Review of the chemistry of α_{S2} -casein and the generation of a homologous molecular model to explain its properties¹

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ABSTRACT

α_{S2} -Casein (α_{S2} -CN) comprises up to 10% of the casein fraction in bovine milk. The role of α_{S2} -CN in casein micelles has not been studied in detail in part because of a lack of structural information on the molecule. Interest in the utilization of this molecule in dairy products and nutrition has been renewed by work in 3 areas: biological activity via potentially biologically active peptides, functionality in cheeses and products, and nutrition in terms of calcium uptake. To help clarify the behavior of α_{S2} -CN in its structure-function relationships in milk and its possible applications in dairy products, this paper reviews the chemistry of the protein and presents a working 3-dimensional molecular model for this casein. The model was produced by threading the backbone sequence of the protein onto a homologous protein: chloride intracellular channel protein-4. Overall, the model is in good agreement with experimental data for the protein, although the amount of helix may be over-predicted. The model, however, offers a unique view of the highly positive C-terminal portion of the molecule as a surface-accessible area. This region may be the site for interactions with κ -carrageenan, phosphate, and other anions. In addition, most of the physiologically active peptides isolated from α_{S2} -CN occur in this region. This structure should be viewed as a working model that can be changed as more precise experimental data are obtained.

Key words: casein structure, casein molecular model, protein functionality, value-added milk product

INTRODUCTION

α_{S2} -Casein (α_{S2} -CN) comprises up to 10% of the casein fraction in bovine milk; it consists of 2 major and several minor components exhibiting varying levels of posttranslational phosphorylation (Swaigood, 1992; Farrell et al., 2004) as well as minor degrees of intermolecular disulfide bonding (Rasmussen et al., 1992). Studies of the association behavior of α_{S2} -CN (Snoeren et al., 1980) and of its secondary structure (Hoagland et al., 2001) are limited compared with other caseins. Also, the role of α_{S2} -CN in casein micelles has not been studied in detail. Interest in the use of this molecule in dairy products and nutrition has been renewed by work in 3 areas: biological activity, functionality, and nutrition.

α_{S2} -Casein peptides derived from its C-terminal section have been found, in in vitro assays, to have unique antibacterial properties (Zucht et al., 1995; Recio and Visser, 1999; McCann et al., 2005; Lopez-Exposito et al., 2006). Moreover, α_{S2} -CN peptides with angiotensin-I enzyme inhibitor properties have been identified (Tauzin et al., 2002). In vitro experiments (Tanabe et al., 2006) suggested that α_{S2} -CN peptides could serve to dampen allergenic responses. Thus, α_{S2} -CN-enriched preparations or proteolytic digests may have use in value-added or health-promoting dairy products (Lopez-Exposito and Recio, 2008). In addition, Kizawa et al. (1996) have purified peptides from α_{S2} -CN that inhibit calmodulin-activation of cyclic nucleotide phosphodiesterase, a major regulatory component of a variety of metabolic pathways. The peptides from α_{S2} -CN apparently bind to the regulatory protein calmodulin interfering with its binding to the enzyme. The physiological implications here are unknown, but other peptides from the same area of the molecule may be involved in promoting intestinal health and well being.

The second area relates to work on the functionality of caprine milks. Caprine caseins, in contrast to bovine caseins, vary considerably in the types of casein present: some are poor in α_{S1} -CN and some are richer in α_{S2} -CN. It has been speculated that the differences in α_{S2} -CN content account for the unique physicochemical

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characteristics of caprine caseins and explain a large part of differences in the technological behavior between caprine and bovine milks (Mora-Gutierrez et al., 1996, 1997; Mora-Gutierrez and Farrell, 2001). In this context, the proportions of α_S -CN fractions are in large part responsible for the differences observed between the 2 milks in cheesemaking (Ambrosoli et al., 1988; Grosclaude, 1988; Remeuf et al., 1995). It has also been suggested that caprine caseins rich in α_{S2} -CN offer enhanced sites for interactions with the common food additive κ -carrageenan (Farrell and Mora-Gutierrez, 2006).

Finally, from a nutritional point of view, a recent study compared native and calcium-fortified bovine and caprine cheeses in terms of calcium absorption and deposition in growing male rats (Mora-Gutierrez et al., 2007a). In the latter study, the caprine milk used was low in α_{S1} -CN but high in α_{S2} -CN. Significant differences in calcium absorption were found among the cheeses with the data yielding the pattern caprine calcium-fortified > caprine = bovine calcium-fortified > bovine > control (Mora-Gutierrez et al., 2007a). The enhanced calcium absorption resulted in the production of greater bone mass and correspondingly increased resistance to bone fracture in the rat femurs (Mora-Gutierrez et al., 2007a). In recent work it has been shown that calcium phosphopeptides (CPP) added to milks enhance calcium adsorption and deposition from added calcium carbonate in growing male rats (Mora-Gutierrez et al., 2007b). In particular, the CPP from caprine casein high in α_{S2} -CN was the most effective.

To help clarify the role of α_{S2} -CN in its structure-function relationships in milk and its possible applications in value-added dairy products, this paper reviews the chemistry of the protein and presents a working 3-dimensional (3D) model for the protein.

MATERIALS AND METHODS

Sequence Alignments

All sequence alignments and homology predictions were obtained using the T-coffee program of Notredame et al. (2000) found on the ExPASy Web site under tools-alignment (<http://tcoffee.vital-it.ch/cgi-bin/Tcoffee/tcoffee.cgi/index.cgi?stage1=1&daction=TCOFFEE::Regular>).

