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Three-Dimensional Molecular Modeling of Bovine Caseins: A Refined, Energy-Minimized κ-Casein Structure[1]

T. F. KUMOSINSKI, E. M. BROWN, and H. M. FARRELL, JR.
US Department of Agriculture
Eastern Regional Research Center
Agricultural Research Service
Philadelphia, PA

ABSTRACT

A refined three-dimensional molecular model of κ-casein has been produced using energy minimization techniques and a Kollman force field on a previously reported predicted three-dimensional structure. This initial model was constructed via molecular modeling techniques from sequence-based secondary structural prediction algorithms. Both the initial and refined structures agreed with global secondary structure analysis from vibration spectroscopy. The refined structure contained many of the features of the initial model, including two sets of antiparallel β-sheet structures containing predominantly hydrophobic side chains, which could form interaction sites with α_{s1}-casein. Two types of energy-minimized dimer and tetramer models are presented: 1) using Cys as potential intermolecular disulfide binding sites and 2) using the two sheets as possible hydrophobic self-association sites, without Cys interactions. All structures yielded good stabilization energies and are in agreement with chemical, biochemical, and physical chemical results obtained for κ-casein.

(**Key words**: casein structure, protein functionality, milk proteins)

Abbreviation key: **BFGS** = Broyden, Fletcher, Goldfarb, and Shanno algorithim; **FTIR** = Fourier transform infrared spectroscopy.

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[1]Reference to a brand or firm name does not constitute endorsement by the USDA over others of a similar nature not mentioned.

INTRODUCTION

The casein micelle represents a unique form of calcium-phosphate transport complex that provides the bulk of these elements required by the neonate (15). The key to the formation of this complex is κ-casein, which provides the stabilizing influence for micelle formation in the presence of physiologically high concentrations of calcium and phosphate (32). The hydrolysis of κ-casein by chymosin initiates the first stages of digestion for release of the nutritionally important elements of the micelle (12, 15, 28). The chymosin reaction is paramount in the first stages of most cheese-making processes. To understand better the role of κ-casein in these physiologically and industrially important processes, we have attempted to refine our knowledge of the molecular structure of κ-casein.

In a previous report (18), a predicted three-dimensional structure of κ-casein was presented. This model was constructed using sequence-based secondary structural prediction algorithms, global secondary structural information from Fourier transform infrared spectroscopy (**FTIR**), and molecular modeling techniques, which partially minimized poor van der Waals' contacts. The predicted structure contained two sets of unstranded antiparallel β-sheets, which are rich in hydrophobic side chains. These sheets are potential sites for complexing with α_{s1}-casein, which was predicted to have similar structures (19). In addition, the model accounted for a variety of functional properties of κ-casein and derived biochemical, chemical, and physicochemical characteristics. However, the initial structure was not energy minimized to reduce interactions resulting from van der Waals' energies, electrostatic interactions, and intramolecular hydrogen bonding. A destabilizing energy of over 2 million kcal/mol (which was due primarily to van der Waals' interactions) was calculated for the initial κ-casein model using a

Kollman force field. Because reproducible crystals have not been obtained for κ-casein, crystallographic structures do not exist. Therefore, we present a structure refined by the use of energy minimization techniques to yield a κ-casein model. This model is compared with the unrefined structure and evaluated with respect to biochemical cleavage sites known for the enzyme chymosin, chemical modification results, and solution physicochemical experiments. Also, interaction sites to mimic the hydrophobically controlled self-association of κ-casein are utilized to construct energy-minimized dimer and tetramer structures.

MATERIALS AND METHODS

Predictions of Secondary Structures

Selection of appropriate conformational states for the individual amino acid residues was accomplished by comparison of the results of sequence-based predictive techniques, primarily those of Chou and Fasman (6), Garnier et al. (13), and Cohen et al. (7, 8). Assignments of secondary structure (α-helix, β-sheet, or β-turn) for the amino acid sequences were made when either predicted by more than one method or strongly predicted by one method but not predicted against by the other methods. In addition, because of the large number of Pro residues in the caseins, Pro-based turn predictions were made using the data of Benedetti et al. (4) and Ananthanarayanan et al. (2).

Energy Minimization: Molecular Force Field

The concept, equation, and full description of a molecular force field were given in a previous communication (20). In those calculations, a combination of the force fields described by Tripos (24) and Kollman (33) was employed. Both force fields used electrostatic interactions calculated from partial charges given by Kollman (33); a united atom approach with only essential hydrogens for reasonable calculation time on the computer was also used. A cutoff value of 8 Å was employed for all nonbonded interactions for both force fields. The Broyden, Fletcher, Goldfarb, and Shanno algorithm (**BFGS**) and conjugate gradient techniques (24) were employed as minimization algorithms when applicable. The Tripos force field (24), unlike the Kollman force field (33), contains fewer parameters (i.e., no added mathematical term for a 10-12 potential function to account for specific hydrogen bonding) and is used in this study to overcome large energy barriers.

Molecular Modeling

The three-dimensional structure of κ-casein was approximated using molecular modeling methods with an Evans and Sutherland PS390 interactive computer graphics display driven by Sybyl (Tripos, St. Louis, MO) software. The initial model was constructed as previously described (18). Briefly, two segments of the protein were selected based on secondary structural predictions (residues 1 to 86 and 87 to 169). Both segments were then built from individual amino acids and assigned ϕ and ψ angles characteristic of the respective predicted structures for each residue (18). The individual pieces were then joined together to produce the total polypeptide model and were energetically adjusted. Other details have been given previously (18, 19, 20).

