

Environmental Influences on Purified κ -Casein: Disulfide Interactions¹

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ABSTRACT

Bovine κ -casein, the stabilizing protein of the colloidal milk protein complex, has a unique pattern of disulfide bonding. The protein exhibits varying molecular sizes on SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis), ranging from monomer to octamer and above in the absence of reducing agents. Heat treatment of the samples with SDS prior to electrophoresis caused an apparent decrease in polymeric distribution: up to 60% became monomers after 30 min at 90°C as estimated by densitometry of SDS-PAGE. In contrast, heat treatment of the samples without detergent at 90 or 37°C significantly increased in high molecular weight polymers, as judged by electrophoresis and analytical ultracentrifugation. In 6 M urea, the protein could be completely reduced by dithiothreitol, but, upon dialysis, varying degrees of polymer reformation occurred, depending on the dialysis conditions. Spontaneous reoxidation to polymeric forms is favored at low pH (<5.15) and low ionic strength. The results are discussed with respect to the influence of the method of preparation on the polymer size of κ -casein and on its resultant physical chemical properties.

(**Key words:** casein structure, molecular mass, disulfide interactions)

Abbreviation key: DTT = dithiothreitol, PIPES = piperazine-N,N'-bis (2-ethanesulfonic acid).

INTRODUCTION

The caseins of milk occur as a unique colloidal calcium phosphate transport complex termed the casein micelle (11). The colloidal stability of these particles is imparted by one particular casein: κ -CN (27). Hydrolysis of κ -CN by digestive enzymes

(chymosin in particular) begins a process of micelle coagulation that ultimately leads to delivery of protein, calcium, and phosphate in a readily digestible form (14, 21).

Because of its importance to milk processing, the best characterized form of κ -CN is that occurring in bovine milk (8, 23). The κ -CN from bovine milk has a monomer molecular weight of 19,000, contains two cysteine residues (Cys¹¹ and Cys⁸⁸), and occurs as a phospho-glycoprotein displaying microheterogeneity with respect to phosphate and carbohydrate (7). As purified from milk, κ -CN contains a unique disulfide bonding pattern that is detected by SDS-PAGE in the absence of reducing agents (13). The molecular weights range from monomer to octamer and above and appear to be rather evenly distributed across this range (13). Rasmussen et al. (19) reported an apparently random distribution of the disulfides in these polymers involving Cys¹¹ and Cys⁸⁸: 88 to 88, 11 to 11, and 11 to 88.

Historically, disulfide bonding has not been considered to be of primary importance in the structure and stability of the casein micelle (8, 23). This concept comes from the early work of Woychik et al. (30), who showed that reduced and alkylated κ -CN had the same propensity to reform model colloids as native κ -CN. The discovery of the unique disulfide bonding properties, coupled with discrepancies in the reported molecular weights for κ -CN aggregates, which could be related to alterations in disulfide bonding patterns (5, 22, 25, 26, 27), prompted a reevaluation of the nature of environmental effects on κ -CN disulfides. In this manuscript, we investigate the effects of heat, reduction, and conditions of reoxidation on the disulfide bonding patterns of κ -CN as they may influence physical properties of the protein.

MATERIALS AND METHODS

Casein was isolated from skim milk of a single cow by precipitation at pH 4.5 to 4.6 and was lyophilized after washing with water. κ -Casein was isolated from whole casein following the method of McKenzie and Wake (16). Preparations were made fat free by ultracentrifugation at 4°C at 100,000 $\times g$ for 1 h.

Fraction III, the final by-product in the κ -CN fractionation of McKenzie and Wake (16), was found by SDS-PAGE to contain a significant amount of κ -CN. To obtain purified κ -CN from this source, fraction III (750 mg) was reduced and fractionated in urea on a DEAE-cellulose column with a salt gradient according to the method of Doi et al. (6). Most of the κ -CN was eluted in three sequential peaks that were designated κ -1 CN (91 mg), κ -2 CN (42 mg), and κ -3 CN (10 mg); these peaks also correspond to fractions κ -I, κ -II, and κ -III of Vreeman et al. (26).

Polyacrylamide gel electrophoresis was according to Weber and Osborn (29) with minor modifications and at 7.5% polyacrylamide. Samples (2 mg/ml) were dissolved in 0.1 M phosphate buffer pH 7.5 in the presence of 2.0% SDS and heated for 2 min in boiling water before they were introduced to the gels; protein concentration was ≤ 0.2 mg per gel. Phast[®] gels (Pharmacia-Biotech, Uppsala, Sweden) were run according to the procedures given by the manufacturer for 7.5% or 20% homogeneous gels. Samples of κ -CN at 5 mg/ml were heated for 15 and 30 min at 90°C in phosphate buffer (pH 7.5) and in piperazine-N,N'-bis(2-ethane sulfonic acid (PIPES) buffer at 35 mM (pH 7.5 and 6.75) containing 80 mM NaCl or 14 mM EDTA. An equal volume of 3.0% SDS was added, and then the samples were subjected to electrophoresis. These buffers were the Na⁺ salts, so that, when an equal volume of 3.0% SDS was added, no potassium was present to precipitate the dodecyl sulfate.

To determine the amino acid composition, protein samples were dissolved in 0.1N HCl. Aliquots were placed in analysis tubes that had been pyrolyzed at 500°C and dried in a Waters Pico Tag[®] work station (Waters-Millipore Corp., Milford, MA). Hydrolysis was carried out in a gas phase at 110°C for 24 h with 6N HCl containing 1.0% (vol/vol) phenol. The amino acids that were liberated were quantitated as their phenylthiocarbonyl derivatives using the Waters Pico Tag[®] HPLC system (Waters, Milford, MA). Cysteine was identified as carboxymethyl cysteine by comparison of retention times with known standards. Reduction and alkylation were used to yield reduced carboxymethylated κ -CN and were carried out according to Schechter et al. (20). Data are reported as molar ratios with phenylalanine fixed at 4 residues per molecule (17).

The N-terminal amino acid sequences of the κ -CN samples exhibiting proteolysis were determined by automated Edman degradation on a pulse-liquid sequencer with online phenylthiohydantoin amino acid analysis (model 473A; Applied Biosystems, Foster City, CA). Note that, as the native N-terminal was

blocked (pyroglutamic acid), only newly liberated residues were observed.

