatography was used to purify the complex between β-casein and reduced α-lactalbumin. A combination of limited proteolysis and mass spectrometry conclusively showed that residues Ala94 to Trp143 encompass β-casein’s chaperone-binding site. In the presence of endoproteinase Glu-C, β-casein bound to α-lactalbumin showed markedly reduced proteolysis at residues Ala91, Ala101 and Glu121 compared with β-casein in the absence of α-lactalbumin, indicating that the bound α-lactalbumin restricted access of Glu-C to residues from Ala91 to Glu121 on β-casein. Chymotrypsin also cleaved within the C-terminal domain of β-casein, with reduced proteolysis at residues between Gly94-Trp143 when β-casein was bound to reduced α-
lactalbumin. Trypsin cleaved at both the N-terminal (Arg$^{25}$ and Lys$^{28}$) and C-terminal (Val$^{170}$-Val$^{209}$) domains of β-casein. However, no difference in proteolysis was observed between β-casein and β-casein bound to reduced α-lactalbumin. Therefore, the polar, phosphorylated residues between Arg$^{1}$ and Lys$^{28}$ are not involved in binding to reduced α-lactalbumin. Instead these residues are likely to assist in solubilising native β-casein and β-casein when it is bound to unfolding target proteins. The results are consistent with experiments performed by Matsudomi et. al. whereby the phosphopeptide Arg$^{1}$-Arg$^{25}$ showed no measurable level of suppression against ovotransferrin aggregation (Matsudomi et al., 2004). The residues from Met$^{170}$ to Val$^{209}$, which are highly hydrophobic, were also determined not to be involved in binding to destabilized and reduced α-lactalbumin, instead they are involved in the hydrophobically driven self-association of β-casein polymers (Thompson et al., 1967; Berry and Creamer, 1975; Andrews et al., 1979).

Interestingly, while caseins are able to inhibit both amorphous aggregation and amyloid fibril formation, both α$_{s2}$- and κ-casein, the minor constituents of milk, are able to form fibrils in vitro (Thorn et al., 2005; Thorn et al., 2008). It is highly likely that α$_{s2}$- and κ-casein are involved in the formation of amyloid fibrillar bodies identified from corpora amylacea (CA) in mammary glands (Claudon et al., 1998; Niewold et al., 1999). As such, in chapter 4, we set out to further our understanding of casein fibril formation. Limited proteolysis and mass spectrometry were used with native and fibrillar RCM κ-casein to determine the regions likely to initiate fibril formation, i.e., the β-sheet core. Evidence was presented that residues from Tyr$^{25}$ to Lys$^{86}$ were incorporated into the core of the fibrils, which is in good agreement with molecular modelling and protein disorder/aggregation algorithms. It is this region, which we predict accounts for the amyloidogenic nature of κ-casein (Ecroyd et al., 2008). Similar experiments were performed with dimeric α$_{s2}$-casein fibrils indicating a protease-resistant
core between Ala\textsuperscript{81} to Lys\textsuperscript{181}, which is consistent with peptide fragments that have been isolated from amyloid deposits associated with mammary CA.

Oxidation of selected methionine residues in various proteins (e.g. Aβ-peptide) is linked to the pathogenicity of several amyloid diseases including Alzheimer’s disease (Dean \textit{et al.}, 1997; Hou \textit{et al.}, 2002). These diseases are characterized, \textit{in vivo}, by the accumulation of insoluble proteinaceous plaques, of which the major constituents are amyloid fibrils. Interestingly, \textit{in vitro}, methionine oxidation has been shown to modulate fibril assembly in several well-characterised amyloid systems (Breydo \textit{et al.}, 2005; Maleknia \textit{et al.}, 2006; Binger \textit{et al.}, 2008). Bovine κ-casein contains two methionine residues (Met-95 and Met-106) and readily assembles, \textit{in vitro} at physiological pH (Thorn \textit{et al.}, 2005), to form amyloid fibrils, thus providing a convenient system to examine the effect of methionine oxidation on amyloid fibril formation. In \textbf{chapter 5}, upon methionine oxidation an increase in the rate of κ-casein fibril-formation and an enhanced level of cellular toxicity was conclusively shown. In addition, β-casein, which is able to inhibit κ-casein fibril-formation, \textit{in vitro}, was observed to be less effective at suppressing fibril-formation of κ-casein upon methionine oxidation. Thus, β-casein is like other chaperones (e.g. the small heat-shock proteins) in that it is a poorer chaperone under conditions of enhanced target protein aggregation (Carver \textit{et al.}, 2002; Treweek \textit{et al.}, 2003). This may have implications \textit{in vitro}, as milk is stored and secreted from the alveolus lumen, an oxidative extracellular environment. Oxidative stress may, therefore, be a causative agent in the pathogenesis of \textit{corpora amylacea}.