In this study, the energy for each of the two pieces of the initial structure was calculated using a Kollman force field. Because of the large destabilizing van der Waals' energy, bond stretching that is due to the high Pro and turn content, and positive hydrogen-bonding energy, each piece was first energy minimized using a Tripos force field without electrostatics. The BFGS technique, which requires a large block of computer memory, was employed as the minimizing algorithm (24). This technique is most useful when many energy barriers are suspected. The pieces were then subjected to a Tripos force field with electrostatics and, finally, a Kollman force field. The conjugate gradient technique was used only when the Kollman force field was employed. The individual pieces were then joined to form a single polypeptide chain, which then was energy minimized to ~ −12 kcal/mol per residue using a Kollman force field.

RESULTS AND DISCUSSION

Generation of Energy-Minimized Three-Dimensional Models

The caseins in general represent a unique class of proteins that are neither globular nor fibrous but are characterized by a high Pro

content. In our initial attempts at undertaking structural motifs for casein (18, 19), we were struck by the fact that FTIR data predicted a high degree of turns and that a correlation apparently existed between the percentage of Pro and the percentage of predicted turns. Proline disrupts regular structure but excels at making turns (2, 4, 6, 27). In globular proteins, these turns are almost always on the surface and are hydrophilic in nature. Caseins, however, have a propensity to self-associate (12, 15, 28), and most data point toward hydrophobic cores in the associated complexes. Thus, Pro-driven turns in hydrophobic areas may serve to facilitate the formation of interaction sites for hydrophobic core formation. Therefore, in the construction of casein monomers, Pro turns in hydrophobic areas can appear on the monomer surface because they probably will be buried in subsequent polymer formation. The absence of a hydrophobic modeling term and the lack of water in these in vacuo calculations may actually be a positive element in these studies. The monomer constructed herein actually could represent the monomer within a polymer for the individual caseins. The exposed hydrophobic sites help explain the nature of casein self-associations. These hydrophobic Pro-based turns could well be the signature of a casein, the structural motif that sets these proteins apart from globular and fibrous proteins.

The work that follows began with secondary structural predictions, which do not have a high degree of certitude but are based on bond angles that occur in aqueous crystals for proteins. The beginning structures, like previously proposed linear models (21, 25), are modified to account for global circular dichroic and FTIR data for κ-casein so that the assigned structures are not altogether arbitrary or without precedent (21, 25). Energy minimization of these structures could trap a less than favorable energetic state, but we have used the BFGS and other algorithms to attempt to avoid this problem. Given the task at hand, de novo calculations from randomized structures also do not guarantee a native structure for a molecule the size of κ-casein. The structure presented herein thus represents a computational short cut and therefore is a working model, subject to change and refinement, not a final exact structure. The model is presented because exact crystal structures will not likely ever be available and is intended to stimulate research and discussion. In that light, the following steps were used to assemble the κ-casein model.

The refined energy-minimized structure of κ-casein was generated from the initial computer model previously presented (18). In the latter paper, two segments, residues 1 to 86 and 87 to 169, were individually built and then joined with appropriate ϕ and ψ angles to produce the polypeptide chain. We energy minimized each of these segments individually in the following manner.

For the first segment, destabilizing energy of over 900,000 kcal was calculated using a Kollman force field. A large portion of this energy resulted from poor van der Waals' and hydrogen-bonding interactions; a smaller contribution was attributed to bond stretching energies. This large energy barrier is most likely due to the large number of Pro residues that are distributed throughout the sequence of κ-casein (Figure 1). Because of these energy values, energy minimization was performed using a Tripos force field, first without electrostatics and then with electrostatic interactions. For these calculations, a BFGS technique was used for the minimization algorithm to avoid high energy wells with local minima; all energies obtained were considerably lower than the initial values. This structure was finally refined by using a Kollman force field in conjunction with the faster conjugate gradient minimization algorithm. The same procedures were applied to the other piece of the initial structure. The two minimized structures were then joined with the appropriate ϕ and ψ angles determined from prediction results based on the secondary structure sequence previously presented (18). This final total polypeptide chain was then energy minimized using a Kollman force field with a conjugate gradient minimizer. The result of this calculation (Table 1, monomer) yields a stabilizing energy of −2134.3 kcal/mol or about −12 kcal/mol per residue. Such values are consistent with those obtained from energy-minimizing structures derived from X-ray crystallography using similar Kollman force fields.

Refined Three-Dimensional Molecular Model of κ-Casein

The energy-minimized structure generated as described for κ-casein is shown in Figure 2. Represented in descriptive terms, the protein