Molecular Modeling of Bovine α_{S2} -CN

A quick route to an approximate 3D model is to use another protein with a strong homology and a known 3D structure (Sawyer et al., 2002) as a guide. Such

structures for the caseins do not appear to be available through conventional database searches. Results from the T-coffee program of Notredame et al. (2000) suggested that the chloride intracellular channel (CLIC) molecules, although not closely related to α_{S2} -CN, had sufficient homology to proceed with the modeling work. The model was generated from the human CLIC-4 model (Q9Y696_C00001) found at http://swissmodel.expasy.org/repository/smr.php?job=2&sptr_ac=Q9Y696&model_name=Q9Y696_C00001. The Q9Y696 model was initially based on the x-ray template of a soluble fragment of human CLIC-1 (O00299; PDB file 1kOm). Initial alignments and set-up for the model were accomplished using the program Deep View/Swiss PDB Viewer 3.7 obtained from the ExPASy site. After initial manipulations by the author, the model was submitted as a project to SWISS-MODEL, an automated homology-modeling server; such modeling is computer based and produced without human intervention (Kopp and Schwede, 2004). Therefore, the model for α_{S2} -CN provides some basis for caution in that it is based on a secondary homology and initially produced without human intervention. Interpretation of the model must be carefully considered in that it has the characteristics of an x-ray structure but lacks the certitude of direct crystallographic data.

After viewing the original model it was apparent that the putative site for casein-casein interactions predicted by Kumosinski et al. (1994) was partially blocked (this could also represent the membrane interaction site in the CLIC proteins). Several manipulations of the bond angles at the base of the proposed site were attempted and the easiest change to accentuate the movement of the interaction site finally proved to be a minor adjustment of the phi/psi angles of Ala 116 from $-60/-60$ to $-60/0$. The pseudo-charge representation of the Ala adjusted molecule used the electrostatic surface calculation of the Deep View program; the parameters for this representation were dielectric constants of the solvent and the protein set at 80 and 5, respectively, the red color represents a value of -1.8 esu (electrostatic units), the white 0, and the blue is $+1.8$ esu.

Energy minimization of the molecule was carried out in vacuo using the program GROMOS96 found within the Deep View program from the ExPASy site (Stocker and van Gunsteren, 2000). The default settings with 20 steps of steepest descent and with a nonbonded cutoff of $10\text{\AA}$ were used with multiple rounds of minimization. The minimization was considered finished when the change between rounds was $<10\%$. Peptide models from α_{S2} -CN were excised from the final energy-minimized model, capped, and again energy minimized as described above.

RESULTS AND DISCUSSION

General Chemical Properties

α_{S2} -Casein was the last of the bovine caseins to be sequenced; it also has the most unique primary structure of the caseins (Brignon et al., 1977). The primary structure of the protein has been established by chemical sequencing (Brignon et al., 1977) and confirmed by cDNA sequencing (Stewart et al., 1987) and genomic DNA sequencing (Groenen et al., 1993). As shown in Figure 1, α_{S2} -CN consists of 207 amino acids with a molecular weight from sequence of 25,226 for the A variant with 11 phosphorylated serine residues (Farrell et al., 2004). There are 4 known genetic variants; the A variant predominates in western cattle (Farrell et al., 2004). Unlike the other caseins, which are rather uniformly phosphorylated, the number of phosphory-

lated residues in α_{S2} -CN varies from 10 to 13 (Farrell et al., 2004). The protein contains no known covalently bound carbohydrate residues, but does have 2 cysteine residues. In contrast to κ -CN, which occurs in milk as a large disulfide linked aggregate (Farrell et al., 2003), α_{S2} -CN appears to occur primarily as a monomer (Snoeren et al., 1980) with an internal disulfide; however, it can form dimers with the chains either parallel or antiparallel to each other (Rasmussen et al., 1992, 1994). In addition, minor amounts of α_{S2} -CN- κ -CN-linked polymers have been isolated (Rasmussen and Petersen, 1991). A convenient large-scale method has been developed for purification of the protein, which may facilitate future research (Vreeman and von Reil, 1990).
Brignon et al. (1977) pointed out that α_{S2} -CN has 2 very large segments of about 80 residues that display

Figure 1. The primary structure of α_{S2} -CN A-11P (Brignon et al., 1977 as modified by Farrell et al., 2004). Serine residues identified as phosphorylated (SeP) are indicated in bold italics.

Table 1. Linear profile of the α_{S2} -CN molecule derived from its primary structure

Residues considered	Net charge ¹	Charge frequency ²	Average hydrophobicity ²
1–41	–7.5	0.39	860
42–80	–14.5	0.51	780
81–125	+2.0	0.15	1,460
126–170	–5.0	0.49	861
171–207	+6.5	0.24	1,674

¹For the form of the molecule with 11 phosphorylated serine residues; serine phosphate = –2, histidine = +0.5.

²Calculated as described by Bigelow (1967).

sequence homology with each other and that may have arisen by gene duplication. Segments of the α_{S2} -CN sequences were reanalyzed using the T-coffee multiple sequence alignment tool (Notredame et al., 2000); the best homologous alignment was for residues 42–122 and 124–207 with a significant score of 50%. Curiously, the last half of the second segment (residues 170–207), although homologous (T-coffee = 50) with the last half of the first segment 86–123, is more hydrophobic than the latter and has a higher positive charge (Table 1). Residues 170–207 represent the largest section of positively charged residues in any of the caseins.

The N-terminal section of the molecule (residues 1–35) not only has a strong homology with all of the other casein phosphopeptide regions but also displays homology with the first half of each of the 2 large segments of α_{S2} -CN (residues 45–80 and 125–149, respectively) with a 3-way T-coffee score of 50%; all are also homologous to other casein phosphopeptides. This being the case, the overall molecule (Table 1) has 5 distinct areas composed of only 2 repeating sequences (a and b): area-a1, residues 1–41: a typical casein phosphopeptide region with high charge and low hydrophobicity; area-a2, residues 42–80: another typical phosphopeptide region; area-b1, residues 81–125: somewhat basic but with a high hydrophobicity; area-a3, residues 126–170: a phosphopeptide homolog with a high negative charge but low phosphate; and area-b2, residues 171–207: high hydrophobicity but with an extremely high positive charge (in summary: a-a-b-a-b).

