

Environmental Effects on Disulfide Bonding Patterns of Bovine κ -Casein

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Bovine κ -casein, the stabilizing protein of the colloidal milk protein complex, has a unique disulfide bonding pattern. The protein exhibits varying molecular sizes on SDS-PAGE ranging from monomer to octamer and above in the absence of reducing agents. Heating the samples with SDS prior to electrophoresis caused an apparent decrease in polymeric distribution: up to 60% monomer after 30 min at 90°C as estimated by densitometry of SDS-PAGE. In contrast, heating the samples without detergent at 90 or 37°C caused a significant increase in high-molecular-weight polymers as judged by electrophoresis and analytical ultracentrifugation. In 6 M urea, the protein could be completely reduced, 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 -caseins and on their resultant physical chemical properties.

KEY WORDS: Casein structure; molecular weight; disulfide interactions.

1. INTRODUCTION

The growth and development of all mammalian neonates is dependent upon an appropriate supply of inorganic calcium and phosphate in a readily assimilable form. The caseins of milk participate in the genesis of a calcium-phosphate transport complex through the formation of colloidal particles termed casein micelles (Farrell and Thompson, 1988). The colloidal stability of these particles is imparted by one particular casein: κ -casein (Waugh and Von Hippel, 1956). Hydrolysis of κ -casein by digestive enzymes (chymosin in particular) begins the process of micelle coagulation which ultimately leads to delivery of protein, calcium, and phosphate to the neonate (Holt, 1992; Schmidt, 1982).

Because of its importance to milk, the best characterized form of κ -casein is that of bovine milk (Swaisgood, 1992; Farrell, 1988). κ -Casein from bovine milk has a monomer molecular weight of 19,000, contains two cysteine residues (Cys-11 and Cys-88), and occurs as a phosphoglycoprotein displaying microheterogeneity with respect to phosphate and carbohydrate (Eigel *et al.*, 1984). As purified from milk, κ -casein contains a unique disulfide bonding pattern detected by SDS-PAGE in the absence of reducing agents (Groves *et al.*, 1991). The molecular weights range from monomer to octamer and above and appear to be rather evenly distributed across this range (Groves *et al.*, 1992). Rasmussen *et al.* (1992) reported an apparently random distribution of the disulfides in these polymers involving Cys-11 and Cys-88 (88-88, 11-11, and 11-88).

Historically, disulfide bonding has not been considered to be of primary importance in casein micelle structure and stability (Farrell, 1988;

Swaisgood, 1992). This concept comes from the early work of Woychik *et al.* (1966), who showed that reduced and alkylated κ -caseins had the same propensity to reform model colloids as native κ -caseins. The discovery of the unique disulfide bonding properties, coupled with discrepancies in the reported molecular weights for κ -casein aggregates, which could be related to alterations in disulfide bonding patterns (deKruif and May, 1992; Thurn *et al.*, 1987; Vreeman *et al.*, 1981; Slattery and Evard, 1973), has prompted a reevaluation of the nature of environmental effects on κ -casein disulfides. In this paper we investigate the effects of heating, reduction, and conditions of reoxidation on the disulfide bonding patterns of κ -casein.

2. MATERIALS AND METHODS

Casein was isolated from 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 at $100,000 \times g$ for 1 hr.

Fraction III, the final byproduct in the κ -casein fractionation of McKenzie and Wake (1961), was found by SDS-PAGE to contain a significant amount of κ -casein. In order to obtain purified κ -casein 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.* (1979). Most of the κ -casein was eluted in three sequential peaks designated κ -1 casein (91 mg), κ -2 casein (42 mg), and κ -3 casein (10 mg); these peaks also correspond to fractions κ -I, κ -II, and κ -III of Vreeman *et al.* (1986).

Polyacrylamide gel electrophoresis was according to Weber and Osborn (1969) 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, heated 2 min in boiling water before introducing them to the gels; protein concentration was 0.2 mg or less per gel. Phast[®] gels (Pharmacia-Biotech, Uppsala, Sweden) were run according to the manufacturer's procedures for 7.5% or 20% homogeneous gels. Samples of κ -casein at 5 mg/ml were heated for 15 and 30 min at 90°C in phosphate

buffer (pH 7.5) and in 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 dodecylsulfate.

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 work station (Waters-Millipore Corp., Milford, MA). Hydrolysis was carried out in gas phase at 110°C for 24 hr 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, Milford, MA). Cysteine was identified as carboxymethyl cysteine by comparison of retention times with known standards. Reduction and alkylation were carried out according to Shechter *et al.* (1973). Data are reported as molar ratios with phenylalanine fixed at 4 residues/molecule (Mercier *et al.*, 1973).

The N-terminal amino acid sequences of the κ -casein samples exhibiting proteolysis were determined by automated Edman degradation on a pulse liquid sequencer with on-line phenylthiohydantoin amino acid analysis (Applied Biosystems 473A, Foster City, CA). Note that as the native N-terminal is blocked (pyroglutamic acid), only newly liberated residues were observed.