Densitometry of the gels was carried out on a Molecular Dynamics gel scanner (Molecular Dynamics, Sunnyvale, CA). Data were analyzed using the Image Quant software programs supplied by the same manufacturer.

For analytical ultracentrifugation, the protein samples were dissolved at concentrations ranging from 1.0 to 3.0 mg/ml at pH 6.75 in 35 mM PIPES containing 80 mM KCl. The samples and solvents were filtered with an HVLP 0.45- μ m membrane filter (Waters). For κ -CN, Phast[®] gel electrophoresis in SDS showed a nearly identical pattern of protein components before and after filtration; <1% of the material was retained on the filter as ascertained by UV spectroscopy. Experiments using sedimentation equilibrium were performed with an XL rotor in an analytical ultracentrifuge (Beckman Optima XL-A; Beckman Instruments, Palo Alto, CA) at speeds ranging from 3000 to 6000 rpm at 25°C. A 12-mm charcoal-epon 6-channel centerpiece was used with quartz windows in a wide aperture window holder. Data were collected at 280 or 292 nm for samples >1.0 mg/ml, using the standard XL-A procedure. The absorption versus radius squared plots were analyzed directly for weight average molecular weight using the program IDEAL1 or ASSOC4 (18).

The ASSOC4 model is for a system with up to four species; here best fits were obtained by assuming that κ -CN exists as a covalently and noncovalently bound monomer that self-associates to polymers of higher order. The equation used is

$$A_r = \text{EXP}[\ln(A_0) + H \times M \times (X^2 - X_0^2)] + \text{EXP}[N_2 \times \ln(A_0) + \ln(K_{a2}) + N_2 \times H \times M \times (X^2 - X_0^2)] + E \quad [1]$$

where

- A_r = total absorbance of all species at radius X ,
- EXP = exponent,
- ln = natural log,
- A_0 = absorbance of the monomer species at reference radius X_0 ,
- H = constant $[(1 - \bar{v}\rho)\omega^2]/2RT$,
- M = apparent monomer molecular weight (covalent and noncovalent polymers),
- X_0 = reference radius,
- N_2 = stoichiometry for species 2 (number of monomers),
- K_{a2} = association constant for the monomer-monomer equilibrium of species 2, and
- E = baseline offset.

TABLE 1. Amino acid composition of κ -CN and reduced carboxymethylated κ -CN and a comparison with sequence data.

Amino acid	Residues per mole		
	Purified κ -CN ¹	Sequence κ -CN A ²	RCM ³ κ -CN
SCM-Cys ⁴	—	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

¹Molar ratio after 24 h of hydrolysis with Phe = 4 as the basis for the ratios; data are the mean of three determinations.

²Numbers in parentheses represent value for κ -CN B.

³Reduced carboxymethylated.

⁴S-Carboxymethyl cysteine.

RESULTS AND DISCUSSION

Characterization of the Purified κ -CN

Alkaline urea-PAGE in the presence of reducing agents showed all preparations to be typical κ -CN containing a number of bands of increasing mobility that were suggestive of a normal distribution of non-glycosylated bands and glycosylated bands (30). The mobility of the nonglycosylated band indicated that the protein is genetic variant A of κ -CN (data not shown).

The amino acid compositions of κ -CN and of reduced carboxymethylated κ -CN are shown in Table 1; composition is based on the amino acid sequence analysis (17). The sequence data shown are for κ -CN A; the composition data for a 24-h hydrolysis time, (which by its nature is incomplete) are consistent with that of κ -CN A, showing apparently equivalent aspartic and isoleucine contents with reduced alanine. This result confirms the alkaline urea-PAGE phenotype. The reduced carboxymethylated sample yielded 1.8 cysteines, showing the presence of the intact sulfur moieties.

Degree of Polymerization of Purified κ -CN in the Presence of SDS

When the bovine κ -CN was subjected to SDS-PAGE in the absence of 2-mercaptoethanol, distinct poly-

mers (at least eight) of orderly increasing size were observed. Polymerization appears to be driven by either one or both of the two cysteines in κ -CN. On electrophoresis of κ -CN after reduction with 1% (vol/vol) 2-mercaptoethanol, the polymers disappear, resulting in a single band representing the κ -CN monomer (Figure 1). In these experiments, the samples (2 mg/ml) were heated in a boiling water bath for 2 min prior to electrophoresis in a standard buffer of SDS (2.0%, wt/vol) with 0.1 M sodium phosphate, pH 7.5. Electrophoresis of the κ -CN under these standard conditions of 7.5% gels at pH 7 and at two loading concentrations show the reduced form (gel a) and polymeric forms (gels b and c). Based on reference to standard molecular weight markers, these bands (1 through >8) appear to be progressive polymers of the κ -CN monomer. The unmarked band at the front of the reduced gel is para- κ -CN, which has been found in nearly all preparations of this protein (7). To gain more information about the relationships between the reduced and oxidized forms of κ -CN, several experiments were carried out.

Most SDS-PAGE protocols require that samples be heated at various temperatures up to 100°C for ≥ 2 min before electrophoresis. A part of the monomer band in Figure 1, b and c, could be a result of the heat treatment. The effects of temperature on κ -CN samples that were heated in SDS without reducing agents at 90°C for 2, 15, and 30 min prior to electrophoresis were tested. Examination of Figure 2 shows a progressive diminution in the number of higher polymers upon continued heating. Densitometric analyses of the gels (Figure 3) showed that the monomeric species increased from 11 to 60% upon heating at pH 7.5 in the presence of SDS and in the absence of reducing agents. There is also a corresponding apparent increase in the amount of the fastest moving component, monomeric para- κ -CN, (Figure 2), but this result was not included in the analysis. Heat treatment for 30 min reduces the most recognizable upper limit of polymers to almost hexamers.