It would appear that the sequences discussed above might give rise to the physical-chemical properties of the α_{S2} -CN molecule. The number of anionic clusters is related to the calcium binding properties of α_{S2} -CN. For example, α_{S2} -CN is more sensitive to Ca^{2+} than α_{S1} -CN (Toma and Nakai, 1973), with almost complete precipitation occurring in 2 mM Ca^{2+} for α_{S2} -CN at pH 7, whereas precipitation of α_{S1} -CN requires 6 mM Ca^{2+} (Aoki et al., 1985). Snoeren et al. (1980) concluded, from a combination of light scattering and viscosity studies, that the self-association of α_{S2} -CN is isodesmic

and produces spherical polymers of about 4 nm at 0.6 M NaCl. The alternating charged areas appear to make this casein the least susceptible to aggregation as initially purified from milk. Snoeren and coworkers (1980) presented a model in which this positively charged tail participates in the self-association of the protein with one of the negative charged sections; they argued for a surface position for the positively charged cluster. As noted above, caprine caseins (in contrast to bovine caseins) vary considerably in the types of casein present: some are poor in α_{S1} -CN and some are richer in α_{S2} -CN (Farrell, 1999). Thus, it is speculated that caprine caseins rich in α_{S2} -CN offer enhanced sites for interactions with negatively charged molecules such as κ -carrageenan (Farrell and Mora-Gutierrez, 2006); again this assumes that this positive cluster is on the surface of associated-whole casein. To test these theories in silico, a 3D model was developed and a preliminary report given (Farrell and Mora-Gutierrez, 2006).

A Homologous Molecular Model for α_{S2} -Casein

The concept that α_{S2} -CN might be involved in the binding of a highly negatively charged species such as κ -carrageenan is supported by an examination of its linear amino acid sequence. As noted above, the molecule contains 3 segments that contain highly charged negative residues due primarily to phosphoserine and glutamyl residues; these segments center on residues 10, 60, and 130. In contrast, the C-terminal 47 residues carry a net charge of +9.5. Because of the net hydrophobicity of the latter segment, it is uncertain whether this segment would be surface-accessible for interaction with the highly negatively charged κ -carrageenan. Hoagland et al. (2001) assessed possible structural arrangements that are in accord with currently available spectral data and one could argue from this analysis that the positively charged section might be surface-accessible. This latter argument is supported by the physical-chemical data of Snoeren et al. (1980). Although 3D molecular models have been developed for the other 3 major caseins (Kumosinski et al., 1994), no models have been published for α_{S2} -CN. One reason for this is the segmental domain nature of the molecule as detailed above and as first noted by Swaisgood (1992). In contrast, the other caseins are more linear amphiphiles with only 2 domains, one hydrophilic and one hydrophobic.

The CLIC molecules form a novel class of recently discovered proteins (Ashley, 2003; Paul and Beitel, 2003). These proteins are often found free in the cytosol of cells, but have the ability to alter their conformation and insert themselves into membranes within the cell (hence the name intracellular); in model systems,

Figure 2. Left: Trace of the backbone structure of α_{S2} -CN derived by homologous modeling. The amino acid sequence of α_{S2} -CN was threaded onto the model of CLIC-4. Right: Manipulation of Ala 116 to produce a hydrophobic core accessible for protein-protein interactions. Coiled ribbons represent α -helix, broad ribbons represent β -sheet, and arrowheads indicate the direction of travel of the chain from N- to C-terminal.

they allow chloride movement through membranes once inserted. The CLIC proteins interact with a variety of intracellular membranes such as mitochondrial, nuclear, Golgi, and sarcoplasmic (Fernandez-Salas et al., 1999; Shanks et al., 2002; Board et al., 2004; Suh et al., 2004). It is not known if the CLIC proteins bind directly to the membranes or to receptors on the membranes, nor is it established which portions of the molecules are inserted into the membranes (Berryman et al., 2004). Within this family there are 5 members termed CLIC proteins 1 to 5 that share strong structural homologies with each other. Known defects in these proteins can cause several molecular disease states (Cromer et al., 2002). Recently it has been shown that the loss of EXC-4, a CLIC protein of *Caenorhabditis elegans*, prevents opening of excretory vesicles (Berry et al., 2003). The interaction of the CLIC proteins by insertion into model bilayers appeared reminiscent of the interactions of β -CN with model membranes as detailed by Casanova and Dickinson (1998). Comparisons of the amino acid sequences of human CLIC proteins with bovine β -CN using the T-coffee program of Notredame et al. (2000) showed only marginal homology. Proteins CLIC-3 and CLIC-4, however, showed good homology with α_{S2} -CN as seen in Table 2. Although the degree of homology between the CLIC proteins and α_{S2} -CN is only about 58%, there was sufficient similarity to proceed with the production of a working 3D model by threading the α_{S2} -CN structure onto a model of CLIC-4. It must be noted that the CLIC-4 model was itself a working model produced from the crystal structure of a soluble monomeric fragment of human CLIC-1 found in the Swiss Model Repository of the ExPASy Web site. A crystal structure for CLIC-4 is now available (Li et

al., 2006), and the ExPASy tool Magic-Fit is able to align the model used to generate the α_{S2} -CN model with the new crystal structure of CLIC-4 with a root mean square (RMS) difference of only 0.86 Å. Thus, little change in the predicted α_{S2} -CN model is expected to occur in future studies. The homologous model produced for α_{S2} -CN is shown as a ribboned backbone trace in Figure 2 (left). In previous models for κ -CN, portions of a hydrophobic section of the molecule (residues 17 to 65) were used for docking sites for other caseins (Kumosinski et al., 1994). This section of κ -CN is also somewhat homologous with the central hydrophobic portion of α_{S2} -CN (residues 77 to 119) and to segments of the CLIC proteins, and could represent a protein-protein or protein-lipid interaction site. The homologous model showed this segment in α_{S2} -CN to be partially exposed for such interactions. The manipulation of only one residue (Ala 116) caused this hydrophobic section to further protrude into space to provide a good site for potential interactions with other caseins (Figure 2, right). This

Table 2. T-Coffee¹ scores for comparison of bovine α_{S2} - and β -CN with human chloride intracellular channel (CLIC) proteins

Protein	β -casein	α_{S2} -casein	CLIC proteins
CLIC-1	39	40	98 ²
CLIC-2	30	45	
CLIC-3	35	57	
CLIC-4	34	58	
CLIC-5	37	35	
α_{S2} -Casein	33		

¹Notredame et al. (2000).
²All human CLIC proteins average a score of 98 in pairwise comparisons.