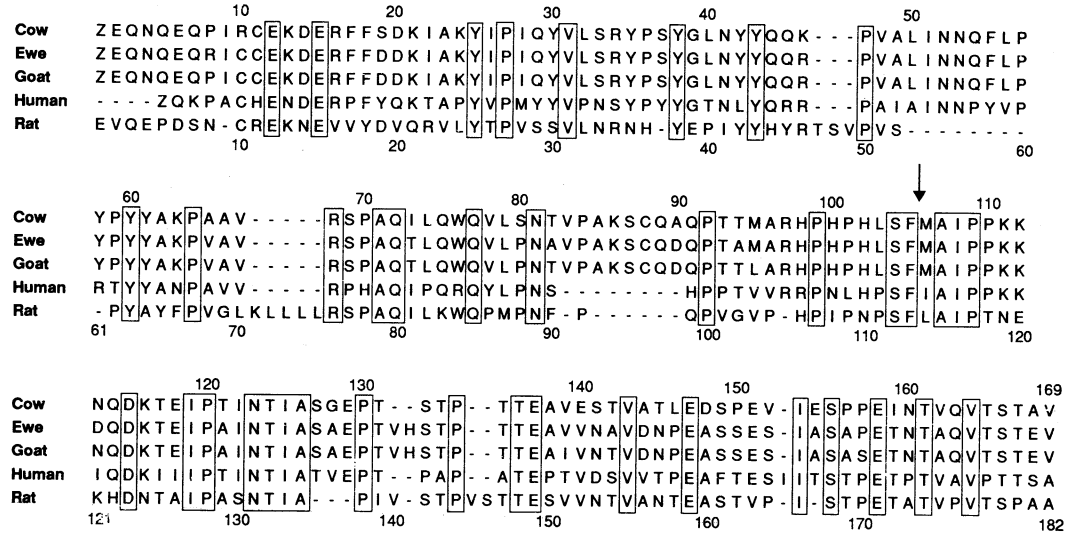

Figure 1. Alignment of the sequences of κ-casein for goat, ewe, cow, human, and rat proteins. Standard one-letter symbols for amino acids. Alignments follow those of Brignon et al. (5) except that the numbers given above the sequences are those of the bovine protein, and numbers below represent the maximum overall chain length regardless of species: □ = Identical residues all species; ↓ is the chymosin-sensitive bond.

can be thought of as a "horse and rider." The amino-terminal 110 to 120 residues represent the "horse," and the carboxy-terminal portion represents the "rider." Two distinct legs are seen in the "horse" portion of the model. These legs are generated by β-sheet regions comprising residues 21 to 25, 29 to 34, 39 to 45, and 49 to 55; these segments are connected by γ- or β-turns, which are centered on Pro-27, Pro-36, Pro-47, and Pro-57 (18, 27).

Considering the known sequences of κ-casein from goats, ewes, cows, humans, and rats (Figure 1), these Pro are invariant, except for Pro-36, which in rats occurs at residue 40. In addition, the relatively hydrophobic character of the segments is preserved. Because the

TABLE 1. Energy of κ-casein hydrophobic aggregates.

Energies	Monomer	Dimer	Tetramer
	(kcal/mol)		
Bond stretching	22.5	43.6	86.4
Angle bending	173.3	337.1	684.9
Torsional	213.1	388.8	791.0
Out of plane bending	10.6	23.3	45.8
1-4 van der Waals'	246.5	503.9	1005.5
van der Waals'	−567.3	−1203.4	−2464.6
1-4 Electrostatic	1932.5	3846.3	7692.0
Electrostatic	−4109.5	−8513.4	−17063.3
H Bond	−56.1	−118.6	−235.1
Total	−2134.3	−4692.4	−9457.4
Dipole moment, Debye units	1644	2866	1759
Net charge	−6.0	−10.0	−20.0
Association energy[1]		−423.8	−920.2

[1]Defined as $E_n - nE_m$ where n is 2 or 4 for the dimer or tetramer, and m is monomer.

caseins generally are not highly conserved evolutionarily across species, such a strong identity for these Pro residues argues for an important biological function. The hydrophobic "legs" generated by these Pro residues may, therefore, serve as a rather universal signature for κ-casein and its role in micelle interactions.

The overall dimensions of the κ-casein monomer predicted by this model are 8.0 × 6.2 × 5.8 nm. The radius of gyration is 20 Å with a dipole moment of 1644 Debye units. In the following section, we attempt to reconcile some known features of the chemistry of κ-casein with this molecular model. To aid in this discussion, the backbone-only model with Pro indicated is given in Figure 3A, along with a stereo view (Figure 3B).

Secondary Structure Analysis

To test experimentally the validity of the energy-minimized three-dimensional structure, global secondary structural analysis was initiated to be compared with reported global analysis obtained from FTIR spectroscopy (Table 2). Figure 4 shows the Ramachandran plot of ϕ and ψ, backbone dihedral angles (open circles) calculated from the refined κ-casein structure using the Tripos' Sybyl molecular modeling software. The bounded areas of