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

For analytical ultracentrifugation, the protein samples were dissolved at pH 6.75 in 35 mM PIPES, 80 mM KCl at concentrations ranging from 1.0 to 3.0 mg/ml. The samples and solvents were filtered with a Waters (Milford, MA) HVLP 0.45- μ m membrane filter. For κ -casein, Phast[®] gel electrophoresis in SDS showed a nearly identical pattern of protein components before and after filtration; less than 1% of the material was retained on the filter as ascertained by UV spectroscopy. Sedimentation equilibrium experiments were performed in a Beckman Optima XL-A (Palo Alto, CA) analytical ultracentrifuge 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 nm or at 292 nm (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 IDEAL 1 or ASSOC4, which are part of the Optima XLA data analysis software.

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

$$A_r = \text{EXP}[\text{LN}(\text{AO}) + \text{H} \cdot \text{M} \cdot (\text{X}^2 - \text{XO}^2)] \\ + \text{EXP}[\text{N2} \cdot \text{LN}(\text{AO}) + \text{LN}(\text{KA2}) \\ + \text{N2} \cdot \text{H} \cdot \text{M} \cdot (\text{X}^2 - \text{XO}^2)] + \text{E} \quad (1)$$

where A_r is the total absorbance of all species at radius x , EXP is the exponent, LN is the natural log, AO is the absorbance of the monomer species at reference radius x_0 , H is the constant $(1 - \nabla\rho)\omega^2/2RT$, M is the "monomer" molecular weight (covalent and noncovalent polymers), XO is the reference radius, N2 is the stoichiometry for species 2 (number of "monomers"), KA2 is the association constant for the monomer-nmer equilibrium of species 2, and E is the baseline offset.

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 is the A genetic variant of κ -casein (data not shown).

The amino acid compositions of κ -casein and RCM- κ -casein (reduced carboxymethylated) are shown in Table I together with their compositions based on the amino acid sequence analysis (Mercier *et al.*, 1973). The sequence data shown are for κ -casein A; the composition data of the sample are consistent with that of κ -casein A, showing apparently equivalent aspartic and isoleucine contents with reduced alanine. This confirms the alkaline urea PAGE phenotype. The reduced carboxymethylated sample yielded 1.8 cysteines, showing the presence of the intact sulfur moieties.

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

Amino acid	Residues/mole		
	Purified κ -casein ^a	Sequence κ -A ^b	RCM κ -casein ^c
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 values for κ -B.

^c RCM, Reduced carboxymethylated.

^d SCM, S-carboxymethyl cysteine.

3.2. Degree of Polymerization of Purified κ -Casein

When the bovine κ -casein was subjected to SDS-PAGE in the absence of 2-mercaptoethanol, distinct polymers (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 -casein. On electrophoresis of κ -casein after reduction with 1% 2-mercaptoethanol, the polymers disappear, resulting in a single band representing the κ -casein monomer (Fig. 1). In these experiments, the samples (2 mg/ml) were heated in a boiling water bath for 2 min prior to electrophoresis in standard buffer, SDS (2.0%) with 0.1 M sodium phosphate, pH 7.5. Electrophoresis of the κ -casein under these standard conditions of 7.5% gels, pH 7, and at several loading concentrations shows the reduced form (gel a) and polymeric forms (gels b-c). To gain more information about the relationships between the reduced and oxidized forms of κ -casein, several experiments were carried out.

Figure 2 shows the patterns of κ -casein in the

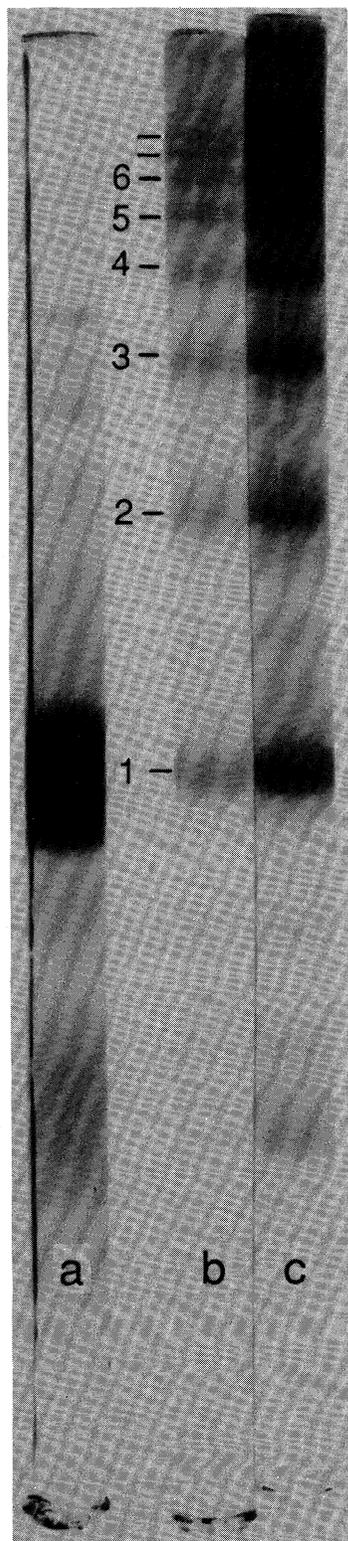


Fig. 1. Electrophoresis in SDS of κ -casein under standard conditions of 7.5% gels, pH 7.0; gel a is reduced. (b, c) Unreduced κ -casein samples with the mobility of the polymers and monomers noted.

