

Particle Sizes of Purified κ -Casein: Metal Effect and Correspondence with Predicted Three-Dimensional Molecular Models

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κ -Casein as purified from bovine milk exhibits a rather unique disulfide bonding pattern as revealed by SDS-PAGE. The disulfide-bonded caseins present range from dimer to octamer and above and preparations contain about 10% monomer. All of these heterogenous polymers, however, self-associated into nearly spherical uniform particles with an average radius of 8.9 nm as revealed by negatively stained transmission electron micrographs. Evidence is presented that multivalent cations play a role in the stabilization of these spherical particles. Treatment with EDTA causes disruption of the κ -casein particles and leads to a broader size distribution as judged by electron microscopy and dynamic light scattering. The size and shape of the particles are in accord with earlier proposed 3D models for κ -casein that actually predicted participation of divalent cations in the structure.

KEY WORDS: Calcium binding; casein structure; Fourier transform infrared spectroscopy.

1. INTRODUCTION

The major proteins of milk, the caseins, form a unique colloidal complex with inorganic phosphate and calcium termed the casein micelle (Farrell, 1988; Holt, 1992; Schmidt, 1982). In bovine milk, this colloidal complex is thought to be composed of submicelles which in turn are aggregates of three calcium-insoluble proteins (α_{s1} -, α_{s2} - and β -casein) and one calcium-soluble protein κ -casein. It is the latter protein which imparts colloidal stability to the system (Holt, 1992; Swaisgood, 1992); indeed, hydrolysis of κ -casein in the complex by proteolytic enzymes triggers a cascade leading to coagulation during digestion and release of the nutritionally beneficial calcium and phosphate.

Studies on purified κ -casein have shown that it has a monomer molecular weight of 19,000 and that it is a phosphoglycoprotein exhibiting a good deal of posttranslational-related heterogeneity (Eigel *et al.*, 1984). In addition, κ -casein contains two cysteine residues, which yield a rather unique disulfide bonding pattern as revealed by SDS-PAGE under nonreducing conditions (Groves *et al.*, 1991). As purified from milk, κ -casein occurs as a high-molecular-weight complex with a Stokes radius of 9.4 nm as determined by gel exclusion chromatography (Pepper and Farrell, 1982). Complete reduction of κ -casein yields a self-associating system which has been characterized by Vreeman *et al.* (1986) as a polymer of $n = 31$ in equilibrium with its component monomer. The latter model has been the subject of some debate and two alternative polymeric models have been suggested (Thurn *et al.*, 1987, deKruif and May, 1991). This paper investigates the overall polymer

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size distribution of purified κ -casein as determined by electron microscopy and correlates these observations with predictive molecular models for κ -casein (Kumosinski *et al.*, 1993). These comparisons and subsequent experimental studies led to the conclusion that metal ions may play a role in stabilizing the structure of purified κ -caseins.

2. MATERIALS AND METHODS

2.1. Characterization of κ -Casein

Casein was isolated from the skim milk of a single cow by precipitation at pH 4.5–4.6. It was recovered by lyophilization after washing with water.

κ -Casein was isolated from whole casein following the method of McKenzie and Wake (1961). Preparations were made fat free by ultracentrifugation at 4°C and $100,000 \times g$.

Polyacrylamide gel electrophoresis was performed according to Weber and Osborn (1969) with minor modifications and at 7.5% polyacrylamide. Samples were heated 2 min in boiling water before introducing them to the gels; the amount of protein was 0.2 mg or less per gel.

For amino acid composition, protein samples were dissolved in 0.1 N HCl. Aliquots were placed in analysis tubes which had been pyrolyzed at 500°C, and dried in a Waters Pico Tag workstation (Waters-Millipore, Milford, MA). Hydrolysis was carried out in gas phase at 110°C for 24 h with 6 N HCl containing 1.0% (v/v) phenol. The amino acids liberated were quantitated as their phenylthiocarbonyl derivatives using the Waters Pico Tag HPLC system (Waters-Millipore, Milford, MA). Cysteine was identified as half-cystine or as carboxymethyl cysteine by comparison of retention times with known standards. Reduction and alkylation were carried out according to Schechter *et al.* (1973). Data are reported as molar ratios with phenylalanine fixed at 4 residues/molecule.

Densitometry of the gels was carried out on a Molecular Dynamics gel scanner. Data were analyzed using software programs supplied by the manufacturer.

A Perkin Elmer Model 1100 atomic absorption analyzer unit was used for this study. Five milligrams of each κ -casein sample was digested in 5% nitric acid for 15 hr on a steam bath, after which samples were analyzed for iron and calcium.

2.2. Electron Microscopy of κ -Casein

Samples of the κ -casein were prepared for electron microscopy by dissolving the casein in PIPES–KCl buffer [25 mM piperazine-N-N'-bis(2-ethanesulfonic acid), pH 6.75, made up to be 80 mM KCl]. The samples were made up to be 30–35 mg/ml and were passed through 0.45- μ m filters. The filtrates were adjusted to 25 mg/ml with filtered buffer and equilibrated at 37°C for 30 min.