Figure 3. A) Stereo view of the C α trace of the homologous model for α_{S2} -CN as given in Figure 2 (right panel); B) mono view of the energy-minimized model with backbone visible and ribboned for defined secondary structures (coiled ribbons represent α -helix, and broad ribbons represent β -sheet), and the free SH groups at residues 36 and 40 are indicated.

manipulation may also shed some light on the CLIC proteins, as the literature suggests the necessity of C- and N-terminal portions in membrane insertion (Paul and Beitel, 2003). Here, a central hydrophobic section is implicated for potential hydrophobic interactions. To understand the relationship of the model to the domains indicated above (Table 1), Figure 3A shows the model as a C α trace in stereo. The 2 phosphopeptide regions 8–21 and 54–63 (areas a1 and a2) represent the negatively charged left wing with the former being toward the front and the latter to the back; area b1, the somewhat basic area with a high hydrophobicity (81–125), represents the lower body of the molecule; and area a3 (126–170) with another phosphopeptide-like region and a high negative charge represents the top of the central body. Finally, area b2 (171–207) with high hydrophobicity and high positive charge is represented on right wing.

Because the FASTA format used by the ExpASY program does not support the amino acid phosphoserine (Pser), Glu was used to replace these residues to mimic the potential charge distribution on the molecule at neutral pH. Eleven Ser that are potentially phosphorylated (Figure 1) were replaced with Glu. The molecule was then energy minimized using the GROMOS program and, after adjusting side chains, the energy minimization was continued until the net total energy decreased by less than 10% of the total of the previous cycle. The energy distribution patterns of the molecule before and after minimization are given in Table 3. Using the ExpASY tool Magic-Fit, the RMS difference between the initial CLIC-based structure and the α_{S2} -CN energy-minimized model was 0.16 Å, indicating little difference between the 2 models. Figure 3B shows a ribboned version of the energy-minimized model, which is quite similar to Figure 2 (right) and shows Cys 36, Phe 39, and Cys 40 side chains.

Table 3. Energy profiles of the α_{S2} -CN molecular models following energy minimization using the GROMOS program

Energy	Energy, KJ/mol		
	Initial molecule before minimization	After minimization	
		11Glu-SH ¹	11Glu-S-S ²
Bond	35,681	165	180
Angle	3,738	1,170	1,207
Torsion	1,024	1,064	1,106
Improper	658	349	353
Nonbonded	21,321	–6,261	–6,272
Electrostatic	–7,060	–7,740	–7,717
Total	55,359	–11,250	–11,140

¹Following conversion of Ser to Glu.

²Following internal disulfide bond formation.

Figure 4. Pseudo charge surface representation of the α_{S2} -CN model. On the right the molecule is in the same orientation as in Figures 2 and 3. The negative charges (red) on the top left and center arise from the anionic sections of the molecule. The positive charges (blue) on the right arise from the C-terminal section of the molecule. The region extending from the center to 6 o'clock represents the central hydrophobic core; it is lightly colored and this section is free to react with other casein molecules as has been speculated for the homologous section of κ -CN (Kumosinski et al., 1994). The molecule on the left is rotated 90° clockwise displaying the C-terminal positively charged areas.

When the α_{S2} -CN working model was fitted with a surface and a pseudo-electrostatic charge calculated, a trefoil-like representation was produced as shown in color (Figure 4, right). Note at 6 o'clock the hydrophobic central core extends downward and on the upper surface, the separation of charges produces both a highly negatively charged domain (red) as well as a unique positive surface (blue) for the potential attachment of κ -carrageenan. With the exception of small portions of κ -CN and α_{S1} -CN (Malin et al., 2005), this represents the most definitive representation of positive charge on the caseins. The model on the left represents a 90° clockwise rotation to emphasize the positive surface charge. Thus, this working model supports the physico-chemical data suggestive of a role for α_{S2} -CN in the interaction of casein and κ -carrageenan (Farrell and Mora-Gutierrez, 2006).

Overall Shape and Size of the α_{S2} -CN Model

The overall dimensions of the molecule (Figure 4) are a vertical distance of 61.4 Å and a horizontal distance of 58.0 Å. The Stokes radius calculated from the longest dimension (sweep out volume) is 30.7 Å. This

yields a hydrated volume of 121,200 Å³. The latter value is smaller than the experimental value of 180,000 Å³ (Farrell et al., 2006b) and considerably smaller than the value of 240,000 from preliminary de novo modeling considerations (Hoagland et al., 2001; Hoagland and Farrell, 2002). Farrell et al. (2006b) argued, using the calculations and nomenclature of Uversky, that α_{S2} -CN, like α_{S1} -CN, would be a natively unfolded coil. The current model at 120,000 Å³ makes the protein more compact but still natively unfolded, and it would be classed as a premolten globule. Note that the α_{S2} -CN model may be more compact because it was threaded onto the CLIC-4 structure. Indeed, the α_{S2} -CN model and the CLIC-4 model have an RMS difference of only 0.16 Å, indicating that the CLIC-4 protein is also a premolten globule based upon its volume. In contrast, a compact globular protein with a sequence length of 205 residues would have a volume of 37,000 Å³ (Farrell et al., 2006b).

An interesting conjecture may be made at this point concerning the highly positive C-terminal section pictured in Figure 4. This segment along with the N-terminal region of α_{S1} -CN is one of the few areas in any of the caseins that could present a positive surface charge

(Malin et al., 2005). If, indeed, the C-terminal segment is surface oriented at physiological ionic strengths, then it may readily react with either the phosphopeptide regions of other caseins or with inorganic phosphate. Experimentally, it has been shown that the presence of calcium enhances phosphate binding to casein (Visser et al., 1979) and that modification of amino groups decreases calcium phosphate binding to α_{S1} -CN (Zhang and Aoki, 1995). The presence, surface accessibility, and stability of this highly positive region could anchor the developing colloidal calcium phosphate during micelle accumulation in the Golgi apparatus (Farrell et al., 2006a).