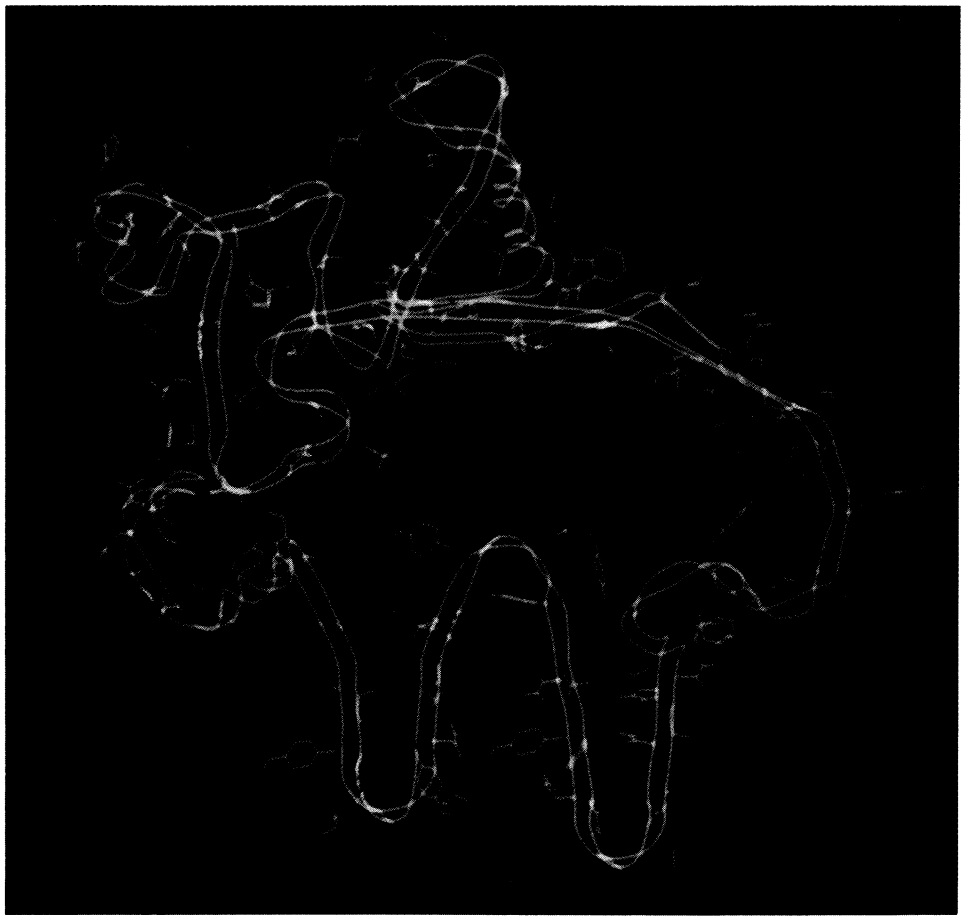

Figure 2. Refined three-dimensional molecular model of κ-casein. The peptide backbone has been replaced by a double yellow ribbon chain trace. Hydrophobic side chains are green; acidic side chains are red; basic side chains are purple; all other side chains are cyan.

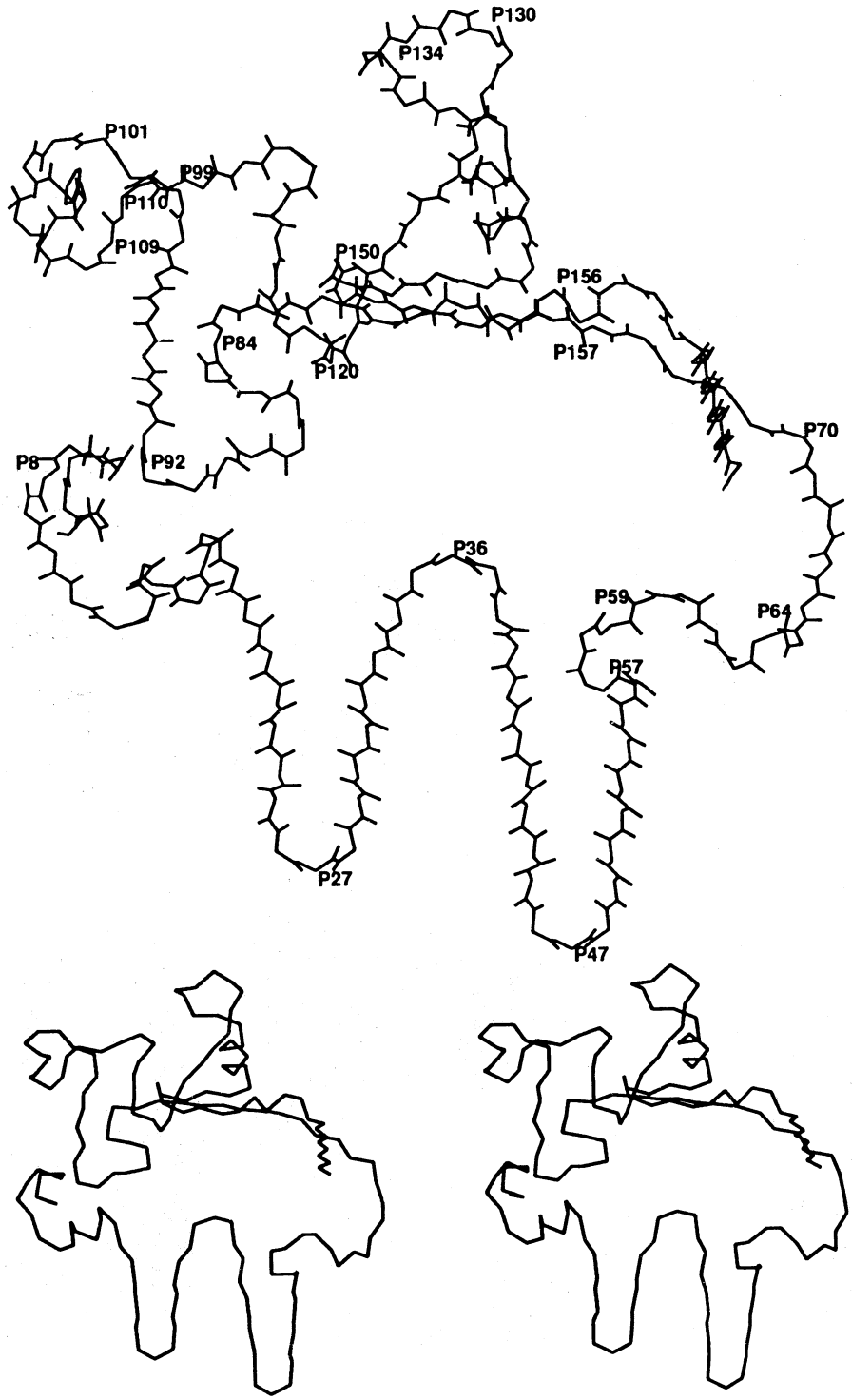

Figure 3. A) Backbone of κ-casein without side chains; Pro (P) indicated. B) Stereo view of the three-dimensional molecular model of κ-casein, showing an α-chain trace; the N- and C-terminal ends of the molecule are to the left and right, respectively.

TABLE 2. Comparison of adjusted κ-casein sequence-based predictions with Fourier transform infrared spectroscopy (FTIR) data.