Thin support films of amorphous carbon were evaporated on strips of cleaved mica and mounted on 400-mesh copper grids. All procedures were carried out on a water bath with samples and reagents at 37°C. Aliquots (10 μ L) of casein in buffered solution were placed on freshly prepared support films for 30–60 sec over a water bath at 37°C; then the sample side of the grid was washed with a controlled stream of 10–15 drops of buffered solution from a disposable Pasteur pipette containing 1% glutaraldehyde at 37°C. This was done to physically stabilize the composition of monomers in the form of polymers and to trap the equilibrium structures, while reducing the protein concentration to produce a discontinuous monolayer of κ -casein particles. Then the adsorbed particles were washed with a similarly controlled stream of 5–10 drops of 2% uranyl acetate solution at 37°C for negative staining. Excess uranyl acetate solution was adsorbed from the grid surface into Whatman #1 filter paper, and grids were allowed to air dry at room temperature.

Images of κ -casein structures in randomly selected fields on grids were recorded photographically at instrumental magnifications of 88,000 \times using a Zeiss Model 10B electron microscope (Thornwood, NY) operating at 80 kV and 97,000 \times using a Philips Model CM12 scanning-transmission electron microscope (Rahway, NJ) operating at 60 kV. Photographic prints were prepared at a magnification of 442,500 \times and the circumferences of individual particles were traced onto transparent overlays. The overlays were digitized and circular diameters of the tracings were calculated and plotted using Imageplus software and a Dapple Microsystems digital image analyzer (Sunnyvale, CA).

2.3. Molecular Modeling Techniques

Construction of Aggregates. The model κ -casein aggregate structure employed the energy-minimized κ -casein tetramer model which was

previously reported by this laboratory (Kumosinski *et al.*, 1993). Octamers were constructed from the tetramer using a docking procedure and the Sybyl molecular modeling software (Tripos, St. Louis, MO). Four possible docking orientations were constructed, energy minimized, and assessed for the lowest energy in order to provide a reasonable sampling of conformational space. The criterion for acceptance of reasonable structures was determined by a combination of experimentally determined information and the calculation of the lowest energy for that structure.

A full description of the concepts behind the use of the molecular force fields (including relevant equations) was given in previous communications (Kumosinski and Farrell, 1994; Kumosinski *et al.*, 1994). In these calculations for protein-protein interactions, a Kollman force field was employed (Weiner *et al.*, 1986; Kollman, 1987). A cutoff value of 0.8 nm was used for all nonbonded interactions. The conjugate gradient technique was also employed as a minimization algorithm for all structures in this study.

Construction of Calcium-Caseinate Aggregates. Calcium-containing structures of the refined, energy-minimized κ -casein octamer model were constructed using the Tripos Sybyl molecular modeling software (Version 6.03) on a Silicon Graphics (Mountain View, CA) Indigo 2 workstation. In this case a Tripos force field was used to achieve minimization of the calcium-caseinate complex (because of its ability to handle nonprotein atoms without having to define a new library entry for calcium "residues"). A nonbonded cutoff of 0.8 nm was used.

2.4. Dynamic Light Scattering

Glassware and quartz cells were cleaned with ultrapure water obtained from a Modulab Polisher HPLC Laboratory Reagent Grade Water System. Samples were prepared as described above for electron microscopy. The quartz cell was rinsed several times with filtered samples prior to capping and measurement of scattering of light. Dynamic light scattering was measured with a Malvern System Model 4700c equipped with a 256-channel correlator. Light at 488 nm was provided by a Spectra Physics Model 2020 5-W laser. The ATTPC6300 computer supplied by Malvern was enhanced with a Sota 386si High Performance

Accelerator card. Solutions were maintained at room temperature with filtered water in the goniometer chamber bath. Dynamic light scattering measurements were made at several angles using a small aperture in the photomultiplier detector. Photon count rates were kept in the low range by controlling the size of the laser beam or by appropriate dilution of the sample. The data were processed by Malvern Automeasure Version 4.12 software. Multiangle analysis by Marquardt minimization was carried out with Malvern software (Cummins and Staples, 1987). The performance of the system and analysis software was tested on latex beads, 91 and 455 nm diameter (Sigma Chemical Co.). The system was tested also by measuring sizes of bovine milk micelles; sizes obtained were in agreement with published values (Horne, 1984).

2.5. FTIR Analysis

Samples of purified κ -caseins were dissolved in 25 mM PIPES containing either KCl (80 mM) or EDTA (14 mM) and prepared as described for electron microscopy. FTIR measurements were made at 25°C in water using a Nicolet 740 FTIR spectrometer. Data accumulation and sample compartment modifications have been described (Kumosinski and Unruh, 1996). Vapor buffer background was subtracted to yield the amide I and amide II bands; deconvolution and summation of bands were attributed to secondary structural elements as previously described (Kumosinski and Unruh, 1996).

3. RESULTS AND DISCUSSION

3.1. Characterization of the Purified κ -Casein

Alkaline-urea PAGE in the presence of reducing agents showed all preparations to be typical κ -caseins containing a number of bands of increasing mobility suggestive of a normal distribution of nonglycosylated and glycosylated bands (Woychik *et al.*, 1966). The mobility of the nonglycosylated band indicated that the protein was the A genetic variant of κ -casein (data not shown).