Correlation of Proteolytic Studies of α_{S2} -CN with the Model

Trypsin cleavage of α_{S2} -CN has been studied by Tauzin et al. (2002, 2003). In the latter paper they studied the time of release of tryptic peptides as a marker of surface accessibility of sections of the α_{S2} -CN molecule. They concluded that the C-terminal sites in the positively charged region (153–207) were released with $t_{1/2}$ values (where $t_{1/2}$ is the time required to release one half of the susceptible peptide bonds) of 22 to 30 min. Next, the N-terminal sites in the 1–90 region were more slowly released (32–35 min), whereas those sites in the center of the molecule (92–150) were the slowest to be hydrolyzed. In contrast, tryptic cleavage of purified κ -CN releases peptides from its hydrophilic C-terminal region with $t_{1/2}$ values of 20 to 40 s (Farrell et al. 1999). The ExPASy Accessibility tool measures the surface accessibility of all residues of a protein model on a color-coded scale (red, orange, yellow, green, and blue), with the estimates ranging from high (red) to low (blue). None of the potential trypsin sites of the α_{S2} -CN model were colored red, indicating that the potential trypsin cleavage sites are not highly surface accessible. A scale of 1 to 5 was assigned for the colored tryptic hydrolysis sites. Trypsin sites with an accessibility value of 1 would be expected to be rapidly released and would have $t_{1/2}$ values of several minutes, so the model is in agreement with the data indicating few if any rapidly released peptides. The C-terminal sites yielded an average numerical value of 3.5, indicating moderate to good accessibility in line with the 22- to 30-min $t_{1/2}$ values for these peptides. The central sites (92–150) had an average accessibility of 4.5, indicating poor accessibility, again in line with the experimental data. Finally, the N-terminal sites had an average accessibility of 4.0 in accord with the intermediate hydrolysis times found by Tauzin et al. (2003). Overall, the α_{S2} -CN model is in relatively good agreement with the average experimen-

tal data; however, the individual regions do contain 2 to 3 outlying points.

Trypsin cleavage of whole caseins over a protracted time yields readily isolated peptide fractions, including the so-called CPP fraction. The CPP fractions can be added to calcium-fortified foods and beverages to improve bone health in individuals with low absorption values (Heaney et al., 1994). It has been presumed that all of the CPP fractions are similar in terms of calcium absorption regardless of the casein fraction from which they are isolated. However, a recent study showed that CPP isolated from goat casein rich in α_{S2} -CN is superior in the delivery of added calcium to developing rats (Mora-Gutierrez et al., 2007b). It is tempting to argue that the helical regions found in the model for 2 of 3 CPP fractions are responsible for this effect. This is, however, not likely for the phosphopeptide regions 8–13 and 54–63, as no experimental evidence supports α -helix in phosphoserine-rich segments (Hoagland et al., 2001; Huq et al., 2003). A more thorough examination of the CPP and their structures is needed before any firm conclusions can be reached regarding this potentially important result.

Plasmin is the major indigenous basic milk protease. This enzyme is responsible for the proteolytic fragments of β -CN known to occur in milk (Eigel et al., 1984). The action of plasmin on purified α_{S2} -CN has been studied by LeBars and Gripon (1989) and by Visser et al. (1989). Both groups showed that α_{S2} -CN is attacked by the enzyme, and similar peptides were released in both studies. Visser et al. (1989) demonstrated by SDS electrophoresis that a substantial amount of the α_{S2} -CN had been broken down to lower molecular weight segment(s) after 1 h of exposure to plasmin at pH 8.4. However, only 8 of about 30 potential cleavage sites were found and the isolated fragments were primarily from the positively charged C-terminal half of the protein. These fragments were similar to the ones detailed above for trypsin by Tauzin and coworkers (2003). Here, cleavages were also found yielding f(1–21) and f(1–24) from the N-terminal section. The latter cleavages yield classical CPP fractions. As is the case with the trypsin cleavage sites, the plasmin cleavage sites reside primarily in the more surface-accessible portions of the α_{S2} -CN model.

Chymosin cleavage of purified α_{S2} -CN was studied by McSweeney et al. (1994). There are 3 primary predicted sites for the cleavage of the protein by this enzyme (88–89, 163–164, and 174–175). All predicted cleavages were found after a period of 20 h. Using the accessibility tool described above, the average value for these residues was 3.4, predicting a moderate time of release for the peptides of 22 to 30 min. It should

be noted that the first susceptible site (88–89) is in the helix-turn-helix motif reminiscent of a membrane insertion protein and therefore may not be available in the self-associated polymers of α_{S2} -CN or in the casein micelles themselves. Molecular dynamics studies of the interaction of chymosin with model substrates show clearly that β -sheet is required for cleavage (Plowman and Creamer, 1995). Here, the model predicts primarily α -helix for 88–89 and 163–164. At first glance, this appears to be contradictory to the α_{S2} -CN model, but the 20-h time of hydrolysis for α_{S2} -CN is quite long (McSweeney et al., 1994) relative to the time of hydrolysis of κ -CN and α_{S1} -CN. It is possible that some slow α_{S2} -CN conformation changes could occur and lead to altered association patterns and hydrolysis. The first of the established sites for chymosin hydrolysis occurs in a region (77–118) homologous to κ -CN (42–84), and it is this section of κ -CN that has been shown to undergo time and temperature conversions to β -sheet with accompanying formation of amyloid bodies (Farrell et al., 2003). Amyloid bodies containing residues 81–125 of α_{S2} -CN have been isolated from bovine mammary gland (Niewold et al., 1999), and Thorn et al. (2008) have shown that a time-dependent conversion of α -helix to β -sheet occurs prior to the formation of amyloid bodies in vitro. Thus, it is possible that the native molecule does contain α -helix, but with time it converts to β -sheet for cleavage by chymosin. Alternatively the α_{S2} -CN model may be incorrect and this area could represent β -sheet. The major segment that is in question occurs in the hydrophobic segment of the molecule, which may represent a major protein-protein interaction site (residues 86–96 with a turn at 97–100 and helix from 101 to 114). For κ -casein, this center has been predicted to be a major interaction site as β -sheet, whereas hydrophobic helices perform the same function in membrane proteins. As noted above, residues 97–100 are turns and, regardless of the surrounding motifs, this would result in a double-sided section with a strong hydrophobic surface structure for protein-protein interactions. Interestingly, the cleavage site 174–175 in the model is in the positive C-terminal region with a β -sheet conformation and would be predicted to be cleaved normally by chymosin in these experiments.