	Helix	β-Structure	Turns	Unspecified
	(%)			
FTIR[1]	8	29	33	30
Initial	16	27	37	20
Final	10	30	32	30

[1]Preliminary data with 10% error (D. Curley et al., 1992, unpublished data).

acceptable ϕ and ψ angles for β-sheet, α-helix, and turn regions are also presented with appropriate labels. When the number of points present within the limits of the particular periodic or turn structure are summed, the global amount of α-helix, β-sheet, or β-turn can be obtained. This type of analysis does not allow for the residue length of the periodic structure or its placement within the sequence structure. Also, ϕ and ψ angles may not exactly represent the secondary structure because of changes in backbone bond lengths. Hence, a visual inspection of the secondary structure by use of a chain trace or ribbon as in Figure 3B should also be employed when the global secondary structure of any model is calculated. Secondary structural analyses, following these procedures, are given in Table 2 for the initial and the refined structures. The global secondary structural results are in good agreement with those obtained from FTIR spectroscopy in D_2O (Table 2). Thus, the refined model does not diverge from experimentally determined secondary structural analysis.

Chemistry of κ-Casein and the Three-Dimensional Model

Chymosin (Rennin) Hydrolysis. The primary phase of the action of chymosin on the bovine casein micelle is the hydrolysis of the highly sensitive Phe-Met peptide bond (residues 105-106) of κ-casein (16, 17). Sequence data (5, 22) show this bond to be located in a Pro-rich region of the molecule. Again, 3 invariant Pro residues occur herein: Pro-92 and Pro-99, which precede the chymosin sensitive bond, and Pro-108, which follows it. Except for the rat protein, Pro-110 is also preserved. In all species, Phe-105 is invariant, but, in humans and rats, κ-casein Met-106 can be replaced by Ile or Leu. In addition, Ser-109, Ala-107, and Ile-108 are conserved. From studies of model peptides, the residues lying between Pro-101 and Pro-109 may occur in a β-sheet structure (25). With other predictive methods, these residues have an equal chance to be in an α-helical conformation (21). In either case, the Pro residues following and preceding the chymosin-sensitive bond cause the formation of a kink, which neatly presents the otherwise hydrophobic Phe-Met on the surface of the molecule. In our model, we show an α-helix that represents the "horse's bit" in our descriptive "horse and rider" model. This segment of α-helix was preserved in the energy minimization procedure. From model building considerations, this sequence represents the minimum number of residues for a stable α-helix. Cleavage of the Phe-Met (105-106) bond would render the helical conformation untenable, the helix would unwind, and a considerable amount of configurational entropy would be added to the hydrolysis reaction. The change from β-sheet to the extended conformation, although significant, would contribute less energy. In either case, helix or sheet, the Pro-based turns present this segment on the surface and make it readily accessible to chymosin. Any model for casein micelle structure must in some way account for this feature of κ-casein.

Sites for Glycosylation and Phosphorylation of κ-Casein. Of the major components of the casein complex, only κ-casein is glycosylated. Nearly all of the carbohydrate and phosphate associated with κ-casein is bound to the macropeptide (11), which is the highly soluble portion formed by chymosin hydrolysis. The major site for glycosylation, Thr-133, is in our refined model on the "rider" and on a β-turn. The sites of phosphorylation, Ser-149 and Thr-145, are also on the "rider" portion and in β-turns.

Hydrophobic Interactions. The amino-terminal fifth of the κ-casein molecule has a relatively high charge frequency [.34]; however, the net charge is zero, and this part of the protein, although it is relatively hydrophobic, is somewhat exposed in the monomer model (Figure 4, A and B). Residues 35 to 68 represent an exceptionally hydrophobic area with almost no charge. It is precisely within this latter region that the majority of the residues found in the "legged" structures of the κ-casein molecule occur. These structures were present in the initial model (18) and persist following energy minimization. They are antiparallel, highly hydrophobic β-sheets, and they make ideal sites for sheet-sheet interactions with either other κ-casein molecules or with hydrophobic domains of α_s- and β-caseins.