The amino acid composition of the κ -casein is shown in Table I together with its composition based on the amino acid sequence (Mercier *et al.*, 1973). The sequence data shown are for κ -casein A;

Table I. Amino Acid Composition of κ - and RCM- κ -Casein; Comparison with Sequence Data

Amino acid	Residues/mole		
	Purified κ -casein ^a	Sequence κ -A ^b	RCM ^c κ -casein
SCM-Cys ^d	—	2	1.8
Asp	11.8	12 (11)	11.9
Thr	12.5	15 (14)	13.1
Ser	11.5	13	12.4
Glu	25.7	27	27.3
Pro	19.9	20	19.9
Gly	2.9	2	2.8
Ala	12.3	14 (15)	13.4
Val	9.6	11	10.2
Met	2.4	2	2.0
Ile	10.3	12 (13)	11.2
Leu	8.6	8	8.9
Tyr	7.7	9	8.4
Phe	4.0	4	4.0
Lys	9.1	9	8.5
His	2.7	3	2.7
Arg	4.1	5	4.6

^a Twenty-four-hour hydrolysis molar ratio, Phe = 4, average of three determinations.

^b Numbers in parentheses represent value for κ -B.

^c RCM, Reduced carboxymethylated.

^d SCM, S-carboxymethyl cysteine.

the composition data of the sample are consistent with that of κ -casein A, showing apparently equivalent aspartic, alanine, and isoleucine contents. This confirms the alkaline-urea PAGE phenotype.

3.2. Degree of Polymerization of Purified κ -Casein

When the bovine κ -casein was subjected to SDS-PAGE in the absence of reducing agents, distinct polymers (at least eight) of orderly increasing size were observed. Polymerization appears to be driven by either one or both of two cysteines in κ -casein (Groves *et al.*, 1991). On electrophoresis of κ -casein after reduction with 2-mercaptoethanol, the polymers disappear resulting in a single band representing the κ -casein monomer (Fig. 1). In these experiments the samples (1 mg/ml) were heated in a boiling water bath for 2 min prior to electrophoresis in standard buffer, SDS (1%) with 0.1 M sodium phosphate, pH 7.0. Rasmussen *et al.* (1992) demonstrated that

these polymers represent a random cross-linking pattern using all three possible combinations of disulfide bonds (11–11, 88–88, and 88–11).

3.3. Electron Microscopy of Purified κ -Casein

When subjected to transmission electron microscopy using uranyl acetate as a negative stain, the purified κ -casein appears to occur primarily as single particles with only a few multiple particles in each field. Close examination of the micrographs shows some particles to have a beanlike shape and some to be spherical, but by and large the particles are rather uniform and spherical in nature. A typical field is shown in Fig. 2. In these experiments the κ -casein was dissolved in PIPES–KCl buffer and the samples tempered at 37°C before fixing as described in Methods and Materials. This was originally done to preserve maximal hydrophobic interactions for whole casein (Kumosinski *et al.*, 1995) and was carried over to these experiments for the same reasons.

Size distributions of the κ -casein particles were determined from 20 electron micrographs. The first sample included 1500 particles and gave a Gaussian but narrow distribution as shown in Fig. 3. For this sample the number average radius was 9 nm, but greater than 86% of the particles counted had a particle radius of 8.8 ± 0.9 nm. A second measurement of size distribution quite similar to the first was obtained by counting 800 particles on 15 fields for another preparation of the protein. This preparation gave an average radius of 9 nm; again, greater than 84% of the particles counted had a particle radius of 9.0 ± 0.7 nm, in good agreement with the first data set. The average radius for the two distributions is 8.9 nm.

Previous studies on whole κ -casein by shadowing yielded ranges of 10–15 nm (Schmidt and Buchheim, 1975) and 18–20 nm (Parry and Carroll, 1969) for the diameters of κ -casein particles. However, few statistical analyses of these particle sizes were given. The particles observed in this study appear somewhat more uniform in distribution, but are in the range of the values previously reported. The rather uniform particle size determined by electron microscopy is somewhat at odds with the apparent random distribution of polymers observed for the κ -casein preparations on SDS-PAGE in the absence of reducing agents. With disulfide bonds ranging from dimers to