Heating in the Absence of SDS

When κ -CN samples were heated at 90°C for 2, 15, and 30 min in the protein solvent (0.1 M sodium phosphate at pH 7.5) in the absence of SDS, instead of degradation to monomers, aggregation to high molecular weight polymers occurred. For these samples, much of the protein did not enter the Weber-Osborn 7.5% gels (without reduction). The relative amounts of κ -CN converted into high molecular weight polymers could be assessed by running the samples (heated in the absence of SDS) on SDS-Phast® gels by which the stacking gel and material in

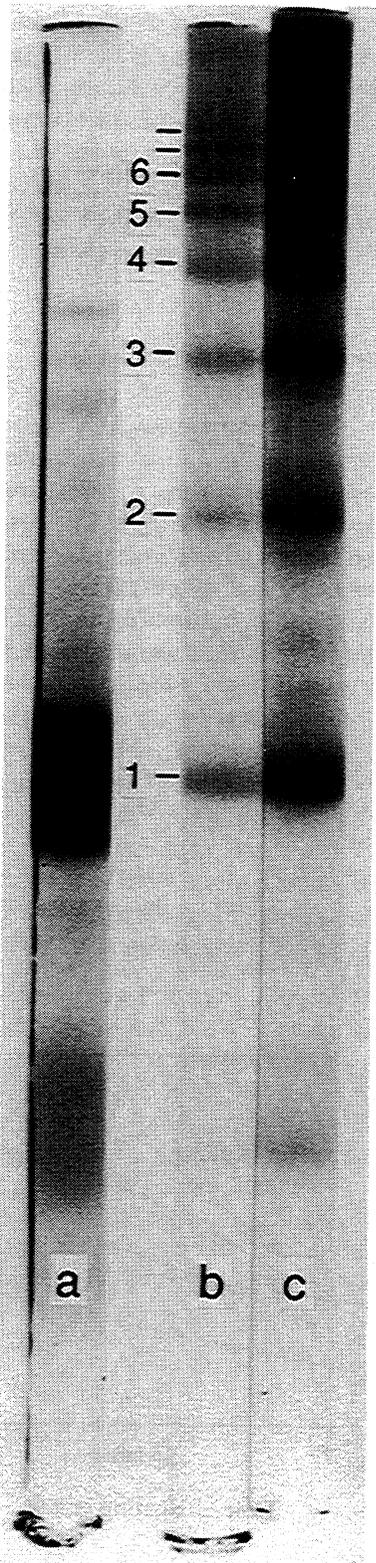


Figure 1. Electrophoresis of κ -CN under standard conditions of 7.5% gels, pH 7.0: gel a is reduced. Also shown are unreduced κ -CN samples with the mobility of the monomer (noted as 1) and polymers (2-etc.) noted; gels b and c. The latter are at two different loading concentrations, 1 and 2 mg/ml, respectively.

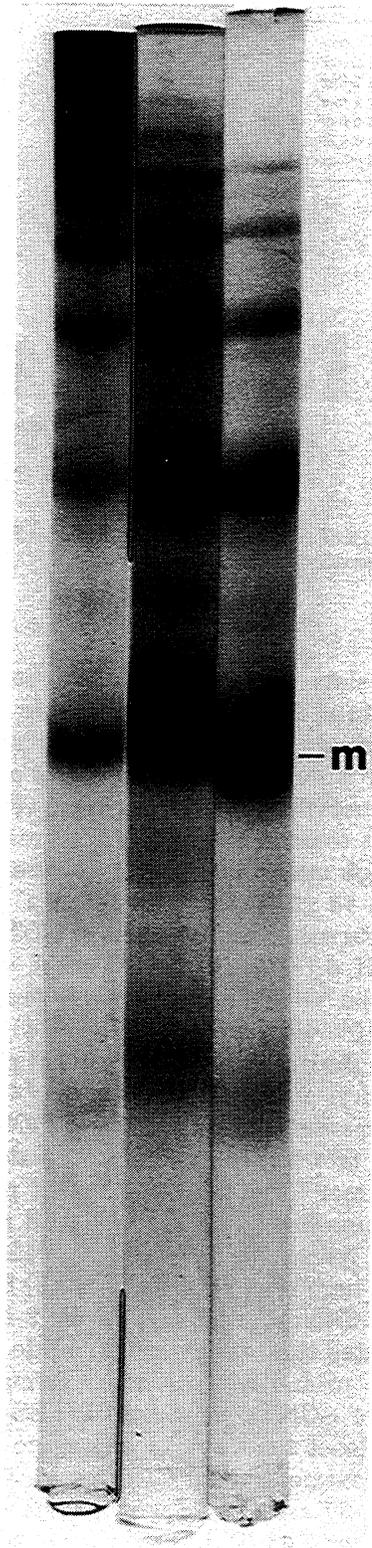


Figure 2. The effect of heat treatment on κ -CN in SDS-protein solvent with no reducing agents. Samples were heated 90°C for 2, 15, or 30 min (left to right); the higher molecular weight polymers are decreased, and the relative amount of monomer (m) is increased.

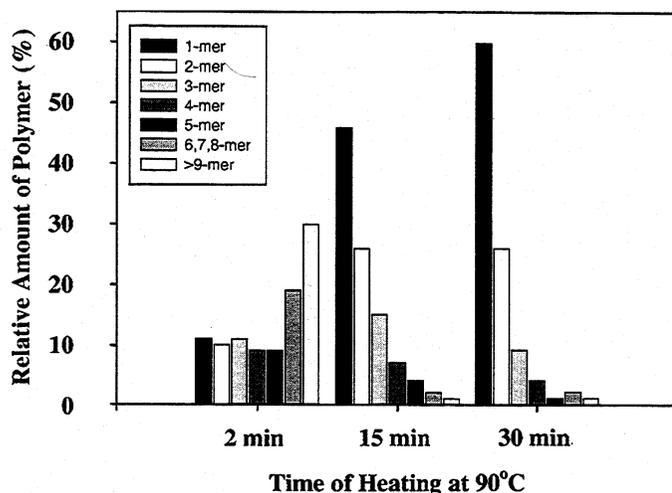
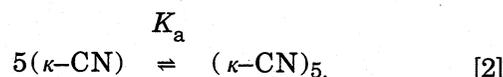


Figure 3. Graphic representation of densitometric traces of gels of Figure 2 demonstrating heat treatment at 90°C for 2, 15, or 30 min.