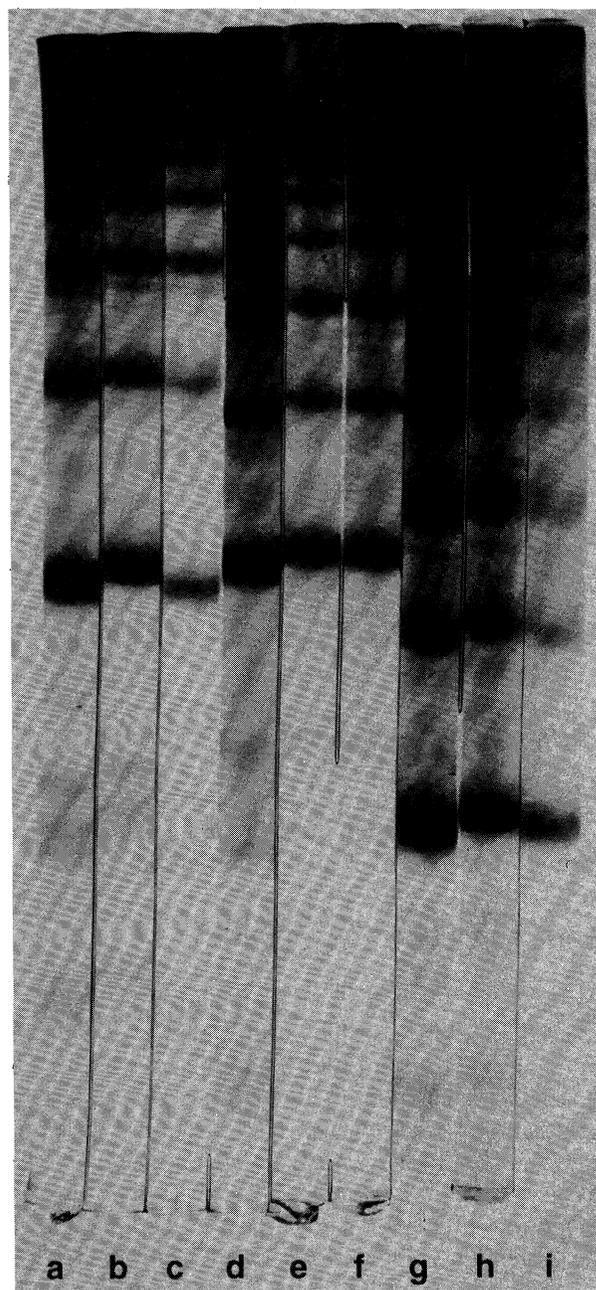


Fig. 2. Electrophoresis patterns of κ -casein in SDS in the presence and absence of 6 M urea. Urea denaturation does not affect the κ -casein gel pattern. (a-c) Standard conditions, 7.5% gels; (d-f) standard conditions with 4 M urea, 7.5% gels; (g-i) standard conditions, except 5% gels.

presence and absence of urea and with variation of gel strength. The presence of 4 M urea (gels d-f) in the 7.5% gels has little effect on the polymeric distribution (gels a-c = standard). However, dropping the acrylamide to 5% does show the upper molecular weight regions in more detail (gels g-i).

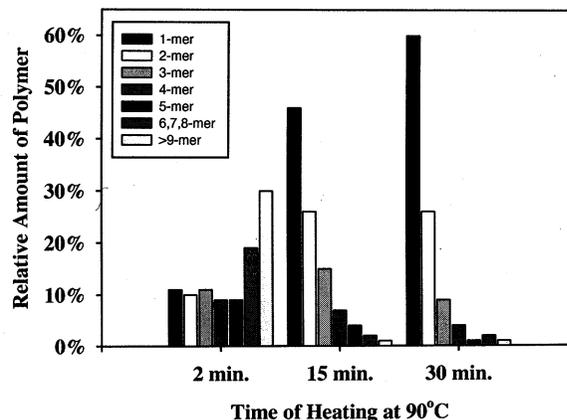
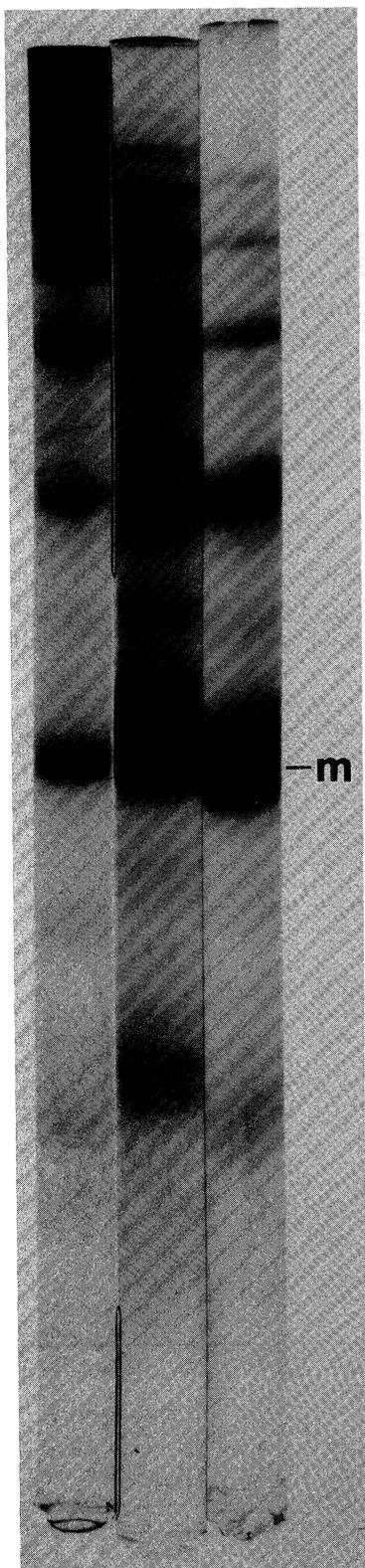