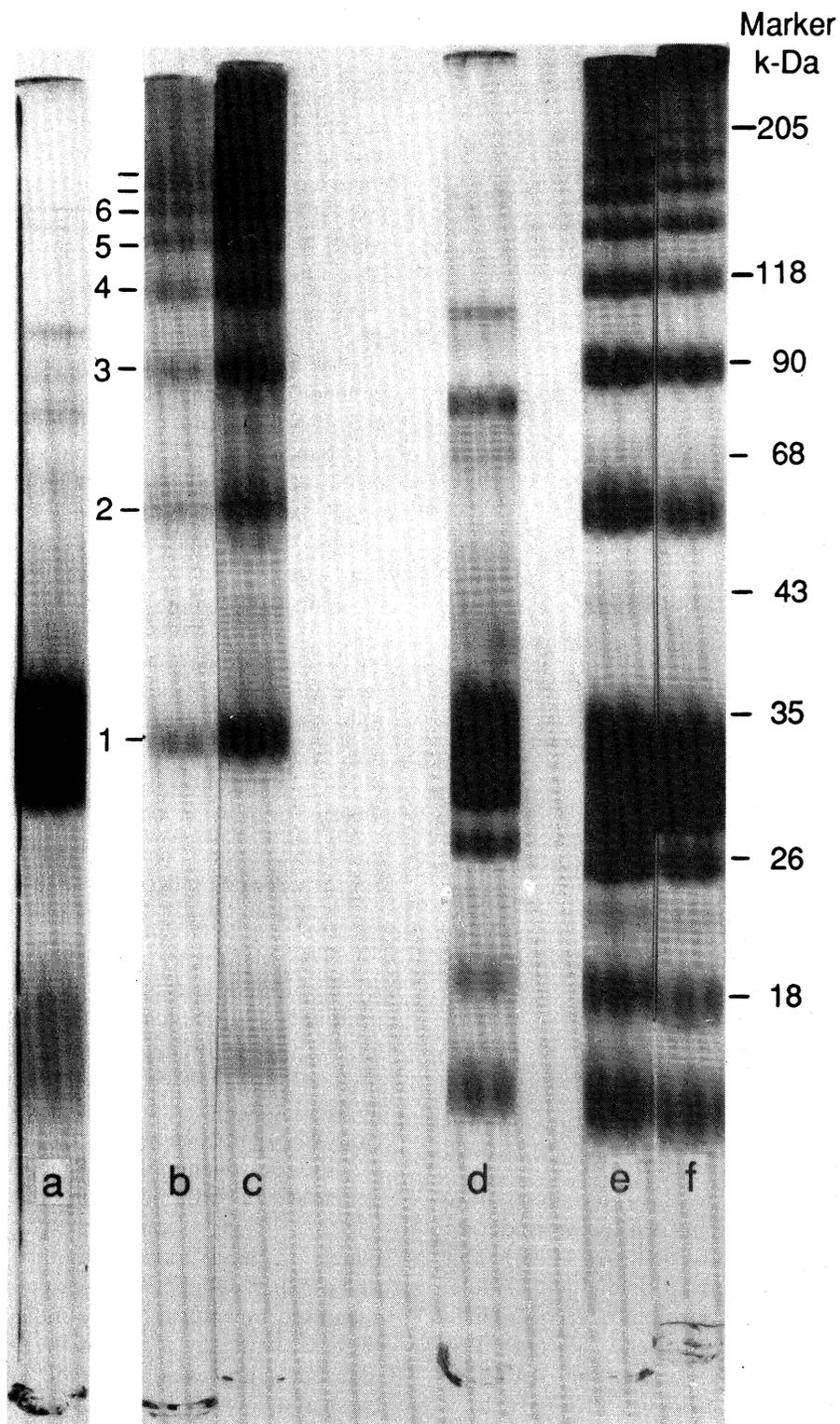


Fig. 1. SDS-electrophoresis of κ -casein under standard conditions of 7.5% gels, pH 7.0, reduced (a) and at two concentrations (b,c) unreduced. Also shown are reduced (d) and unreduced κ -casein samples (e, f) which are partially purified.

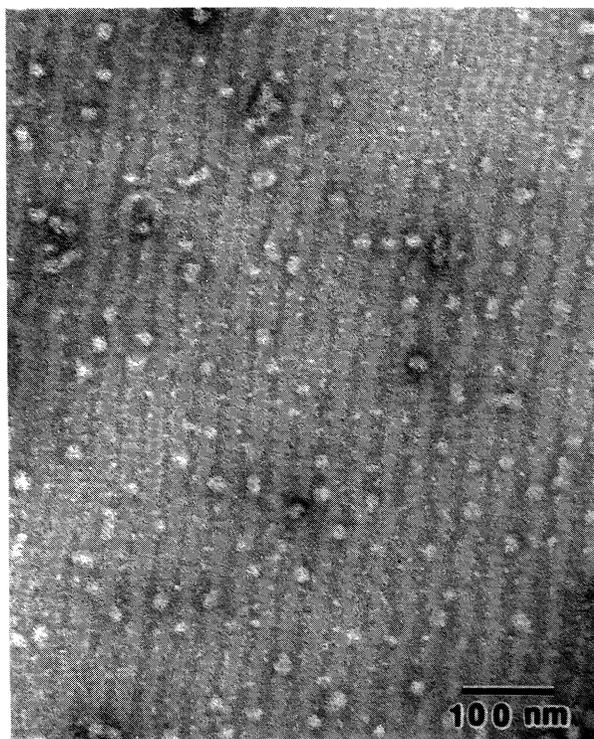


Fig. 2. Transmission electron micrograph of a general field of negatively stained (uranyl acetate 2%) κ -casein.

octamers and above, a more heterogeneous population could be anticipated. However, the complexes are quite highly associated and in fact do not form reversible associating systems in the absence of reducing agents (Pepper and Farrell, 1982). The Stokes radius determined by gel chromatography for these maximally associated κ -casein particles was 9.4 nm. This value is in good agreement with the average value of 8.9 nm obtained by electron microscopy in this study.

3.4. Molecular Modeling of the κ -Casein Polymers

Previous studies from this laboratory have generated a three-dimensional molecular model for κ -casein monomers (Kumosinski *et al.*, 1993). This monomer model was used to assemble disulfide-linked tetramers with an asymmetric arrangement of disulfides (11–88, 11–11, and 88–11). This tetrameric species is shown in Fig. 4A. The calculated radius of gyration for this particle is 4.7 nm, which converts to a hydrodynamic radius of 6.1 nm, somewhat smaller than the experimentally observed particles and than the experimental molecular weight [150,000–600,000 (Swaisgood *et*

al., 1964)]. To simulate more fully the experimental data, two structures similar to Fig. 4A were docked at various angles relative to each other and then energy minimized. The apparent best fit, based on minimized energy, was about a 90° angle. A small degree of energy reduction (–237 kcal/mol) occurred as shown in Table II. The calculated hydrodynamic radius for this molecule was 8.0 nm, which is more in agreement with the experimental data. On observation of the first octamer molecular model, small pockets of net negative charge on the adjacent tetramers which could deter docking were observed. Four Ca^{2+} ions were added to the octamer, two each to bridge Glu-151 to Glu-151 and Glu-12 to Glu-12. Addition of four Ca^{2+} ions to the model reduced the total energy of the system by 1441 kcal/mol as measured during 20-psec molecular dynamics trajectories at 50°K using the Tripos force field. These molecular dynamics calculations yielded a radius of gyration of 5.4 nm or a hydrodynamic radius of 7.0 nm. Thus the divalent cations could lend stability to the κ -casein particles. It should be kept in mind that the model systems employed here do not contain water, and thus the energies reported are relative to each other and have only qualitative significance. The final model is shown in Fig. 4B. Note that one Ca^{2+} has migrated toward a backbone carbonyl.