Diaz et al. (1996) and Gagnaire and Leonil (1998) studied the release of tryptic peptides from micellar casein. The latter researchers found that for α_{S2} -CN, only 10 of 30 potential sites were cleaved by the enzyme. Short peptides from Lys residues 21 and 24 were cleaved but not readily released from the micelles; in contrast, a limited number of peptides from the positive C-terminal were released. Moreover, Diaz et al. (1996) found that peptides from β -CN and α_{S1} -CN were released quickly, but those of α_{S2} -CN were released more slowly and in

lesser amounts. The slow release of these peptides suggested that, of the calcium-sensitive caseins, α_{S2} -CN is the least accessible in the micelles. Most of the short peptides identified were from the C-terminal portion of the molecule. This region, although relatively hydrophobic, carries a high net positive charge. The α_{S2} -CN model as noted above does not show these cleavage sites to be exceptionally accessible; apparently they are less so in the micelles. However, the electrostatic calculation of Figure 4 shows that this region is dominated by the charges from these Lys and Arg residues. This too raises the question of whether or not these residues have a specific function in the micelle; that is, phosphate binding as noted above. Binding to calcium phosphate by the Lys and Arg residues in the positively charged C-terminal of α_{S2} -CN would reduce their accessibility to trypsin in the micelle.

The time-dependent cleavage of caseins in Cheddar cheese was studied by Basch et al. (1989) who found that the cleavage of α_{S2} -CN was slowest of all caseins with a half-life of 20 wk. For comparison, the chymosin cleavage of α_{S1} -CN is complete in less than 1 d, and residual plasmin/trypsin cleavages of β -CN occur in 3 wk. Thus, the chymosin and plasmin/trypsin cleavage sites of α_{S2} -CN are not readily accessible within the cheese matrix.

Secondary Structure Predictions and the α_{S2} -CN Model

Several groups have published secondary structure predictions for α_{S2} -CN and these have been summarized by Tauzin et al. (2003) who also presented the results of a consensus algorithm for the secondary structure of the protein. In past de novo modeling studies, the method of Garnier and coworkers (1978) was used to help generate casein working models such as those presented by Kumosinski et al. (1994). Therefore, for comparison with the model, its predictions for α_{S2} -CN will be presented along with the predictions of Hoagland et al. (2001) who attempted to correlate the predictions with spectroscopic evidence as well as the predictions of Tauzin and coworkers (2003). Because these predictions as well as the de novo models of Kumosinski and coworkers (1994) and the homologous model presented herein are meant to serve as guides to further research, the secondary structure results for the model will be compared with the predictions only in terms of overall percentages, and only gross deviations from the predictions will be discussed in detail. In addition, the polyproline II (**PPII**) predictions of Adzhubei and Sternberg (1993) are included because the importance of these structures in caseins has only recently been recognized (Farrell et al., 2001; Barron et al., 2002).

Table 4. Summary of the overall percentage of secondary structure of the α_{S2} -CN model and comparison with secondary structure prediction algorithms and with experimental data

Structure	Model	Tauzin et al. (2003)	Hoagland et al. (2001)	Garnier et al. (1978) ¹	Polyproline II ²	Experimental ³
α -Helix	46	45	33	54		24 to 32
β -Sheet	9	6	26	15		27 to 37
All turns	12			19		24 to 31
Polyproline II	7				15	
NC α - or β - ⁴	19					
Unspecified	7	49	13	13		9 to 22

¹Values calculated for α_{S2} -CN using the method of Garnier et al. (1978).

²Adzhubei and Sternberg (1993).

³Hoagland et al. (2001).

⁴Noncontinuous α -helix or β -sheet as described in the text.

The results from analysis of the phi/psi angles of the model are compared with both experimental data and the above predictions in Table 4.

For the α_{S2} -CN model the Ramachandran plot is extremely simple with about 60% of the residues in the α -helical region, 2 residues in the lower left “forbidden zone,” a few in the turns region, and the remainder in the upper left quadrant or β -sheet/extended region. However, the actual continuous β -sheet found in the model is quite low compared with most of the predicted values and the experimental data given in Table 4. One reason for this is that PPII would fall in this region and be included as sheet in the Fourier transform infrared data of Hoagland et al. (2001); using the predictions of Adzhubei and Sternberg (1993), 15% PPII was predicted for α_{S2} -CN. However, analysis of the α_{S2} -CN model showed that only 7%, residues 123–128, 174–177, and 200–202, are in the PPII configuration (continuous left-handed helix). In addition, there are considerable segments of the molecule that have 3 to 4 continuous sheet phi/psi angles that are interrupted by 1 or 2 α -helical angles resulting in loops or turn-like structures. In Table 2 these are termed noncontinuous α -helix and β -sheet and account for 19% of the α_{S2} -CN model.