Fig. 4. Graphic representation of densitometric traces of gels of Fig. 3 for heating at 2, 15, and 30 min.

Here it is possible to discern bands corresponding to at least dodecamers.

It is of interest to note that most SDS-PAGE protocols call for samples to be heated at various temperatures up to 100°C for up to 2 min or more before electrophoresis. The effect of temperature on κ -casein samples which were heated in SDS without reducing agents at 90°C for 2, 15, and 30 min prior to electrophoresis was tested. Examination of Fig. 3 shows a progressive diminution in the number of higher polymers upon continued heating. Densitometric analyses of the gels (Fig. 4) show that the monomeric species increases from 11 to 60% upon heating at pH 7.5 in the presence of SDS and the absence of reducing agents. There is a corresponding apparent increase in the amount of monomeric para- κ -casein as well (Fig. 3), but this was not included in the analysis. Heating for 30 min reduces the most recognizable upper limit of polymers to about hexamers.

3.3. Heating in the Absence of SDS

When κ -casein samples were heated at 90°C for 2, 15, and 30 min in the protein solvent (0.1 M sodium phosphate, pH 7.5) in the absence of SDS, instead of degrading to monomers, aggregation to very high molecular weight polymers occurred. For these samples much of the protein did not enter the Weber-Osborn 7.5% gels (without reduction). The

Fig. 3. The effect of heat on κ -casein in SDS-protein solvent, no reducing agents. Samples were heated at 90°C for 2, 15, and 30 min (left to right); the higher molecular weight polymers are decreased, while the relative amount of monomer (*m*) increases.

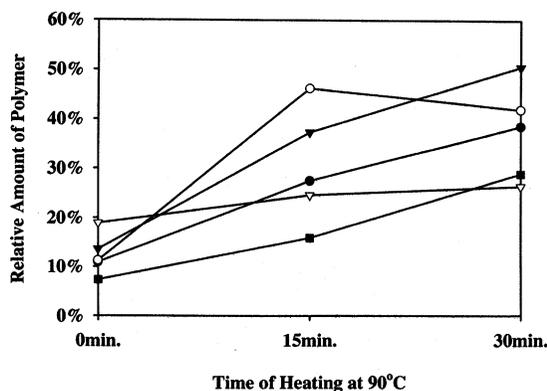


Fig. 5. Relative amount of high-molecular-weight polymers generated by heating κ -casein with various buffers in the absence of detergent at 90°C for 0, 15, and 30 min: (▼) phosphate 0.1 M, pH 7.5; (●) PIPES + 80 mM NaCl, pH 7.5; (○) PIPES + 14 mM EDTA, pH 7.5; (▽) PIPES - 35 mM NaCl, pH 6.75; (■) PIPES + 14 mM EDTA, pH 6.75.

relative amounts of κ -casein converted into high-molecular-weight polymers could be assessed by running the samples (heated in the absence of SDS) on SDS-Phast gels where the stacking gel and material in the slots are retained. For these gels the amounts of stained protein in the sample slot plus the amount in the stacking gel as detected by densitometry were taken as a measure of high-molecular-weight-polymer formation (Fig. 5). In general, heating at pH 7.5 increased high-molecular-weight-polymer formation from 10% (no heat) to $44 \pm 5\%$ (90°C, 30 min), with more rapid increases in the presence of EDTA and phosphate. At pH 6.75 (90°C, 30 min) only 22% high-molecular-weight polymer was generated, compared to 10% with no heat (Fig. 5). Thus heating in the absence of detergent at pH 7.5 where some sulfhydryl anion may be present (Darby and Creighton, 1995) increases polymer formation. Addition of EDTA or phosphate (both of which may chelate divalent cations) hastens polymer formation. Heating at pH greater than 7.5 may cause β -elimination of OH and SH and result in the formation of lysinoalanine cross links (Feeney, 1977). The polymers formed here were reversed (>80%) by treatment with 40 mM DTT for 2 min at 90°C; this indicates that primarily sulfhydryl-disulfide interactions are involved in polymer formation.