3.5. Divalent Metal Ion Contents of the Purified κ -Casein and Effect of EDTA

The McKenzie and Wake (1961) method of κ -casein preparation involves a number of classical protein isolation techniques such as acid, salt, alcohol, and urea fractionation. The κ -casein preparations were subjected to atomic absorption analysis and were found to contain two major cations: calcium and iron. For three preparations, 0.22 mol of calcium and 0.19 mol of iron were found per mole of protein monomer with about 20% variance from preparation to preparation. This equals on the average 1.8 mol of Ca^{2+} and 1.5 mol of Fe^{2+} per octamer of κ -casein. It is possible, as suggested by analysis of the molecular models, that these cations play a role in the stabilization of the κ -casein complexes, observed in Fig. 2, and as measured by gel permeation chromatography (Pepper and Farrell, 1982). To test this hypothesis, κ -casein was dissolved in the PIPES buffer at pH 6.75 as before but with added 14 mM trisodium EDTA to replace the KCl while maintaining

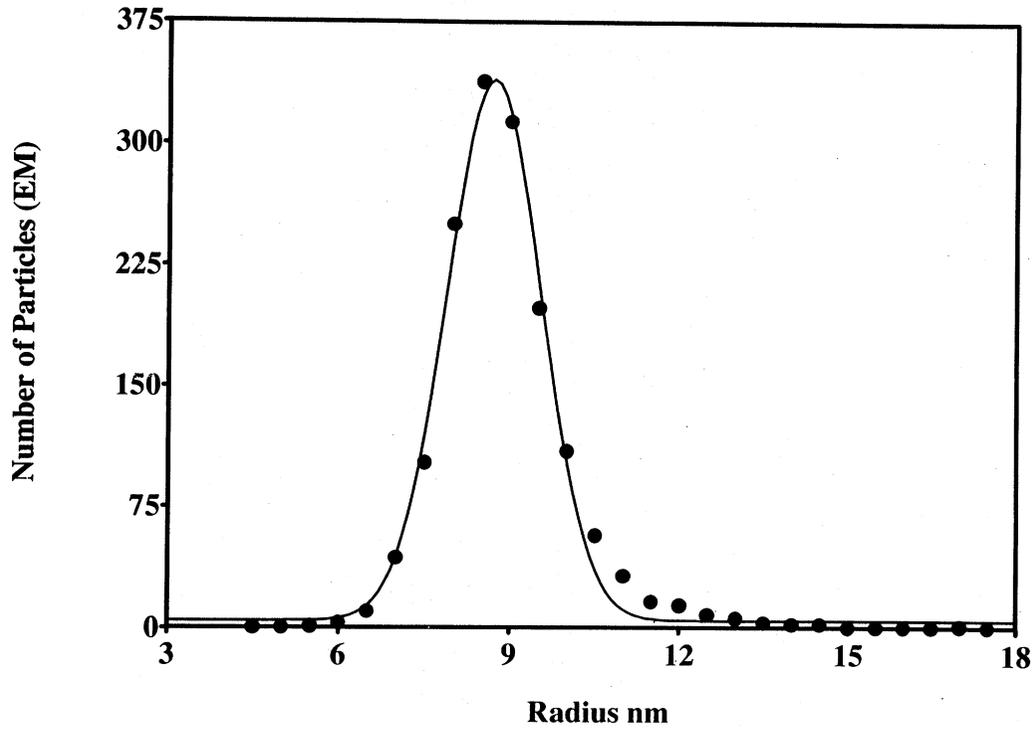


Fig. 3. Size distribution of κ -casein particles counted and sized by electron microscopy as described in Section 2.



Fig. 4. (A) Three dimensional model for κ -casein tetramer linked by an asymmetrical arrangement of disulfides, 11–88, 11–11, 88–11.