6.2 \textbf{Future studies}

This study has conclusively shown the importance of phosphate groups in casein chaperone activity (\textbf{chapter 2}). A potential avenue for future research would be to investigate the effect of hyper-phosphorylation on casein chaperone action as α\textsubscript{s}, β- and κ-casein all have
additional serine residues that are not post-translationally modified. Hyper-phosphorylation could be achieved either chemically (e.g. via the addition of phosphorus oxychloride, phosphoric acid, sodium trimetaphosphate) or enzymatically (protein kinases) (Wold, 1981; Matheis and Whitaker, 1984). Depending on the target protein and the phosphorylating reagent the functional properties of proteins can be significantly altered upon chemical phosphorylation (Matheis and Whitaker, 1984). For example, when phosphorus oxychloride (POCl$_3$) is used as the phosphorylating reagent, a decrease in water solubility and an increase in gel-forming properties of phosphorylated proteins are observed (Matheis and Whitaker, 1984). These changes in physical properties are attributed to protein cross-linking which occurs upon phosphorylation with POCl$_3$ (Matheis and Whitaker, 1984). In contrast, the phosphorylation of soy protein using sodium trimetaphosphate led to much improved functional properties of the protein including solubility, water-holding capacity, emulsifying activity and foaming properties (Hsien-Yi et al., 1983). Whether, phosphorylation of food proteins modifies their properties, e.g. increases the chaperone activity of casein proteins, is still to be determined. However, a recent study on the role of phosphorylation on the chaperone action of $\alpha$B-crystallin has been reported (Ecroyd et al., 2007). Recombinant $\alpha$B-crystallin was mutated to mimic its natural serine phosphorylation by replacement of the relevant serine residues with negatively charged aspartic residues (Ecroyd et al., 2007). Mimicking phosphorylation of $\alpha$B-crystallin resulted in more efficient chaperone activity against heat- and reduction-induced amorphous aggregation of target proteins and also against $\kappa$-casein, an amyloid-forming target protein (Ecroyd et al., 2007). Likewise, elevated phosphorylation of caseins may lead to an increase in chaperone activity which has commercial potential, e.g. a lower concentration of casein proteins could be use to stabilise a higher concentration of whey protein isolate under stress conditions, e.g. elevated temperature.
This study involved a thorough investigation into the chaperone binding region of β-casein in the presence of reduced α-lactalbumin an amorphously aggregating target protein (chapter 3). Additional experiments could involve determining the chaperone binding region of β-casein in the presence of amyloid-forming target proteins such as κ-casein and α-synuclein. Conceivably, different regions could be involved in the binding of amorphously aggregating and fibril-forming target proteins as may occur in sHsps (Ecroyd et al., 2007). Similarly, the chaperone binding region of αs-casein could also be investigated against amorphously aggregating or amyloid-forming target proteins. Identifying peptide regions involved in chaperone binding increases our understanding of the mechanism of chaperone action. In addition, it may also assist in the development of small proteins/peptides or other analogues to act as molecular chaperones, thereby mimicking the actions of chaperones in vivo with possible therapeutic applications.

In chapter 4 of this thesis, we identified residues from Tyr25 to Lys86 to be incorporated into the core of κ-casein fibrils. While three-dimensional modelling and protein disorder/aggregation algorithms were in concordance with this result (Kumosinski et al., 1993; Ecroyd et al., 2008), additional experiments are needed to determine whether this region (Tyr25 to Lys86) forms fibrils itself. Previous experiments on the fibril-forming propensity of para-κ-casein (Glu1-Phe105, resulting from the specific cleavage of the Phe105-Met106 bond by chymosin, as in cheese manufacture) has been hampered by the insolubility of this highly hydrophobic polypeptide in phosphate buffer (pH 6) (Leonil et al., 2008). However, with appropriate solution conditions, e.g. change in buffer, the addition of organic solvent or salt, the fibril forming propensity of para-κ-casein could be investigated and tested against full-length κ-casein. Preparation of the polypeptide Tyr25-Lys86 could also be achieved via recombinant protein expression, or purification from trypsin-treated fibrils and its fibril-forming propensity investigated against full-length κ-casein. In addition, peptide fragments
(e.g. 10 amino acids each) Tyr^{25}-Lys^{86} could be synthesised and their fibril-forming propensity investigated. This approach has been used to localise specifically the fibril-forming region in apolipoprotein C-II (Wilson et al., 2007).