With regard to α -helix, the overall predictions in the 50% area are closer to the α_{S2} -CN model and sequence predictions are quite good for residues 81–207 of the model. Although the total percentage helix predicted is similar to the α_{S2} -CN model for residues 1–89, there is a lack of any agreement between the actual residues predicted to be in α -helical structures and those found in the model. The percentage α -helix may be over-reported in the model because the template used (CLIC-4) has a high degree of α -helix (42%), which in turn may guide the threading experiment and influence the α_{S2} -CN model. Indeed, the ExpASY tool Magic-Fit is able to align the parent and homolog molecules with an RMS difference of only 0.16 Å, but the experimental data for α -helix do not support the high degree of helix found in

the model. Because of the consensus in the C-terminal regions between the predictions and the model, the N-terminal areas of α -helix in the model must be viewed with suspicion. This is particularly true for the helices found in the model for the phosphopeptide regions 8–13 and 54–63, as no experimental evidence supports α -helix in phosphoserine-rich segments. Hoagland et al. (2001) calculated that residues 1–7 could exist in a helical conformation because of paired ions in the sequence. However, although Huq et al. (2003) have shown the potential in residues 1–41 of α_{S2} -CN, actual helix was observed only in the presence of added calcium. In fact, riboflavin binding protein contains a phosphopeptide region homologous to those of the major caseins; in the crystal structure of riboflavin binding protein (Monaco, 1997) the phosphopeptide region is missing because of extremely high temperature factors that produce too much motion and uncertainty. Thus, as noted above, predicted helical segments for the phosphopeptides in all of the caseins must be viewed with suspicion.

Finally, turn regions account for 12% of the α_{S2} -CN model; there are 6 regions where the polypeptide chain reverses direction near the surface of the molecule and twists toward the more interior regions. These regions are 46–49, 65–68, 97–100, 148–151, 171–174, and 191–194. Interestingly, the predictions of Garnier et al. (1978), although close in percentage, are in accord only with the last 2 of the 6. The experimental predictions of turns found in Table 4 are greater; however, the databases used had no sequences present similar to the noncontinuous regions noted above.

Sulfhydryl-Disulfide Interactions

α_{S2} -Casein has 2 Cys residues: 36 and 40. Experimental evidence (Rasmussen et al., 1992, 1994) has disclosed that in whole casein, >85% of the protein exists with an intramolecular disulfide between these 2 residues. The remainder of the α_{S2} -CN appears limited

Figure 5. Tight loop introduced by the internal disulfide bond of α_{S2} -CN between Cys 36 and Cys 40. The disulfide bond lies between a stretch of helix and one of sheet. The Phe 39 side chain is shown as a point of reference.

to dimers connected in a parallel or antiparallel fashion (Rasmussen et al., 1992, 1994). Unlike κ -CN where multiple polymers exist between its 2 Cys residues (Farrell et al., 2003), the number of α_{S2} -CN polymers is small in both number and size. In the α_{S2} -CN model the 2 Cys residues can be found in the upper left corner of the molecule (Figure 3B) in a surface-oriented position where they are readily surface accessible and where the 2 side chains can be rotated outward to facilitate docking of a second monomer. Docking in a parallel manner (36–36 and 40–40) allows for both hydrophobic tails to be orientated in the same direction, possibly sharing a common hydrophobic environment. Docking in an antiparallel fashion (36–40 and 40–36) causes the hydrophobic tails to be oriented away from each other. These latter interactions should occur between 2 molecules not sharing a common hydrophobic center, and therefore arise from surface interactions between 2 protein aggregates (submicelles). Such interactions must be limited, because, as noted above, the majority of the protein (85%) is monomeric.

With only a minor degree of side-chain manipulation, a disulfide bond was put in place between Cys 36 and Cys 40. This short distance forms a very tight loop, energy minimization readily removed any constraints, and the disulfide model had a total energy equivalent to the original –SH model (Table 3). Using the ExpASY Magic-Fit tool the RMS difference between the 2 models was 0.03 Å, indicating little divergence between the 2 models. The tight loop introduced by this disulfide is shown in Figure 5. Therefore, the model is in accord with the experimental data regarding the sulphhydryl-disulfide interactions of α_{S2} -CN.

Figure 6. Bioactive peptides from the C-terminal of α_{S2} -CN. A) Molecular model for the peptide fragment of α_{S2} -CN (Kizawa et al., 1996) homologous to calmodulin-binding protein domains f (183–207). In the model the latter segment is primarily composed of α -helix (residues 183–190, 194–199, and 204–207). The 3 sections of helix are separated by 2 nontraditional turns or tight loops. The overall structure can be characterized as an open hairpin; B) molecular model for α_{S2} -CN f (165–203) called casocidin I by Zucht et al. (1995). In this case the latter 2 sections of helix are again separated by a nontraditional turn or tight loop, but the first 2 are separated by a section of the molecule (172–180) that is linear in structure and is primarily in a polyproline II configuration (173–178). This makes the peptide quite planar with the overall appearance of a Greek letter Ω ; C) molecular model for the major angiotensin I converting enzyme (ACE) inhibitor in tryptic digests of α_{S2} -CN f (174–181) (Tauzin et al., 2002). Residues 173–180 are in the polyproline II conformation.

Predicted Structures of the Biologically Active Peptides of α_{S2} -CN

Calmodulin is a rather ubiquitous, low molecular weight protein with multiple cellular functions ranging from regulating cell division to activation of membrane enzyme cascades; it was purified from bovine mammary gland by Thompson et al. (1987). It is thought that calmodulin acts by binding to positively charged areas on its target proteins; for example, calmodulin stimulates the activity of membrane-bound cyclic nucleotide phosphodiesterase. The work of Kizawa et al. (1996) clearly demonstrates that the peptide fragments of α_{S2} -CN, which are homologous to calmodulin binding enzyme domains, bind to calmodulin and prevent its activation of the phosphodiesterase. These fractions represent f(164–179) and f(183–206/207); f(183–207) is the most reactive with a K_i of 2.6 μM for inhibition of calmodulin activation of the enzyme. This is in the range required to exert required physiological activity. In the α_{S2} -CN model this latter segment (Figure 6A) is primarily composed of α -helix (residues 183–190, 194–199, and 204–207). The 3 sections of helix are separated by 2 nontraditional turns or tight loops. Note the high preponderance of hydrophobic residues in this section. The overall structure can be characterized as a flat open hairpin (Figure 6A). This is precisely the structure predicted to be necessary for calmodulin binding by Kizawa et al. (1996).