Samples of κ -casein at pH 6.75 in PIPES-NaCl buffer showed the least tendency to aggregate to polymers upon heating at 90°C. κ -Casein was

dissolved at pH 6.75 in 35 mM PIPES with 80 mM KCl and subjected to analytical ultracentrifugation at 25°C. The weight-average molecular weight at equilibrium was 1.2 million using the program IDEAL1. Better fits to the data were obtained by using the program ASSOC4, which, as described in Section 2, calculates the "apparent monomer" molecular weight of associating species, the association constant K_a for the reaction, and an apparent stoichiometric number N (Fig. 6). The values obtained for these parameters are given in Table II. A good deal of preparation variation is seen in the estimate of the association constants. It can be calculated from the following Equation that, on the average, 35% of the apparent 650,000-MW "monomers" are present as higher order polymers,



The rotor and fresh samples were prepared at 25°C; on heating for 3 hr in the chamber at 3000 rpm, the average temperature in the XLA came to 37°C.

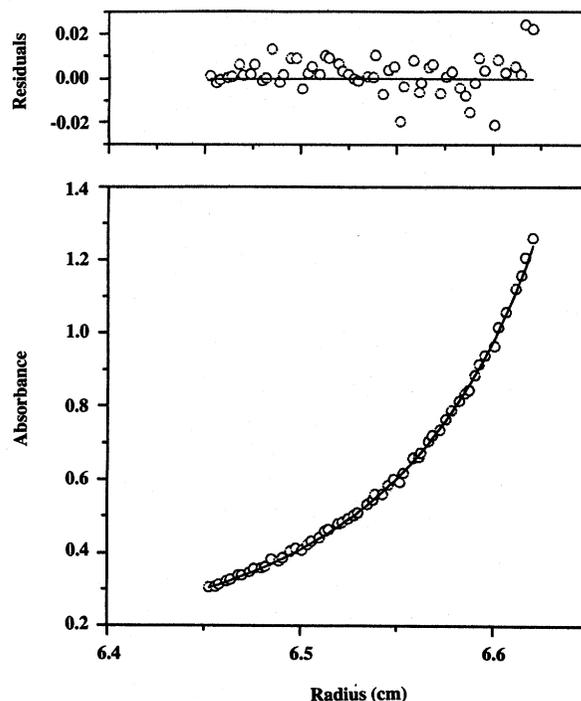


Fig. 6. Analytical ultracentrifugation analysis of κ -casein at 25°C in PIPES (35 mM) with 80 mM KCl. The lower graph shows the fit to the absorbance versus radius plot for Eq. (1). The upper plot shows the residuals for the fit, showing no pattern. The chi-square value for this fit was 1.0×10^{-4} ; average values for the parameters K_a , N , and MW "monomer" are given in Table II.

Table II. Analytical Ultracentrifugation Analysis of Temperature-Induced Changes in κ -Casein^a

Temperature (°C)	MW (MDa)	K_a	MW "monomer (kDa)	N
25 ^b	1.18 ± 0.13	5.0 ± 2.2	648 ± 44	5 ± 1
37 ^c	1.55 ± 0.09	6.4 ± 3.1	895 ± 90	7 ± 1

^a MW, weight-average molecular weight; data were analyzed as in Section 2 (MDa, million daltons; association constant K_a , MW "monomer" (kDa, kilodaltons), and N calculated using ASSOC4 as described in Section 2.

^b Four analyses with three preparations 1–3 mg/ml.

^c Three analyses with three preparations 1–3 mg/ml.

Scans of the samples during this time showed thermal gradients which disrupted the boundaries. After 12 hr, equilibrium was attained at 37°C and after an additional 6 hr postheating the data showed that the weight-average molecular weight increased to 1.6 million. Using analyses by the program ASSOC4, there was no significant change in K_a or N , but the "apparent monomer" increased from 650,000 to 895,000, indicating increased polymer formation at 37°C. This represents a change from a 34-mer to a 47-mer. Such time-temperature effects for κ -casein were noted by Thurn and co-workers (1987) but not quantified. Thus, heating at 37°C can cause increased weight-average molecular weights. However, one κ -casein sample held in buffer at pH 6.75 for 24 hr at 37°C showed a change in its reduced SDS pattern indicating limited proteolysis (about 30%) to a species with a molecular weight of 16,900; three others showed lesser amounts of change (~10%). Sequence analysis of the most effected sample showed that the newly liberated N-terminal residues were Ile₂₂ and Tyr₂₅, indicating proteolysis by a trypsin or plasmin-like enzyme at Lys₂₁ and Lys₂₄, respectively. κ -Casein has generally been considered to be resistant to this type of proteolytic activity at neutral pH (Bastian and Brown, 1996). No proteolysis was observed in samples heated at 90°C with or without detergent. At 25°C the degree of proteolysis after 24 hr ranged from 0 to 10% for all preparations.