the sample slots are retained. For these gels, the amounts of stained protein in the sample slot plus the amount of protein in the stacking gel as detected by densitometry were taken as a measure of the formation of high molecular weight polymers (Figure 4). In general, heat treatment at pH 7.5 increased the formation of high molecular weight polymers from 10% (no heat) to $44 \pm 5\%$ (90°C for 30 min); more rapid increases occurred in the presence of EDTA and phosphate. At pH 6.75 (90°C for 30 min), only 22% of high molecular weight polymer was generated compared with 10% with no heat (Figure 4). Thus, heat treatment in the absence of detergent at pH 7.5, when some sulfhydryl anion may be present (4), increased polymer formation. The addition of EDTA or phosphate (both of which may chelate divalent cations) hastens polymer formation. Heating at pH >7.5 may cause β elimination of OH and SH and result in the formation of lysinoalanine crosslinks (12). The polymers formed here were reversed (>80%) by treatment with 40 mM dithiothreitol (DTT) for 2 min at 90°C, which indicates that sulfhydryl-disulfide interactions are involved primarily in polymer formation.

Samples of κ -CN at pH 6.5 in PIPES-NaCl buffer showed the least tendency to aggregate to polymers upon heat treatment at 90°C. κ -Casein was dissolved at pH 6.75 in 35 mM PIPES with 80 mM KCl and was subjected to analytical ultracentrifugation at 25°C. The weight average molecular weight at equilibrium was 1.2 million using the program IDEAL1 (18). Better fits to the data were obtained by using the program ASSOC4, which calculates the apparent monomer molecular weight of associating species, the

association constant (K_a) for the reaction, and an apparent stoichiometric number (N) (Figure 5). The values obtained for these parameters are given in Table 2. Variation of sample preparation is evident in the estimate of the association constants. On average, 35% of the apparent 650,000 molecular weight monomers are present as higher order polymers, which can be calculated from Equation [2].



The rotor and fresh samples were prepared at 25°C; upon heat treatment for 3 h in the chamber at 3000 rpm, the mean temperature in the analytical ultracentrifuge was 37°C. Scans of the samples during this time showed thermal gradients that disrupted the boundaries. After 12 h, equilibrium was attained at 37°C, and, after 6 h more of postheating, the data showed that the weight average molecular weight increased to 1.6 million. Analyses using the program ASSOC4, showed no significant change in the association constant or apparent stoichiometric number, but the apparent monomer increased from 650,000 to 895,000, indicating increased polymer formation at 37°C, which represents a change from a 34-mer to a 47-mer. Such effects of time and temperature for κ -CN were noted by Thurn et al. (25) but not quantified. Thus, heat treatment at 37°C can cause

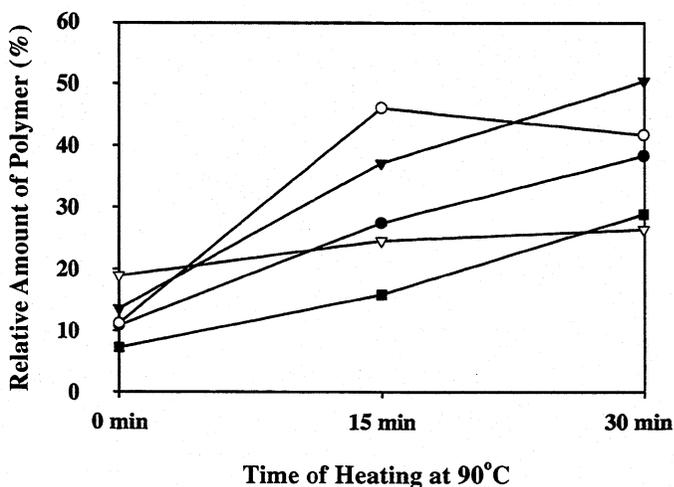


Figure 4. Relative amount of high molecular weight polymers generated by heat treatment of κ -CN with various buffers in the absence of detergent at 90°C for 0, 15, and 30 min: phosphate 0.1 M pH 7.5 (▼); 35 mM PIPES [piperazine-N,N'-bis(2-sulfonic acid)] + 80 mM NaCl, pH 7.5 (●); 35 mM PIPES + 14 mM EDTA, pH 7.5 (○); 35 mM PIPES + 80 mM NaCl, pH 6.75 (▽); and 35 mM PIPES + 14 mM EDTA, pH 6.75 (■).

increased weight average molecular weights. However, one κ -CN sample held in buffer at pH 6.75 for 24 h at 37°C showed a change in its reduced SDS pattern (Figure 6), indicating limited proteolysis (about 30%) to a species with an apparent molecular weight of 16,900; three other samples showed lesser amounts of change (~10%). Sequence analysis of the most affected sample showed that the newly liberated N-terminal residues were Ile₂₂ and Tyr₂₅, indicating proteolysis by a trypsin or a plasmin-like enzyme at Lys₂₁ and Lys₂₄ respectively. The calculated weight of the peptide 1 to 24 is 2740 in good agreement with the SDS gel estimation of 16,900 for the larger fragment of the parent. κ -Casein (2) generally has been considered to be resistant to this type of proteolytic activity at neutral pH. No proteolysis was observed in samples that were heated at 90°C with or without detergent. At 25°C, the degree of proteolysis after 24 h ranged from 0 to 10% for all preparations; all native

TABLE 2. Analytical ultracentrifugation analysis of temperature-induced changes in κ -CN.

(Temp)	MW ¹	SE	K _a ²	SE	MW ²	SE	N ²	SE
	— (MDa) —		— (L/g) —		— (kDa) —			
25°C ³	1.18	0.13	5.0	2.2	648	44	5	1
37°C ⁴	1.55	0.09	6.4	3.1	895	90	7	1

¹Weight average molecular weight. Data were analyzed as noted in Materials and Methods.

²K_a = Association constant, MW = molecular weight of the apparent monomer, and N = stoichiometry calculated using ASSOC4 as described in Materials and Methods (Equation [1]).

³Four analyses with three preparations 1 to 3 mg/ml.

⁴Three analyses with three preparations 1 to 3 mg/ml.

preparations showed some proteolysis after 24 h at 37°C.