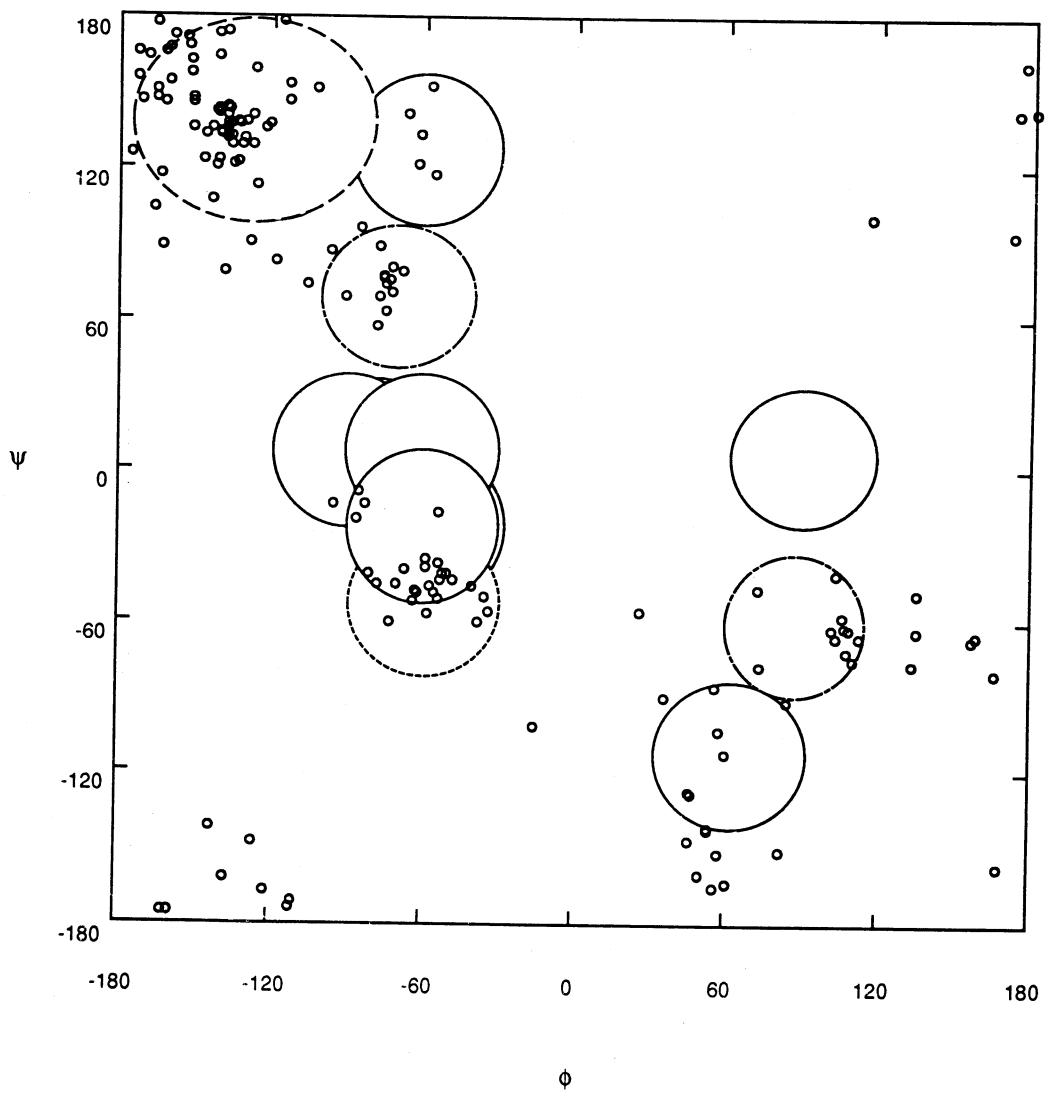

Figure 4. Ramachandran plot of ψ and ϕ angles calculated from energy-minimized structure of κ-casein. Area for β-sheet structure (---), α-helix solid lines, β-turns (····), and γ-turns (——).

Several investigators (1, 9, 10) have noted that β- and κ-caseins diffuse out of the casein micelle at low temperatures. As the temperature is lowered, hydrophobic stabilization energy decreases, and κ-casein is able to dissociate from the micelle. The importance of the hydrophobic residues in casein-casein interactions can be imputed by comparison of κ-casein sequences (Figure 1). Residues Tyr-25, Tyr-38, Tyr-43, and Tyr-60 are invariant across the species studied, as is Val-31. In addition, the other 5 Tyr residues are present in relatively the same position in 3 to 4 of the species. Of 9 Tyr residues in bovine κ-casein, 7 are located between Tyr-35 and Tyr-68. Woychik and Wondolowski (35) showed that nitration of 7 Tyr residues of κ-casein severely inhibited its ability to stabilize α_{s1}-casein (35). As can be seen in Figure 3, the "legged" structures are constituted from this region and contain 7 Tyr residues. Another important feature of this region could be the invariant nature of the positive charge at residue 46 (Figure 1).

Sulfhydryl-Disulfide Interactions. κ-Casein contains 2 Cys residues. Whether these residues can form intra- or intermolecular disulfide bonds and what effects such bonding can have on micelle stabilization have not been clearly established. The occurrence of free sulfhydryl groups in the milk-protein complex has been reported by Beeby (3), but not by others (14, 29). Swaisgood et al. (29) reported significant S-S crosslinking in purified κ-casein; however, Woychik et al. (34) demonstrated that reduced and alkylated κ-casein stabilized α_{s1}-casein against calcium precipitation as well as native κ-casein. Pepper and Farrell (23) found that, in soluble whole casein and in the absence of Ca^{2+}, κ-casein occurred as a high molecular mass polydisperse complex with a Stokes radius of 94 Å; only by addition of reducing agents (i.e., converted to the sulfhydryl form) did κ-casein exhibit concentration-dependent associations, both with itself and with other caseins. Groves et al. (14) have recently shown by SDS-PAGE that, without reduction, purified bovine κ-casein occurs with a characteristic pattern of aggregates of varying molecular mass ranging from monomer to octamer in integral steps. Thus, although κ-casein represents only 13% of the casein, many κ-casein molecules must be somewhat contiguous in the micelle in order to form these high molecular mass disulfide-linked aggregates. Such patterns are peculiar to ruminant milks (Figure 1) because only those milks contain two Cys residues. Residues Cys-10 and Cys-11 appear to be conserved across species, but Cys-88 occurs only in the ruminants. Of interest are the locations of Cys-11 and Cys-88 (see Figure 3B). The former is located between two segments predicted to be in α-helical conformations and in the stereo view is on the "rider's" right, whereas Cys-88 is located in a predicted β-turn on the opposite side. In our model, both of these residues are located near the surface of the monomer molecule and are directed away from each other at a distance of over 33 Å. This distance could account for the ability of the κ-casein molecule to form the interchain disulfide-bonded polymers discussed rather than an intramolecular disulfide bond. Groves et al. (14) have quantitatively measured the number and amount of disulfide-linked aggregates of bovine κ-casein and shown that bovine κ-casein naturally occurs as eight equal weight fractions of monomer, dimer, trimer, . . . to octamer. To mimic that study (14) and to test our refined model, we have built two disulfide-linked tetramers of κ-casein and energy minimized their structures. Figure 5A shows a chain trace model of a symmetric disulfide-linked tetramer in which all three disulfide links are between Cys-11 and Cys-88. This structure shows a linear arrangement and allows easily for the building of larger disulfide-linked aggregates. In Figure 5B, a tetramer with an assymmetric disulfide arrangement was built, i.e., 11 to 88, 11 to 11, and 88 to 11. This structure, although not as linear as the symmetric structure, still allows for increased aggregates, i.e., at least to an octamer. Both structures were easily energy minimized and showed energies equivalent to a monomer that is not disulfide bonded. Hence, no destabilization energy is observed. The structures shown in Figure 5, A and B, have calculated radii of gyration of 42.6 and 47.0 Å, respectively. Thurn et al. (30) studied calcium-induced aggregates of purified κ-casein; they interpreted their small angle neutron-scattering data as the sum of κ-casein subaggregates of ~nine monomers each. Individual subaggregates are thought to be starlike. The radius of gyration for the average subaggregate calculated by Thurn et al. (30)