The occurrence of free sulfhydryl groups in the milk-protein complex was reported by Beeby (1964) but not by others (Swaisgood *et al.*, 1964; Jolles *et al.*, 1962). However, heating apparently causes rapid disulfide interchange, resulting in more monomeric species (in the presence of SDS) or more polymeric species (no SDS). In the latter case, the pH of the buffer and close proximity of the bonds in these polymers could easily promote

polymerization reactions at 2–5 mg/ml. Removal of bound divalent cations by EDTA or phosphate (Farrell *et al.*, 1996b) may accelerate polymer formation, but similar final yields are obtained with NaCl (Fig. 5).

3.4. Reduction of κ -Casein to Monomers

Preparations of κ -casein have been reduced for physical chemical studies by a variety of methods (Farrell *et al.*, 1996a). To determine the requirements for reduction of the κ -casein polymers, several approaches were tested. Purified κ -casein samples at 1 mg/ml were dissolved in 2.0% SDS, 0.1 M phosphate, pH 7.5, and exposed to 5 mM DTT for 16, 6, and 1 min prior to electrophoresis with no heat. Only the 1-min sample showed a lack of complete reduction (octamers and below); the 6- and 16-min samples contained mostly monomers and about 10% dimers and trimers. Heating for 2 min at 90°C in SDS with 40 mM DTT always yielded >95% monomers with some residual dimer.

Purified κ -casein was dissolved at 3 mg/ml in 20 mM imidazole-HCl, pH 6.7, 100 mM NaCl, and treated at room temperature with 5 mM DTT for various times; an equal volume of SDS (3.0%) was added and the samples 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 -casein was converted to monomer, 78% at 15 min, and 90% after 2 hr. κ -Casein samples (1 mg/ml) were treated with 40 mM DTT at pH 7.5 in 0.1 M sodium phosphate buffer containing 0–6 M urea for 2 min (no SDS). The samples were then ultrafiltered (10K cutoff; this takes about 2 hr), taken up in 3.0% SDS-phosphate, and electrophoresed with no heating. All samples were completely reduced to monomer regardless of urea content. Thus, neither

urea nor SDS is needed to reduce κ -casein to monomers within 2 hr with 40 mM DTT. In general at concentrations between 1 and 3 mg/ml, κ -casein is fairly readily reduced in the presence of urea or SDS by modest (5 mM) DTT concentrations, but in the absence of urea or SDS, higher concentrations (40 mM) of reductant are necessary to facilitate 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 -casein; reduction is accelerated by ratios >100:1.

3.5. Reoxidation of Reduced κ -Casein

To gain more information regarding the disulfide patterns of κ -casein generated by various treatments, the protein was thoroughly reduced for 1 hr and allowed to spontaneously reoxidize under a variety of conditions. Reduction with 33 mM DTT was carried out in 0.1 M TRIS (1 ml) 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 against 100 ml of selected buffers, and samples were withdrawn hourly for electrophoresis. Results of the degree of spontaneous reoxidation are summarized in Table III.

When the dialysis buffer was 0.1 M phosphate, pH 7.0, 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 even after an additional 3–4 hr with no DTT. The

urea could prevent the proper protein–protein interactions necessary to promote disulfide formation or yield a different conformation incapable of polymeric disulfide association. When the above experiment was repeated at pH 7.4 with urea but no DTT in the dialysis buffer, again no polymeric species were observed upon electrophoresis (Table III); elimination of the urea did not enhance reoxidation.

Some spontaneous reoxidation (tetramers) occurred by dialysis at pH 6.8 in 0.005 M NaCl (4 M urea), as seen in Fig. 7 (gels b and d). This indicates that the spontaneous reoxidation to polymers is apparently favored at lower pH rather than at higher 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 with 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 IV). 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 hr at 5°C.

κ -Caseins 1, 2 and 3 were prepared as described in Section 2. These samples were dialyzed for 3 days against distilled water (20 ml of sample against 2.0 L of water, 3 changes/day). The internal

Table III. Summary of Results of the Reoxidation of κ -Casein to Polymeric Structures