The occurrence of free sulfhydryl groups in the milk protein complex has been reported by Beeby (3) but not by others (15, 24). However, heat treatment apparently caused a rapid disulfide interchange resulting in more monomeric species (in the presence of SDS) or more polymeric species in the absence of detergent. In the latter case, the pH of the buffer and the close proximity of the bonds in these polymers could easily promote polymerization reactions at 2 to 5 mg/ml. Removal of bound divalent cations by EDTA or phosphate (10) may accelerate polymer formation, but similar final yields of polymer are obtained with NaCl (Figure 4) at the same pH levels.

Reduction of κ -CN to Monomers

Preparations of κ -CN have been reduced for physical chemical studies by a variety of methods (9). To determine the requirements for the reduction of the κ -CN polymers, several approaches were tested. Purified samples of κ -CN were dissolved at 1 mg/ml in 2.0% SDS containing 0.1 M phosphate, pH 7.5, and were exposed to 5 mM DTT for 16, 6, and 1 min prior to electrophoresis at ambient temperature. Only the 1-min sample showed incomplete reduction (octamers and below); the 6- and 16-min samples contained mostly monomers and about 10% dimers and trimers. Heat treatment for 2 min at 90°C in SDS with 40 mM DTT always yielded >95% monomers with some residual dimers.

Purified κ -CN was dissolved at 3 mg/ml in 20 mM imidazole-HCl at pH 6.7 containing 100 mM NaCl and was treated at room temperature (22°C) with 5 mM DTT for various times; an equal volume of SDS

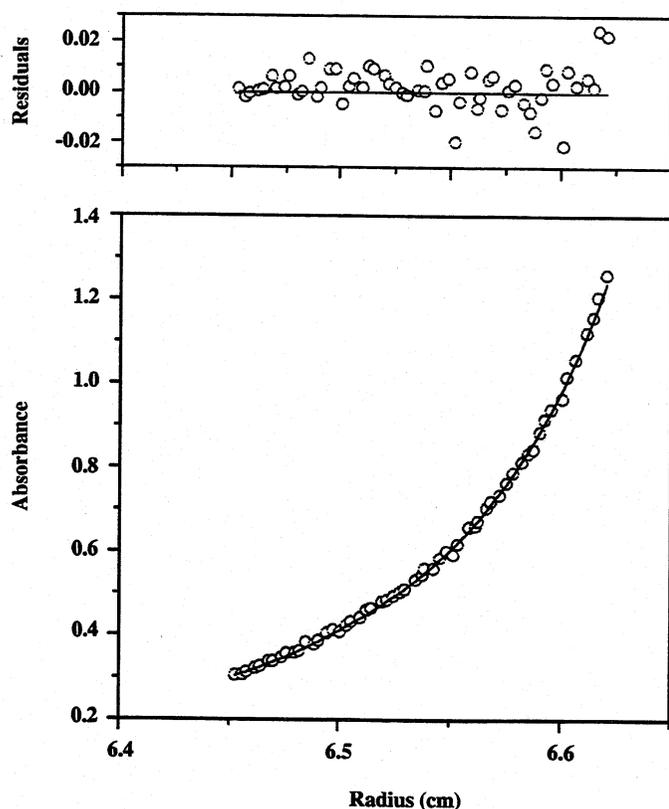


Figure 5. Analytical ultracentrifugation analysis of κ -CN at 25°C in PIPES [piperazine-N,N'-bis(2-ethanesulfonic acid)] (35 mM) with 80 mM KCl. The lower graph shows the fit to the absorbance versus the radius plot for Equation [1]. The upper plot of the residuals for the fit shows no pattern. The chi-square value for this fit is 1.0×10^{-4} ; mean values for the association constant, stoichiometry, and the molecular weight of the apparent monomer are given in Table 2.

(3.0%) was added, and the samples were subjected to Phast[®] gel electrophoresis without heating. Exposure to 5 mM DTT with no detergent and no denaturants yielded fairly rapid reduction. After 5 min, 68% of κ -CN was converted to monomers, 78% was converted at 15 min, and 90% was converted after 2 h. The κ -CN samples (1 mg/ml) were treated at room temperature (22°C) with 40 mM DTT at pH 7.5 in 0.1 M sodium phosphate buffer containing 0 to 6 M urea (1 M increments) for 2 min (no SDS). The samples were then diafiltered (10 K cutoff; in about 2 h), resuspended in 3.0% SDS-phosphate, and electrophoresed with no heating. All samples were completely reduced to monomers, regardless of urea content. Thus, neither urea nor SDS is needed to reduce κ -CN to monomers within 2 h when 40 mM DTT is used. In general, at concentrations between 1 and 3 mg/ml, κ -CN is fairly readily reduced in the presence of urea or SDS by modest concentrations (5 mM) of DTT, but, in the absence of urea or SDS, higher concentrations (40 mM) of reductant are necessary to facilitate the complete conversion of polymers to monomers within minutes. In the absence of denaturants, the most important factor is the molar ratio of DTT to κ -CN; reduction is accelerated by molar ratios >100:1.

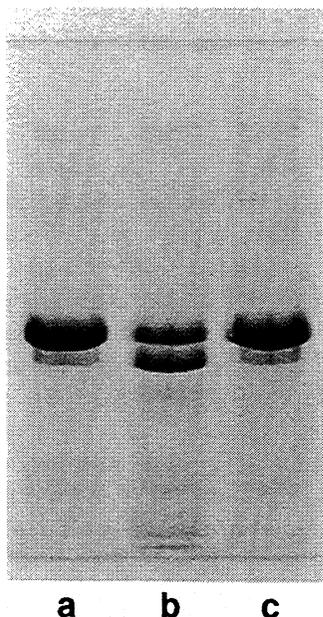


Figure 6. Phast[®] gel electrophoresis (Pharmacia-Biotech, Uppsala, Sweden) of bovine κ -CN showing degradation by protease after holding for 24 h at pH 6.75. Left to right: 0 h, 24 h at 37°C, and 24 h at 25°C. This sample showed the highest degree of proteolysis.

TABLE 3. Summary of results of the reoxidation of κ -CN to polymeric structures.