was 70 Å, which is in moderately good agreement with a hypothetical structure that could be built from two of the tetramers shown in Figure 5, A or B. The apparent Stokes radius of disulfide-linked κ-casein polymers was 94 Å (23). The structures of Figure 5, used as "building blocks," suggest the formation of a high molecular mass polymer of κ-casein, as

Figure 5. Chain trace of disulfide-linked tetramer of κ-casein with labeled Cys. A) Symmetric arrangement of disulfides, 11-88, 11-88, 11-88. B) Asymmetric arrangement, 11-88, 11-11, 88-11.

predicted by Thurn et al. (30), that resembles a "rooted tree."

In contrast to the models shown in Figure 5, disulfide-linked structures between Cys-88 on adjacent molecules could not readily be built without a large increase in destabilizing energy. However, such disulfide linkages have been reported (26) to account for 32% of the S-S linkages of κ-casein. Such S-S bonds could arise from adjacent submicelle contacts, which are favorable only within the total micellar structure where κ-casein may be interacted with α_{s1}- or β-casein. Here, interactions are only for κ-casein polymers, and this model mimics the constraints built in by disulfide formation.

Correlation with Physical Chemical Studies. The radius of gyration of the refined κ-casein is 20 Å, but no in-depth small angle X-ray or neutron scattering studies have been carried out on any reduced and alkylated κ-casein, especially at low temperatures when κ-casein may disaggregate. Because Thurn et al. (30) studied only high molecular mass aggregates of κ-casein by small angle neutron scattering, no correlation between an experimental radius of gyration for the monomer and the value calculated from the refined structure is possible. However, light-scattering studies exist on reduced κ-casein from which a stoichiometry of the κ-casein self-association has been obtained at a variety of ionic strengths. These results (31) indicate that κ-casein undergoes a concentration-dependent, reversible association from monomer to polymers of up to 30 molecules, depending on the ionic strength. The concentration-dependent reaction profile of the reduced form of purified κ-casein can be fitted with a model for polymerization at a critical micelle concentration of .05% (31). To determine the reliability of the refined three-dimensional structure presented in this paper, we attempted to construct energy-minimized dimer and tetramer structures that could undergo such reversible aggregation using only hydrophobic and charged sites. This theoretical structure will of necessity not be equivalent to the disulfide-linked molecular mass aggregate formed in the native micelles and depicted in Figure 5. Reoxidized κ-casein on SDS-PAGE shows a preference for lower aggregates in which >50% are trimers or less (14).

The first step was to create a dimer from the smaller pair of antiparallel stranded β-sheet centered on Pro-27, i.e., residues 22 to 33. The side chains are predominately hydrophobic, but the ruminant forms carry a net positive charge. Hydrogen bonding of the antiparallel sheet secondary structures imparts rigidity to this site. As seen in Figure 6A, a dimer can easily be formed if two of these stranded sheets are docked in an antiparallel manner. Such an asymmetric arrangement could maximize the attractive dipole-dipole interaction of the backbone while allowing the hydrophobic side chains to interact freely. After energy minimization, this structure (Figure 6A) exhibits a stabilizing energy of –423.8 kcal/mol over the sum of two monomers (Table 1). The positive charge conserved at residue 49 is thus brought into proximity with the conserved glutamic residues 12 and 15. As seen in the chain trace of the orthogonal view, the two longer hydrophobic "dog legs" are still free for interaction with another similar dimer unit. Construction of such a tetramer unit from two dimer units for which the larger "dog legs" are docked in an antiparallel manner yields the compact structure shown in Figure 6B. This structure, when energy minimized, yields an interaction energy of –920.2 kcal/mol (Table 1). The interaction energies are the difference between the energy of the aggregate and the product of the energy of the monomer times the aggregation number. As seen in Table 1, this greater stabilizing energy for the dimer and tetramer are due primarily to electrostatic and van der Waals' interactions. This energy change is a product of the assymmetric docking of the antiparallel sheets and side chain ion pair interactions between the conserved charged residues. This ionic bond formation is indicated by the loss of net charge with dimer and tetramer formation, i.e., 2 for the dimer and 4 for the tetramer (Table 1), calculated from $-10 - [2(-6)] = 2$ and $-20 - [4(-6)] = 4$, respectively. As also seen in Figure 6, A and B, larger linear aggregates can be easily formed by following the docking procedure. Also, the dipole moment of the dimer is greater than that of the monomer, whereas the tetramer is comparable with the monomer value. The radius of gyration of this tetramer was 42.6 Å.