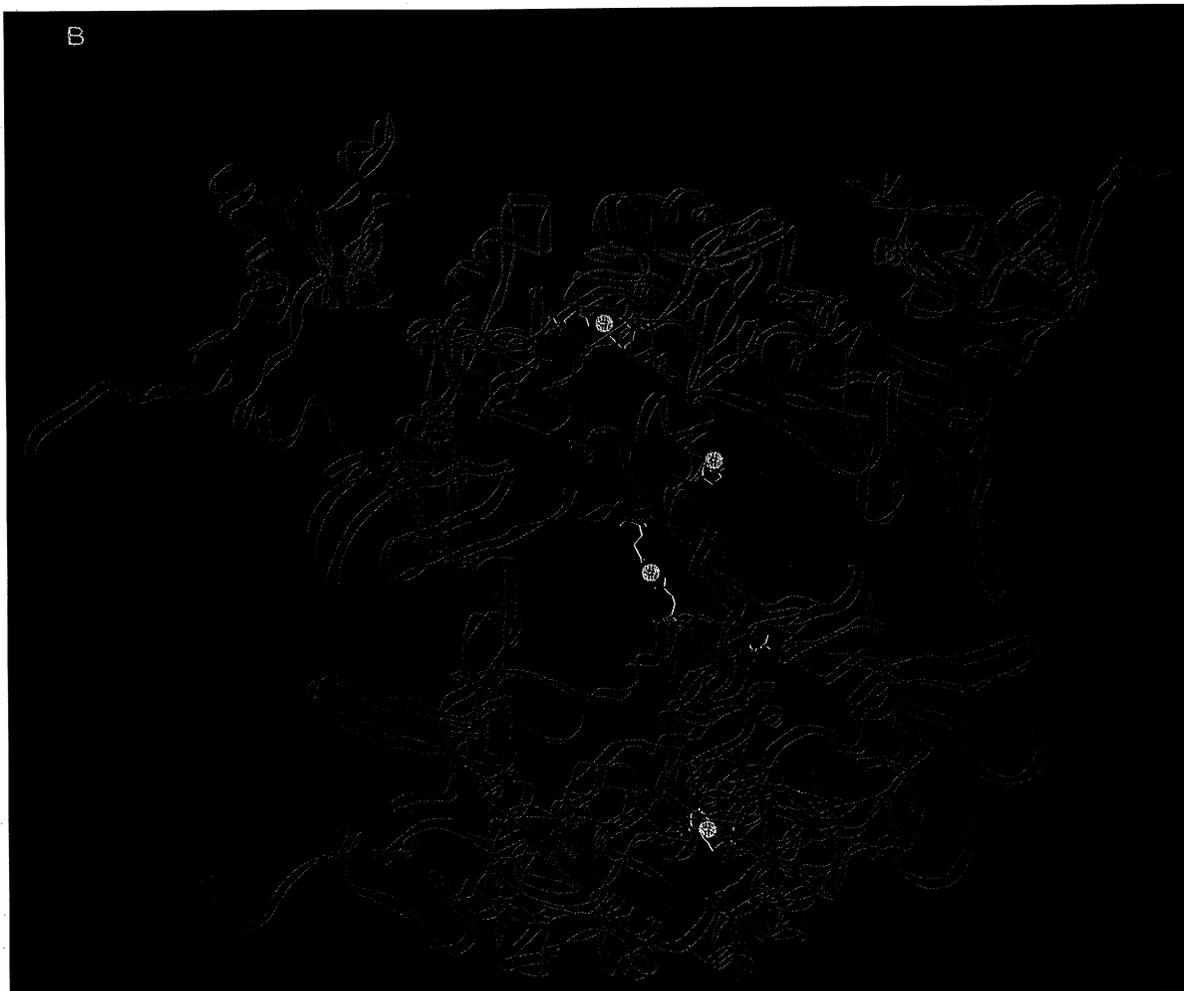


Fig. 4. (B) Three-dimensional model for κ -casein octamer formed from two tetramers with added Ca^{2+} (four) after minimization and 20 psec of molecular dynamics simulation.

Table II. Energy of κ -Casein Aggregates

Structure	Energy for given Tripos force field (kcal/mol)		ΔE^b
	Tetramer	Octamer ^a	
Bond stretching	247	532	38
Angle bending	1,757	3,653	139
Torsional	916	1,871	39
Out-of-plane bending	80	169	9
1-4 van der Waals	173	343	-3
van der Waals	-1,986	-4,034	-62
1-4 Electrostatic	13,275	26,642	92
Electrostatic	-18,947	-38,383	-489
Total	-4,485	-9,207	-237

^a Molecular dynamics for 5 psec.

^b Defined as $E_{\text{OCTAMER}} - 2E_{\text{TETRAMER}}$.

constant ionic strength. Transmission electron microscopy was carried out in parallel on both KCl and EDTA samples above. The κ -casein particles were visualized by negative staining. The net effect of the EDTA was to partially disrupt the uniform distribution seen in Fig. 2; many of the κ -casein particles are seen as diffuse and poorly organized or partially aggregated. The κ -particles seem to be flatter in EDTA (Fig. 5A) and their uniform character and nature in KCl (Fig. 5B) are dramatically altered.

To characterize more quantitatively this shift in size distribution, κ -casein was dissolved in PIPES-KCl or EDTA as described above at concentrations of 4 g/L. These samples were analyzed for particle size at 30, 60, 90, and 120° by dynamic light scattering. The treatment with EDTA yielded an