Dialysis condition ¹	Maximum polymer size ²
Whole κ -CN	
pH 7.0, 4 M urea, 0.1 M sodium phosphate DTT gradually reduced, 5 h	None
pH 7.4, \pm 4 M urea, no DTT, 0.1 M sodium phosphate, 5 h	None
pH 6.8, 4 M urea, no DTT, 0.005 M NaCl, 5 h	Tetramers
pH 6.0, no urea, no DTT, 0.1 M sodium phosphate, 5 h	Hexamers
pH 6.6, no urea, no DTT, 0.1 M sodium phosphate, 6 h air, 5°C	Pentamers
pH 5.15, no urea, no salt, no DTT, 1 wk 5°C	Octamers and above
κ -CN 1, 2, and 3 ³	
pH 5.3, no urea, no salt, 3 d, 5°C	Hexamers
pH 5.2, no urea, 0.01 M NaCl, 1 wk, 5°C	Octamers and above ⁴

¹Samples defined in text; dialysis at room temperature (22°C), except as noted.

²Polymer size estimated by SDS-PAGE in the absence of added reducing agent.

³ κ -I, κ -II, and κ -III as designated by Vreeman et al. (26, 27).

⁴Figure 8.

Reoxidation of Reduced κ -CN

To gain more information regarding the disulfide patterns of κ -CN that were generated by various treatments, the protein was thoroughly reduced for 1 h at room temperature (22°C) under N₂ and allowed to reoxidize spontaneously under a variety of conditions. Reduction with 33 mM DTT was carried out in 1 ml of 0.1 M Tris at pH 8.0 in the presence of 6 M urea and EDTA (10 mg/100 ml) and a protein concentration of 1 mg/ml. The samples (1 ml) were then transferred to acid-washed dialysis tubing (3500 MW cutoff) and dialyzed at room temperature (22°C) against 100 ml of selected buffers; samples were withdrawn hourly for electrophoresis. Results of the degree of spontaneous reoxidation are summarized in Table 3.

When the dialysis buffer was 0.1 M phosphate at pH 7.0 containing 4 M urea and the DTT concentration of the dialysate was reduced from 5 to 0 mg/ml at hourly intervals, only monomeric species remained after an additional 3 to 4 h of dialysis without added DTT. The urea could prevent the proper interactions between proteins that are necessary to promote disulfide formation or could yield a different conformation that is incapable of polymeric disulfide association. When this experiment was repeated at pH 7.4 with urea but without DTT in the dialysis buffer, no polymeric species were observed upon electrophoresis

(Table 3); finally, elimination of the urea did not enhance reoxidation at pH values between 7.0 and 7.4.

Some spontaneous reoxidation (tetramers) occurred during dialysis at pH 6.8 in 5 mM NaCl (4 M urea) (Figure 7, gels b and d). The spontaneous reoxidation to polymers is apparently favored at lower pH even in the presence of urea (gel e was thoroughly reduced prior to electrophoresis). Gels a and c show that the monomer bands are not discrete, as though the samples are partially denatured but not forming disulfides (compare with gel e, reduced just prior to electrophoresis). When the pH of dialysis was reduced to 6.0 (0.1 M sodium phosphate), spontaneous reoxidation occurred over time in the absence of urea and DTT in the dialysate, and a number of polymeric species were observed as revealed by densitometric traces (Table 4). These bands, however, did not go beyond hexamers. Similar results (bands no greater than pentamers) were obtained at pH 6.6 (0.1 M sodium phosphate) when air was bubbled gently through the solution for 6 h at 5°C.

As described in the Materials and Methods section, κ -1, κ -2, and κ -3 were prepared. These samples were dialyzed for 3 d against distilled water (20-ml sample against 2.0 L of water, three changes per day). The internal pH of these solutions averaged 5.3 ± 0.1 , and electrophoresis revealed spontaneous reoxidation up to hexamers (data not shown). Table 4 shows that, when dialysis was continued for 1 wk but with 0.01 M NaCl, octamers were achieved at pH 5.2 (see also, Figure 8). Thus, κ -CN association and subsequent reoxidation are promoted at lower pH values (Table 3). Finally, whole κ -CN was reduced with Tris, EDTA, urea, and DTT, as described, and dialyzed at 5°C for 1 wk against distilled water (multiple daily changes). After this period (without buffer or NaCl), precipitation began, and the internal pH was 5.15. The resulting material contained a distribution of molecular weight polymers that was somewhat similar to those observed in the native species of protein.

Under these latter conditions (reduction, fractionation in urea, and dialysis), many preparations of κ -CN have been prepared for physical chemistry studies, and changes in the dialysis conditions could give rise to the apparent discrepancies reported for the molecular sizes of κ -CN polymers. Vreeman et al. (26) studied the concentration dependence of the molecular weight of κ -CN (κ -I = carbohydrate free) by Trautman analysis of sedimentation velocity experiments and concluded that κ -CN aggregates to polymers containing 31 monomers; κ -CN I was prepared in the manner of the κ -1 that was used in this study.