In chapter 4, we also determined that residues from Ala^{81} to Lys^{181} are incorporated into the core of α_{s2}-casein fibrils. However, smaller peptide fragments, i.e. Ala^{81}-Lys^{113}, Ala^{81}-Lys^{114}, and Thr^{138}-Lys^{181} in α_{s2}-casein were also determined to be resistant to trypsin proteolysis. Preparation of these various peptides (via recombinant protein expression, purification or synthesis) and examination of their fibril forming propensity is needed to determine the exact residues responsible for α_{s2}-casein fibril formation. Qin et al., (2007) using limited proteolysis and MS determined that residues ~32-102 comprise the β-sheet core of α-synuclein fibrils. However, while truncated Syn30-103 formed fibrils, a larger truncation (Syn30-140) that included the C-terminal region was necessary for the formation of mature, full length α-synuclein fibrils (Qin et al., 2007). Similarly, while peptides Ala^{81}-Lys^{113}, Thr^{138}-Lys^{181}, and Ala^{81} to Lys^{181} were determined to be resistant to proteolysis, it may be that the longer peptide fragment, Ala^{81} to Lys^{181}, is necessary for the formation of stable, mature α_{s2}-casein fibrils.

Amyloid fibrils have attracted considerable interest because of their intimate association with amyloid-fibril diseases including Alzheimer’s disease, Parkinson’s disease, Huntington’s disease senile systemic amyloidosis, the spongiform encephalopathies (e.g Creutzfeld-Jakob disease) and other prion-related diseases (Dobson, 1999; Dobson, 2003; Stefani and Dobson, 2003; Ecroyd and Carver, 2008). However, the occurrence of amyloid fibrils is not limited to disease and several fibrillar species have been discovered that have positive functions, for example, the extracellular amyloid fibrils known as curli which occur on the surface of Escherichia coli, chaplins from Streptomyces coelicolor, malarial coat protein, spider silk
(some but not all spiders) and mammalian melanosomes (Gras, 2007; Hammer et al., 2008). These species are known as functional amyloid. The highly structured nature of amyloid fibrils, their ability to self-assemble, their inherent strength and their stability have elicited considerable research interest of late in their potential use as bionanomaterials, for example as drug delivery agents and in the field of bioelectronics (Gras, 2007; Ecroyd and Carver, 2009). Amyloid fibrils have a size and structure comparable with man-made nanoscale structures, such as carbon nanotubes and bucky balls (Katz and Willner, 2004). However, from a synthetic point of view, bionanomaterials which are prepared from amyloid fibrils have advantages over man-made nanomaterials in that they are biocompatible and in particular, the protein backbone of amyloid fibrils can be functionalised with additional peptides, protein groups or entities such as fluorophores and metalloporphyrins (Baxa et al., 2003; Baldwin et al., 2006).

While protein nanofibres have great potential within the nanotechnology industry, to realise this potential, nanofibres need to be manufactured on a commercial scale. The majority of protein nanofibres with desirable bionanotechnological properties have only been produced using expensive, and time-costly, small-scale methods (Garvey et al., 2009). However, Garvey et al., (2009) recently produced protein nanofibres from semi-pure and crude mixtures of bovine crystallin proteins, which are readily available and are currently a waste product in the meat industry (Garvey et al., 2009). Moreover, the morphology of these fibrils, varying between short and curly and closed loops to straight, can be altered significantly depending on the solvent conditions (Ecroyd and Carver, 2009; Garvey et al., 2009).

Amyloid fibrils can also be inexpensively produced from both κ-casein and to a lesser extent, \( \alpha_{s2} \)-casein, making them a potential industrial source of protein nanofibres. The difference in the morphology of the fibrils formed by \( \alpha_{s2} \) and κ-casein (RCM κ-casein assembles into rod-
like structures measuring up to 600 nm in length (Thorn et al., 2005), while αs2-casein forms twisted, ribbon-like fibrils that have a tendency to circularise to form closed loop structures (Thorn et al., 2008), could be exploited to make nanofibres of different shapes and sizes. Using different buffers and the presence of various exogenous compounds, e.g. lipids and crowding agents, will also modify fibrillar structure (Garvey et al., 2009). Furthermore, trypsin-treated αs2-casein fibrils exhibit a short and straight morphology, rather than the twisted ribbon-like morphology observed for untreated αs2-casein fibrils (Figure 4.9). Therefore, fibril-forming peptide fragments from αs2- and κ-casein could also be used to make various morphologies of amyloid fibrils. However, given κ-casein fibrils are toxic even at low concentration (Figure 5.7, Hudson et al., 2009) further research is needed to explore the possibility of utilising casein fibrillar species in the emerging field of bionanomaterials.