With the presentation of such possible structures, investigators will be motivated to perform detailed chemical or enzymatic

modifications and small angle X-ray scattering experiments at a variety of conditions to confirm or to disprove the nature of these aggregates and to bring about a fuller understanding of κ-casein and its chemistry.

CONCLUSIONS

We have presented an energy-minimized, predicted, three-dimensional structure of κ-casein using a combination of sequence-based secondary structural prediction algorithms, global secondary structural results from FTIR spectroscopy, and molecular modeling techniques for energy minimization. This structure retains many of the features of the initial model (18), which are in agreement with biochemical and chemical properties of κ-casein, including the ready availability of the major chymosin cleavage site. It also is in agreement

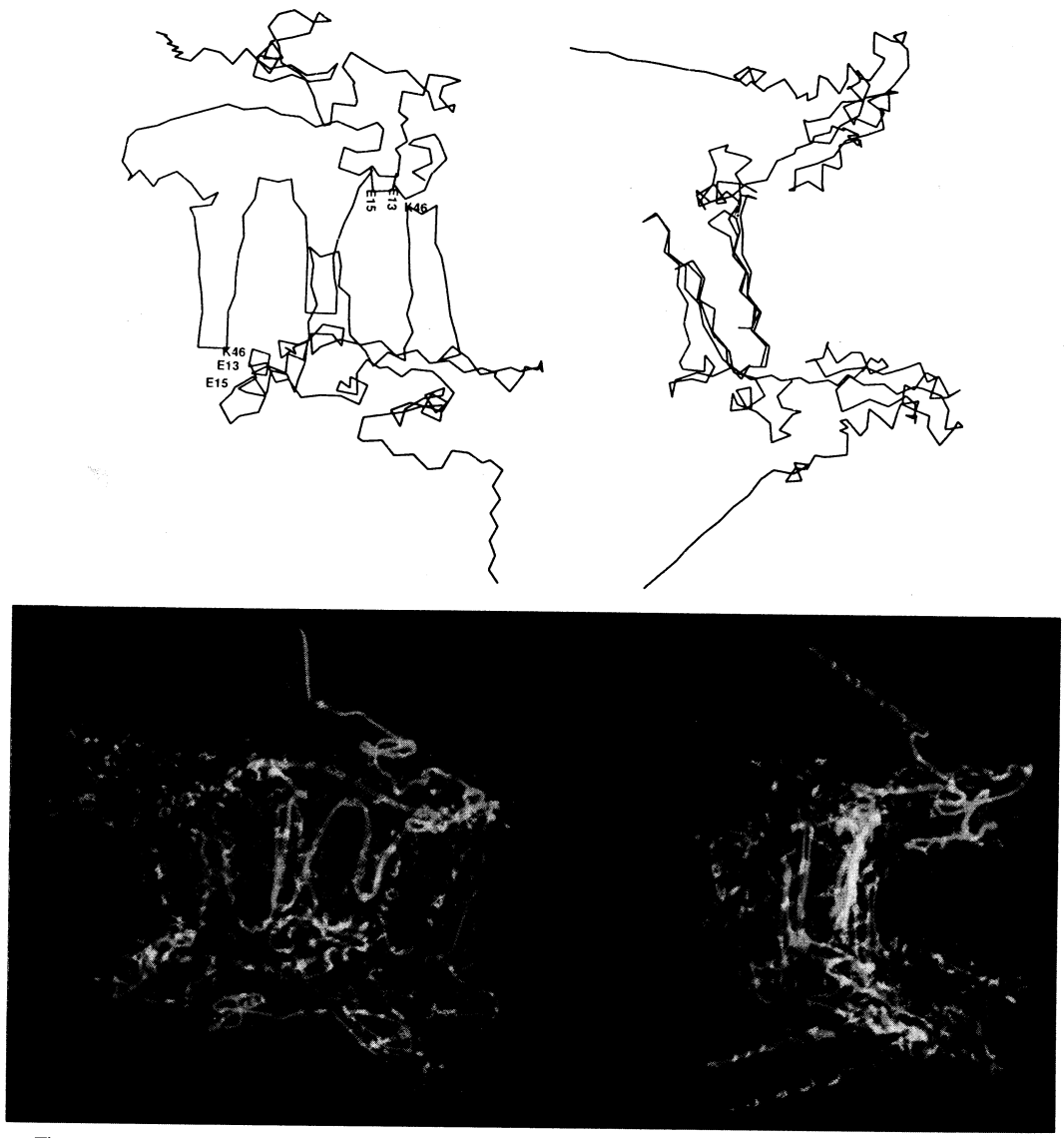

Figure 6. A) Chain trace structure (orthogonal view) of hydrophobic stabilized antiparallel sheet dimer; selected charged residues are labeled. B) Chain trace structure (orthogonal view) of hydrophobic tetramer of κ-casein. Individual monomer backbones are red, purple, yellow, and orange. Hydrophobic side chains are green; acidic side chains are red; basic side chains are purple; all other side chains are cyan.

with other experimentally derived results from solution physicochemical experiments and provides a molecular basis for the temperature-dependent self-association characteristics of κ-casein. However, this structure should be considered to be a working model, to be changed as more precise experiments are performed to ascertain the validity and predictability of this three-dimensional structure. In further studies, calculations of molecular dynamics will be performed on this structure to test its stability when a kinetic energy equivalent to the temperature in bulk solution is applied. In addition, it may be possible in the future to ascertain how the α_{s1}- and β-casein molecules specifically interact with κ-casein to produce synthetic submicelle structures with an average stoichiometry of 4 α_{s1}-casein molecules to 4 β-casein molecules to 1 κ-casein molecule. Such an energy-minimized synthetic submicelle structure is presently being constructed at this laboratory.

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