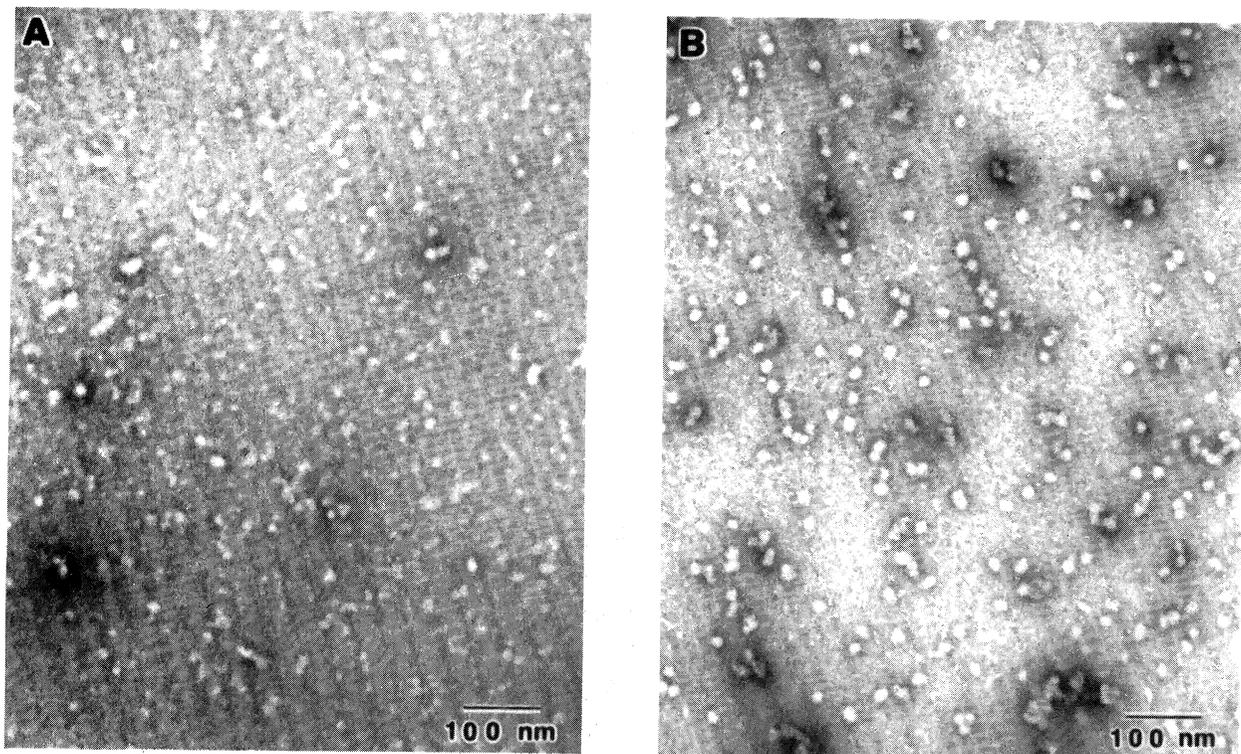


Fig. 5. Comparison of size and shape of κ -casein particles in (A) PIPES-EDTA and (B) PIPES-KCl as determined by transmission electron microscopy.

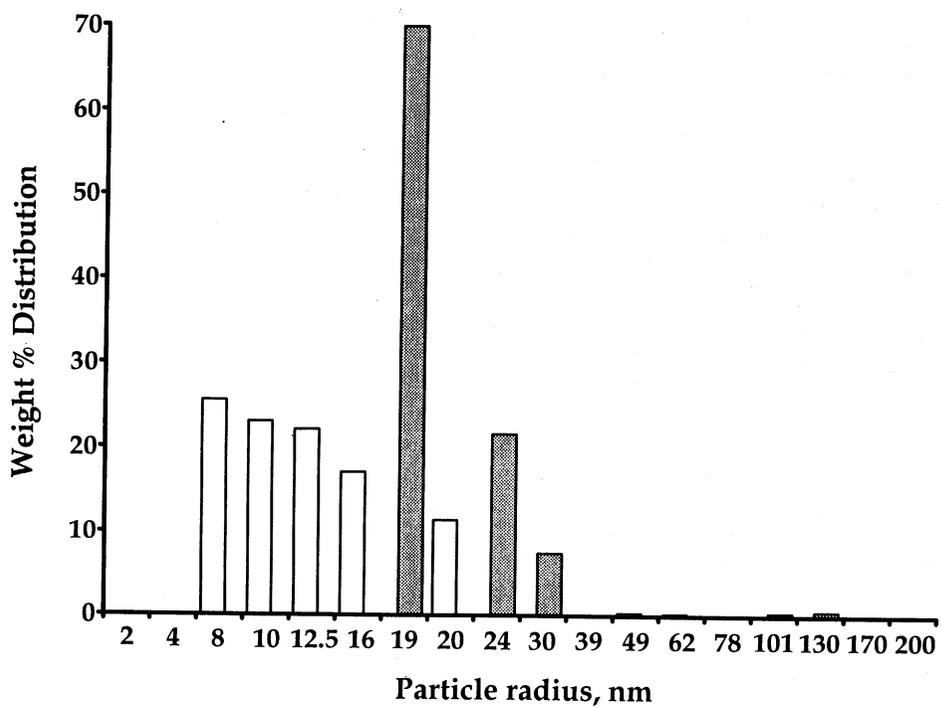


Fig. 6. Comparison of angle-dependent weight percent particle size distributions for κ -casein in PIPES-KCl (clear) and EDTA (shaded) as determined by dynamic light scattering.

increase in overall size, accompanied by a shift to a somewhat less asymmetrical weight percent distribution (Fig. 6). This weight percent distribution could be influenced by the larger sizes generated by EDTA; for each angle, the Z-average diameters increased more dramatically than the mean diameters. Overall, the weight percent diameter shifted from 24.9 to 42.9 nm. The number-average radius shifted from 9.6 ± 2.5 nm in KCl to 19.9 ± 2.4 nm in EDTA; similar results were given by a second preparation. Thus the data from both electron microscopy and light scattering argue for altered particle size and shape on treatment of purified κ -casein with EDTA. This indicates that the metal ions which are either normally associated with κ -casein or become associated with it during purification play a rather distinct role in the particle distribution observed for the purified protein. Because of the variance in the type and amounts of ions present, it is likely that the binding represents a series of multiple equilibria, rather than specific binding sites.