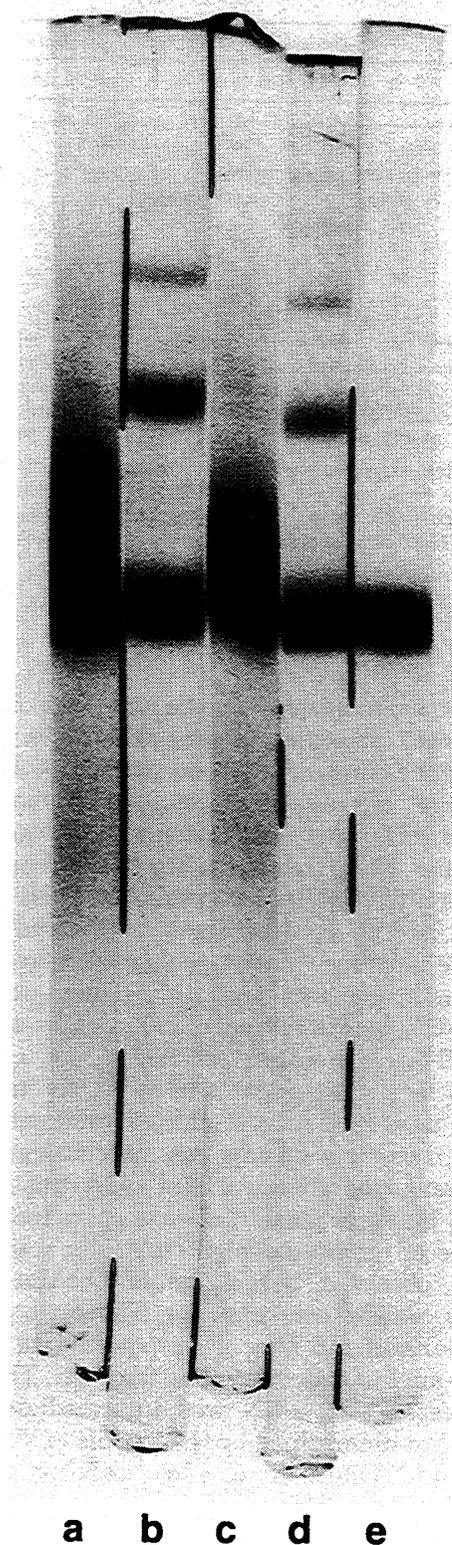


Figure 7. Gels of reduced κ -CN showing limited spontaneous reoxidation on dialysis at pH 6.8 in 4 M urea and 0.005 M NaCl. Experiment 1: gel a = 0 h; gel b = 1 h. Experiment 2: gel c = 0 h; gel d = 5 h. Gel e is the standard reduction prior to electrophoresis.

TABLE 4. Relative areas for each oligomeric form of κ -CN after reoxidation as determined by densitometry.

Band ³	Whole κ -CN ¹					Coomassie stain	
	0 h	1 h	4 h	6 h	24 h	κ -CN 1 ²	κ -CN 2 ²
	(% of total area)						
1	100	65	60	58	44	31	43
2	...	15	17	15	17	16	16
3	...	9	13	16	19	10	10
4	...	6	5	7	10	7	7
5	...	3	3	3	6	6	5
6	...	2	2	2	3	5	3
7	3	2
8	2	2

¹McKenzie-Wake whole κ -CN, Coomassie stain. Mean values of three scans of each sample reduced and then reoxidized during dialysis at pH 6.0 in 0.1 M phosphate at room temperature (22°C) for 6 h and at 5°C for 18 h and studied by SDS-PAGE.

²Designated as κ -I and κ -II by Vreeman et al. (26, 27); these fractions are reduced for DEAE chromatography but apparently reoxidize during exhaustive dialysis against distilled water containing 0.01 M NaCl and lyophilization.

³Band numbers correspond to those of Figure 1.

In our experiments, when dialysis was continued without NaCl and the pH was allowed to approach the isoelectric point, as noted by McKenzie and Wake (16), large aggregates formed that were often difficult to redissolve. This latter process was accelerated by the heat treatment in the present study. Vreeman et al. (26) reported that, for their samples, reduction had to proceed for 1 wk to reduce completely the polymers that were formed on reoxidation after chromatographic separation. Thurn et al. (25) studied κ -CN by static and dynamic light scattering and small-angle neutron scattering and calculated that κ -CN formed aggregates of up to 130 monomers. The κ -CN used by Thurn et al. (25) was prepared by reduction, two steps of urea column chromatography, dialysis, and lyophilization. The κ -CN was then dissolved in buffer without subsequent reduction. The occurrence of unreduced high molecular weight polymers, as observed herein, could account for the molecular weights reported by Thurn et al. (25). Finally, deKruif and May (5) reported data gathered via small-angle X-ray scattering for κ -CN. Their analysis indicated that κ -CN was intermediate in size between that determined by the models of Vreeman et al. (26) and Thurn et al. (25). deKruif and May, however, used samples of casein, which they obtained from Vreeman; these samples were dissolved in D₂O, DTT (5 mM) and imidazole without undergoing extensive reduction. In our studies in H₂O, some disulfide-bonded polymers would remain under these latter conditions, as the ratio of DTT to κ -CN would

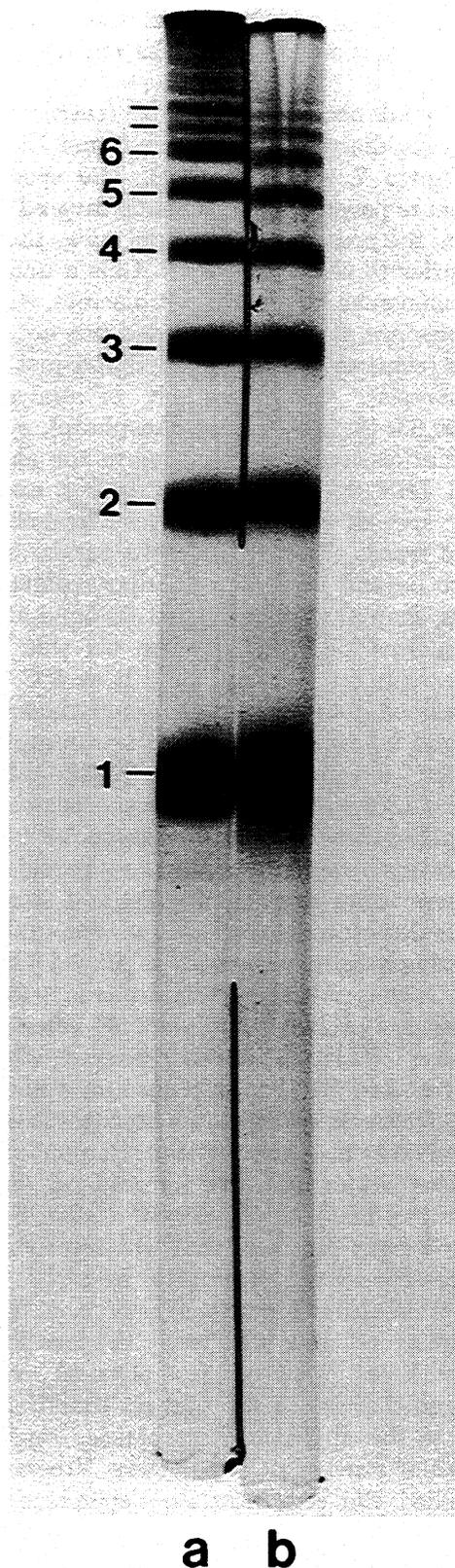


Figure 8. Gels of unreduced κ -CN showing variable reoxidation after 1 wk of extended dialysis at 5°C, pH 5.2, and 0.01 M NaCl; a is κ -1; b is κ -2.