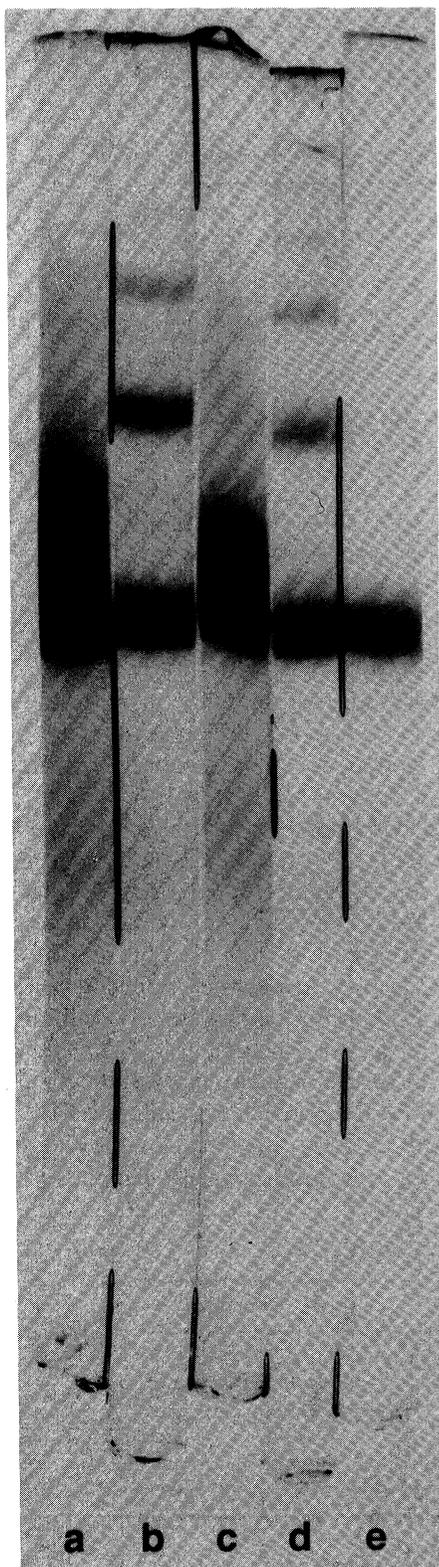
Dialysis conditions ^a	Maximum polymer size ^b
Whole κ -Casein	
pH 7.0, 4 M urea, 0.1 M NaPi DTT gradually reduced, 5 hr	None
pH 7.4, 4 M urea, no DTT, 0.1 M NaPi, 5 hr	None
pH 6.8, 4 M urea, no DTT, 0.005 M NaCl, 5 hr	Tetramers
pH 6.0, no urea, no DTT, 0.1 M NaPi, 5 hr	Hexamers
pH 6.6, no urea, no DTT, 0.1 M NaPi, 6 hr, air, 5°C	Pentamers
pH 5.15, no urea, no salt, no DTT, 1 week, 5°C	Octamers and above
κ -Caseins 1, 2, 3 ^c	
pH 5.3, no urea, no salt, 3 days, 5°C	Hexamers
pH 5.2, no urea, 0.01 M NaCl, 1 week, 5°C	Octamers and above ^d

^a Samples defined in text; dialysis at room temperature, except where noted. NaPi, sodium phosphate.

^b Polymer size estimated by SDS gel electrophoresis in the absence of added reducing agent.

^c κ -I, κ -II, and κ -III as designated by Vreeman *et al.* (1986).

^d Figure 8.



pH of these solutions averaged 5.3 ± 0.1 . Electrophoresis of these samples revealed spontaneous reoxidation up to hexamers (data not shown). Table IV shows that when dialysis was continued for 1 week but with 0.01 M NaCl, octamers were achieved at pH 5.2 (Fig. 8). Thus even lower pH values promote κ -casein association and subsequent reoxidation (Table III).

Finally, whole κ -casein was reduced with Tris, EDTA, urea, and DTT as above and dialyzed at $5^{\circ}C$ for 1 week against only distilled water (multiple daily changes). After this period of time (no buffer, no NaCl) precipitation began and the internal pH was 5.15. The resulting material contained a distribution of molecular weight polymers somewhat similar to those observed in the native species of protein.

It is under these latter conditions that many preparations of κ -caseins have been prepared for physical chemical studies, and changes in the dialysis conditions could give rise to the apparent discrepancies reported for the molecular sizes of polymeric κ -caseins. Vreeman *et al.* (1981) studied the concentration dependence of molecular weight of κ -casein (κ -I = carbohydrate-free) by Trautman analysis of sedimentation velocity experiments, and concluded that κ -casein aggregates to polymers containing 31 monomers; κ -casein I was prepared as was the κ -1 used in this study. In our experiments, when dialysis was continued without NaCl and the pH allowed to approach the isoelectric point, as noted by McKenzie and Wake (1961), large aggregates form which are often difficult to redissolve. This latter process is accelerated by heat in the present study. Vreeman *et al.* (1981) reported that for their samples, reduction had to proceed for 1 week to absolutely reduce the polymers formed on reoxidation after chromatographic separation. Thurn *et al.* (1987) studied κ -casein by static and dynamic light scattering and small-angle neutron scattering (SANS); these workers calculated that κ -casein formed aggregates of up to 130 monomers. The κ -casein used in the Thurn study was prepared by reduction, two column chromatography steps,

Fig. 7. Gels of reduced κ -casein in SDS showing limited spontaneous reoxidation on dialysis at pH 6.8 in 4 M urea, 0.005 M NaCl. Experiment 1: gel a = zero time; gel b = 1 hr. Experiment 2: gel c = zero time, gel d = 5 hr. Gel e is standard reduction prior to electrophoresis.