The first antibacterial peptide isolated from α_{S2} -CN was called Casocidin I by Zucht et al. (1995). This peptide inhibited the growth of both *Escherichia coli* and *Staphylococcus carnosus* in a dose-dependent manner at concentrations above 300 ng. The active peptide was identified as f(165–203). In the α_{S2} -CN model this segment is also primarily composed of α -helix (residues 165–171, 181–190, and 194–199). In Figure 6B it can be seen that the latter 2 sections of helix are separated by a nontraditional turn or tight loop, but the first 2 are separated by a section of the molecule (172–180) that is a linear structure and is primarily in a PPII configuration (173–178). This makes the peptide quite planar with the overall appearance of a Greek capital letter Ω as seen in Figure 6B. Note again the high preponderance of hydrophobic residues. Recio and Visser (1999) found 2 antibacterial peptides in peptic digests of α_{S2} -CN representing sequences similar to those found by Zucht and coworkers (1995); namely, f(164–179) and f(183–207). The latter fraction had consistently higher activity. Finally, McCann et al. (2005) identified 5 antibacterial peptides from chymosin digests of α_{S2} -CN; all were from the C-terminal end with f(164–207) and f(172–207) being most active against *Listeria innocua*. The activities of the latter peptides were equivalent to

the bacteriocin nisin. Note that the structures would be nearly identical to that of Figure 6B. Lopez-Esposito et al. (2006) studied ovine α_{S2} -CN peptides with antibacterial action against *E. coli*. The highest potency was found for f(165–181), which again would be predicted to be structurally similar to Figure 6B.

Tauzin et al. (2002) discovered inhibitors of the angiotensin-I-converting enzyme in tryptic digests of α_{S2} -CN. The fragments were identified as f(174–181) and f(174–179). These segments are contained within the N-terminal of the Casocidin I structure noted above. Here, the motif is predicted to be only the extended planar structure discussed above. Residues 173–180 are in the PPII conformation (Figure 6C). It is interesting that these segments contain the PPII conformation. It has been proffered that this conformation may be highly involved in protein-protein interactions regulating several metabolic pathways and protein-mediated intracellular cytoskeleton changes (Kay et al., 2000). Once more, the molecule is flat with a high level of hydrophobic residues.

Intestinal absorption can be studied in vitro by the use of Caco-2 cell cultures. These cells, derived from a colon carcinoma line, form monolayers in culture and mimic intestinal brush borders (Tanabe et al., 2006). One potential route of food allergy is the permeation of the intestinal cell wall by intact proteins. Nondenatured ovalbumin can permeate Caco-2 cells in culture. Tanabe and coworkers (2006) discovered that a small peptide from α_{S2} -CN f(102–111) can prevent ovalbumin permeation of Caco-2 cells in vitro. Although not predicted to be helical by the programs cited above (Table 4), this segment of the α_{S2} -CN model is a tightly wound helix (Figure 7A, bottom). It contains the correctly aligned H-bonds for an α -helix as calculated by the ExPASy tool, and is highly hydrophobic. In Figures 3 and 4 it represents the right side of the hydrophobic foot at 6 o'clock, which has been hypothesized to be related to protein-protein interactions between α_{S2} -CN and other caseins. Its hydrophobic residues (perhaps stabilized by the internal H-bonds) may bind to pores in the cell membranes and prevent permeation by ovalbumin. Alternatively, once excised from the molecule it could unfold to β -sheet as seen in Figure 7A (top); this segment, as discussed above, is a portion of the α_{S2} -CN found in amyloid bodies in mammary gland f(81–112) by Niewold et al. (1999), and amyloid bodies are generally β -sheet-based structures.

Overall intestinal health and well-being is the result of interactions between the cells of the intestinal mucosa and the bacterial flora that surrounds them. In the normal course of events proteins called defensins are the first line of surveillance; they are secreted by the Paneth cells of the intestinal mucosa and bind to

Figure 7. Peptides from α_{S2} -CN potentially active in the intestine. A) Molecular model for the potential antiallergenic peptide from α_{S2} -CN f (102–111) as discovered by Tanabe et al. (2006). Note the tight helical conformation and the presence of Trp (bottom) and the segment as a potential β -sheet (top); B) molecular model for α_{S2} -CN f (34–59) as excised from the total model (right) and compared with the crystal structure (left) for defensin 6 (Szyk et al., 2006).

and monitor helpful and potentially harmful bacteria. These defensins have low molecular weight and have known crystal structures (Szyk et al., 2006). Defensin 6 has an overall homology to α_{S2} -CN of 68% using the T-coffee program; this is for the pre-pro-defensin. When the latter is cleaved by proteases to the active form of defensin 6, the homology score remains the same, but only the first 21 residues remain in alignment with the α_{S2} -CN residues f(34–59), the remainder form a gapped alignment with α_{S2} -CN f(164–174). Nevertheless, the molecular model for the first section of α_{S2} -CN f(34–59) is strikingly similar to the total crystal structure of defensin 6 (Figure 7B). Thus, α_{S2} -CN may be predicted to yield peptides with defensin-like activity and aid the immune system in fighting bacteria.

CONCLUSIONS

A 3D molecular model for α_{S2} -CN has been produced by threading the backbone sequence of the protein onto a homologous protein, CLIC-4. In this model Glu was used in place of Ser to mimic phosphoserine in the overall size and charge distribution on the protein. Overall, the model is in good agreement with experimental data for the protein; however, the amount of helix may be over-predicted. The model does, however, offer a unique view of the highly positive C-terminal portion of the molecule as a surface accessible area. This region may be the site for interactions with κ -carrageenan, phosphate, and other anions. In addition, many of the physiologically active and antibiotic peptides isolated from α_{S2} -CN occur in this region; others come from the functional hydrophobic foot as well as the center of the molecule. Based on molecular similarities, α_{S2} -CN is predicted to yield peptides with defensin-like activity, which may aid the immune system in fighting bacteria. This structure should be viewed as a working model with the ability to be changed as more precise experimental data are obtained. As such, the model completes the series of working models for the caseins (Kumosinski et al., 1994).

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