SYMPOSIUM: CASEIN MICELLE STRUCTURE

TABLE 5. Summary of physical data on κ -CN.

Source	MW	Radius (nm) ¹	Method	Reduction
Vreeman et al. (26)	600,000	11.1	Sedimentation	
Slattery and Evard (22)	600,000	11.2	Velocity	1 wk, 2-ME ²
deKruif and May (5)		14.7	Sedimentation	1 h, DTT (40 mM)
Thurn et al. (25)	2,000,000	7.0 ⁴	SANS ³	DTT (5 mM)
This study	1,180,000	12.5 ⁵	SANS	None
			Sedimentation	None
			Equilibrium	

¹Radius type varies with method.

²2-Mercaptoethanol.

³Small-angle neutron scattering.

⁴Internal submicellar particle of larger high molecular weight aggregate.

⁵Weight average radius by dynamic light scattering (10).

be low. The results from the current study (Table 3) indicate that, for κ -CN (after reduction and spontaneous reoxidation), the degree of polymerization achieved can be quite variable, depending upon treatments. Correspondingly, the degree of reduction or the lack of reduction of highly polymerized samples could lead to the various results reported for molecular weights and summarized in Table 5. Interestingly, as determined by the sedimentation equilibrium for the preparations of κ -CN used in these studies, the weight average molecular weight at 25°C in PIPES-KCl is 1.18 ± 0.13 million. When the samples were heated to 37°C, after 24 h, the weight average molecular weight increased to 1.55 ± 0.09 million (Table 2). Correspondingly, the monomer of Equation [1] increased from 648,000 to 895,000. These values for native κ -CN that is unreduced are between the values determined for various chromatographed samples (Table 5); the values of the chromatographed samples appear to depend on the degree of reoxidation and subsequent reduction prior to analysis.

It may be of interest to consider the results obtained here in terms of modern theories of protein folding (1) or disulfide bonds and stability (4). Small globular proteins tend to form different folding intermediates (and, hence, disulfides), depending on their native structural stabilities; buried S-S bonds are the slowest to form, and surface bonds form more rapidly. The two-cysteine system of κ -CN is not as complex as the six-cysteine system of bovine pancreatic trypsin inhibitor, but the innate structure of κ -CN is more flexible (14), as judged by the many intermolecular disulfides in the gels, but the particles themselves are quite spherical (Figure 9). With respect to reduction, κ -CN polymers are fairly readily reduced even in the absence of denaturants, which could mean that either the disulfides are close to the surfaces or that the

polymeric aggregates are highly porous. Results of the study (25) using small-angle neutron scattering would argue for the latter case. Conversely, the proteolysis observed for the samples held at 37°C indicated that residues 21 and 24 (lysine) are relatively

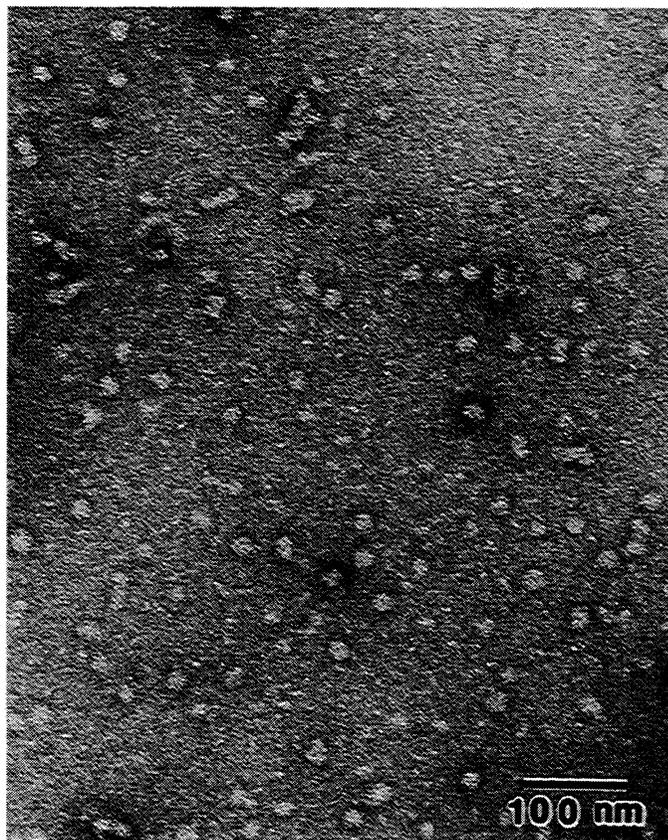


Figure 9. Transmission electron micrograph of a general field of negatively stained (uranyl acetate 2%) κ -CN particles (10).

near the surface of the polymer and that Cys₁₁ could be somewhat close to the surface as well. With respect to reoxidation, native-like polymers (partially buried S-S) apparently form only when the κ -CN is highly aggregated (pH 5.2). Perhaps the combination of other caseins and the acidic pH present in the Golgi apparatus during micelle assembly (8, 14) help to dictate the conformation of the protein, which, in turn, leads to the disulfide patterns achieved in native κ -CN. Thus, κ -CN disulfide patterns report upon κ -CN within the casein polymeric complex as a whole, and the nearest neighbor to the majority of κ -CN monomers is another κ -monomer.

In summary, κ -CN as isolated from bovine milk contains a wide range of polymeric species. Sample preparation even in SDS-PAGE can influence this distribution. The degree of disulfide exchange, which occurs on spontaneous reoxidation, is also dependent upon environmental conditions and can lead to polymers of differing sizes. The degree of disulfide bonding, as assessed by SDS-PAGE, should be taken into account in evaluating the properties of κ -CN.

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