3.6. Effects of EDTA on Secondary Structural Elements of κ -Casein as Determined by FTIR

FTIR Measurements were made on purified κ -casein dissolved in water in PIPES-KCl or EDTA. Deconvolution of the amide I and II bands and summation of the areas attributed to prominent structural elements yielded the results of Table III. The results for κ -casein in KCl are in accord with earlier secondary structural analysis of casein by Raman spectroscopy (Byler *et al.*, 1988). The repeatability of the FTIR methodology as shown in Table III is similar to that found for globular proteins (Kumosinski and Unruh, 1996). Thus changes in extended (β -) structures and in α -helical or "bent-sheet" structures are most likely significant; there appears to be a loss of the former and a gain in the latter on treatment with EDTA. While these are not dramatic, some secondary

Table III. FTIR Estimations of the Secondary Structural Elements of κ -Casein

Structural element	KCl %	EDTA %
Turns	25.2 ± 1.8	21.2 ± 1.6
Helix ^a	16.7 ± 2.1	24.5 ± 2.9
Extended ^b	35.2 ± 3.2	28.0 ± 2.5
Irregular	23.0 ± 3.9	26.2 ± 4.4

^a Includes 3–10 helix and "bent-sheet" structures.

^b Includes β -sheet.

structural change accompanies the overall alteration of κ -casein particles by EDTA. It is interesting to note that the β -sheet (extended) structures which are prominent in the interactions used to dock the 3D models (Fig. 4) are decreased in the EDTA-treated samples and more bent-sheet (or possibly helical) elements are formed.

4. GENERAL CONCLUSIONS

κ -Casein as purified from bovine milk exists as rather uniform spherical particles of 8.9 nm radius. This is at odds with some physical chemical data for κ -casein (Table IV). It must be noted that the more recent data on the size distributions of κ -casein were not taken on the type of κ -casein used here. All of these data (deKruif and May, 1991; Thurn *et al.*, 1987; Vreeman *et al.*, 1986) were collected on samples which were reduced, purified on DEAE media in urea to remove glycosylated forms, dialyzed, lyophilized (Vreeman *et al.*, 1981), and then redissolved and reduced to varying extents (Table IV). The process of further isolation by use of urea and reducing agents may yield differently sized particles. In this study the average radius for bovine κ -casein particles was 8.9 ± 1.1 nm, which is similar to the value of 7.9 nm found for whole casein submicelles by identical electron microscopy techniques (Kumosinski *et al.*, 1995). Few higher order particles were observed in these studies perhaps because the McKenzie-Wake method includes a $100,000 \times g$ centrifugation to remove fat-protein complexes.

An overall view of κ -casein as purified from bovine milk is that the protein contains a series of disulfide-bonded polymers, ranging from monomers to octamers and above (Fig. 1). These polymers are most likely distributed among three possible arrangements of disulfides (11–88, 88–88, and 11–11) according to Rasmussen *et al.* (1992). The apparent heterogeneity of the individual κ -casein chains, however, is overcome through protein-protein interactions which yield rather uniform particles with radii of 8.9 nm as revealed by electron microscopy or 9.6 nm (number-average) by dynamic light scattering. The shape and size of the κ -casein particles may be mediated in part by metal cations such as calcium and iron. However, no specificity for these ions can be implied from the data. Removal of multivalent cations by EDTA yields more-heterogeneous particles which could be anticipated by the SDS-PAGE data. Finally the previous models for κ -casein (Kumosinski *et al.*,

Table IV. Summary of Physical Data on κ -Caseins^a

	MW	Radius (nm) ^b	Method	Reduction
Vreeman <i>et al.</i> (1986)	600,000	11.1 ^c	Sedimentation	1 week, 2-ME
Slattery and Evard (1973)	600,000	11.2 ^d	Sedimentation	1 h, 40 mM DTT
deKruif and May (1991)	—	14.7 ^c	SANS	5 mM DTT
Pepper and Farrell (1982)	—	9.4 ^d	GPC	None
Thurn <i>et al.</i> (1987)	2,000,000	7.0 ^{c,e}	SANS	None
This study	—	8.9 ^d	EM	None
		9.6 ^d	DLS	None

^a 2-ME, 2-Mercaptoethanol; SANS, small-angle neutron scattering; GPC, gel permeation chromatography; EM, electron microscopy; DLS, dynamic light scattering, number average.

^b Radius type varies with method.

^c DEAE purified K-I casein.

^d Whole κ -casein.

^e Internal "submicellar" particle of larger aggregate.

1993) suggested possible internal and external sites for divalent cation binding to enhance closer approach and yield the more compact particles observed by electron microscopy in KCl. Subsequent measurements in EDTA support this notion.

In its native environment within the colloidal casein complex, κ -casein most likely occurs in close association with the more phosphorylated caseins. During the isolation procedure such heteropolymeric interactions are most likely replaced by homopolymeric associations which could lead to sequestration of multivalent ions. It may be speculated, however, that the multivalent cations replace the interactions between cationic (Lys, Arg) groups on the other caseins and κ -casein. Thus both hydrophobic and ionic interactions could play a role in the protein-protein interactions within the casein micelle.

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