CHANAT, E., MARTIN, P. & OLLIVIER-BOUSQUET, M. 1999. Alpha(S1)-casein is required for the efficient transport of beta- and kappa-casein from the endoplasmic


Appendix 7.2 A: MS spectrum of native $\alpha_s$-casein in acetonitrile: 0.1% formic acid (1:1, v/v)
Appendix 7.2 B: MS spectrum of dephosphorylated $\alpha_s$-casein in acetonitrile: 0.1% formic acid (1:1, v/v)
Appendix 7.3 A: MS/MS spectrum from a Glu-C digest. Abs.Int. Absolute Intensity (arbitrary units)
2107.2 = LLYQEPVLGPVRGPFPIIV (L191-V209)

Appendix 7.3 B: MS/MS spectrum from a GluC-digest. Abs.Int. Absolute Intensity (arbitrary units)
Appendix 7.3 C MS/MS spectrum of an α-lactalbumin peptide from a GluC-digest. Absolute Intensity (arbitrary units)
Appendix 7.3 D: MS/MS spectrum from a trypsin digest. Abs.Int. Absolute Intensity (arbitrary units)
1590.9 = VLPVPQKAVPYPQR (V170 - K176)

Appendix 7.3 E: MS/MS spectrum from a trypsin digest. Abs.Int. Absolute Intensity (arbitrary units)
2908.5 = DMPIQAFLLYQEPVLGPVRGPFPIIV (D184 – V209)

Appendix 7.3 F: MS/MS spectrum from a trypsin digest. Abs.Int. Absolute Intensity (arbitrary units)
2970.6 = GVSKVKEAMAPKHKEMPFPKYPVEPF (G94 - F119)

Appendix 7.3 G: MS/MS spectrum from a chymotrypsin digest. Abs.Int. Absolute Intensity (arbitrary units)
Appendix 7.3 H: MS/MS spectrum from a chymotrypsin digest. Abs.Int. Absolute Intensity (arbitrary units)
3024.6 = SLSQSKVPVPQKAVPYPQRDMPIQAF (S164 - F190)

Appendix 7.3 I: MS/MS spectrum from a chymotrypsin digest. Abs.Int. Absolute Intensity (arbitrary units)
Appendix 7.3 J MS/MS spectrum from a chymotrypsin digest. Abs.Int. Absolute Intensity (arbitrary units)
Appendix 7.3 K: MS/MS spectrum from a chymotrypsin digest. Abs.Int. Absolute Intensity (arbitrary units)
Appendix 7.3 L: MS/MS spectrum from a chymotrypsin digest. Abs.Int. Absolute Intensity (arbitrary units)
Appendix 7.3 M: MS/MS spectrum from a chymotrypsin digest. Abs.Int. Absolute Intensity (arbitrary units)
Appendix 7.4 A: MS/MS spectrum of trypsin-treated RCM κ-casein fibrils. Abs.Int. Absolute Intensity (arbitrary units)
Appendix 7.4 B: MS/MS spectrum of trypsin-treated RCM κ-casein fibrils. Abs.Int. Absolute Intensity (arbitrary units)
Appendix 7.4 C: MS/MS spectrum of trypsin-treated RCM κ-casein fibrils. Abs.Int. Absolute Intensity (arbitrary units)
Appendix 7.4 D: MS/MS spectrum of proteinase K-treated RCM κ-casein fibrils. Abs.Int. Absolute Intensity
Appendix 7.4 E: MS/MS spectra of trypsin-treated $\alpha_{s_2}$-casein fibrils. Abs.Int. Absolute Intensity (arbitrary units)
2725.6 = FPQYLQYLQGPIVLNPWDQVK (F92 - K113)

Appendix 7.4 F: MS/MS spectra of trypsin-treated $\alpha_{s2}$-casein fibrils. Abs.Int. Absolute Intensity (arbitrary units)
Appendix 7.4 G: MS/MS spectra of trypsin-treated α_{s2}-casein fibrils. Abs.Int. Absolute Intensity (arbitrary units)
979.6 = FALPQYLK (F174 - K181)

Appendix 7.4 H: MS/MS spectra of trypsin-treated $\alpha_\text{s2}$-casein fibrils. Abs.Int. Absolute Intensity (arbitrary units)
Appendix 7.4 I: MS/MS spectra of trypsin-treated αs₂-casein fibrils. Abs.Int. Absolute Intensity (arbitrary units)
Appendix 7.4 J: MS/MS spectra of trypsin-treated α₂-casein fibrils. Abs.Int. Absolute Intensity (arbitrary units)
Appendix 7.5 A: MS spectrum of native κ-casein in acetonitrile: 0.1% formic acid (1:1, v/v)
Appendix 7.5 B: MS spectrum of oxidized κ-casein in acetonitrile: 0.1% formic acid (1:1, v/v)
Appendix 7.5 C: MS spectrum of native and oxidised κ-casein in acetonitrile: 0.1% formic acid (1:1, v/v)