Table IV. Relative Areas for Each Oligomeric Form of κ -Casein as Determined by Densitometry After Reoxidation

Band ^c	Percentage of total area						
	Whole κ -casein ^a					κ -Casein 1 ^b	κ -Casein 2 ^b
	0	1 hr	4 hr	6 hr	24 hr		
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

^a McKenzie–Wake whole κ -casein, Coomassie stain; dialysis against 0.1 M sodium phosphate at room temperature (6 hr) and 5°C (18 hr), pH 6.0.

^b κ -I and κ -II as designated by Vreeman *et al.* (1986); these fractions are reduced for DEAE chromatography, but apparently reoxidize during exhaustive dialysis against distilled water and lyophilization (Groves *et al.*, 1992). Coomassie stain; average values three scans of samples reduced and then reoxidized by dialysis against 0.01 M NaCl, and studied by SDS-PAGE.

^c Band numbers correspond to those of Fig. 1.

dialysis, and lyophilization. The κ -casein was then dissolved in buffer without subsequent reduction. The occurrence of unreduced high-molecular-weight polymers as observed here could account for the molecular weights reported by Thurn *et al.* (1987). Finally, deKruif and May (1991) reported SANS data for κ -casein. Their analysis indicated κ -casein was intermediate in size between the Vreeman and Thurn models. In this case, samples (obtained from Vreeman) were dissolved in D₂O, DTT (5 mM), and imidazole without extensive reduction. In our studies in H₂O, some disulfide-bonded polymers would remain under these conditions, as the ratio of DTT to κ -casein would be low. The results from the current study summarized in Table III indicate that for κ -casein

(after reduction and spontaneous reoxidation) the degree of polymerization achieved can be quite variable, depending upon treatments. Correspondingly, the degree of re-reduction (or its lack) of highly polymerized samples could lead to the various results reported for molecular weights and summarized in Table V. It is interesting to note that, as determined by sedimentation equilibrium for the preparations of κ -casein 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 the weight-average molecular weight increased to 1.55 ± 0.09 million (Table II). These values for “native” unreduced κ -casein are between the values found for various chromatographed samples (Table V);

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

	MW	Radius (nm)	Method	Reduction
Vreeman	600,000	11.1	Sedimentation velocity	1 week, 2-ME
Slattery	600,000	11.2	Sedimentation	1 hr, DTT (40 mM)
deKruif		14.7	SANS	DTT (5 mM)
Thurn	2,000,000	7.0 ^b	SANS	None
This study	1,180,000	12.5 ^c	Sedimentation equilibrium	None

^a Radius type varies with method; 2-ME, 2-mercaptoethanol; SANS, small-angle neutron scattering.

^b Internal “submicellar” particle of larger high-molecular-weight aggregate.

^c Weight-average radius by dynamic light scattering (Farrell *et al.*, 1996b).

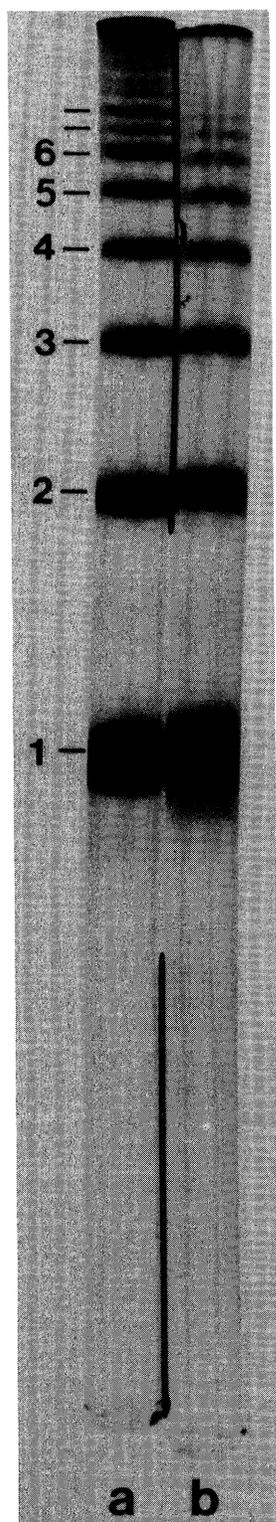


Fig. 8. Gels of unreduced κ -casein in SDS showing variable reoxidation after 1 week extended dialysis at 5°C, pH 5.2, and 0.01 M NaCl; (a) κ -1; (b) κ -2.

the values of the chromatographed samples appear to depend on the degree of reduction prior to analysis.

It may be of interest to consider the results obtained here in terms of modern theories of protein folding (Baldwin, 1995) or disulfide bonds and stability (Darby and Creighton, 1995). Small globular proteins tend to form different folding intermediates (and hence disulfides) depending on their native structural stabilities, and buried S-S bonds are slowest to form, while surface bonds form more rapidly. The two-cysteine system of κ -casein is not as complex as the six-cysteine system of bovine pancreatic trypsin inhibitor, but the innate structure of κ -casein is more flexible (Holt, 1992) as judged by the many intermolecular disulfides seen in the gels. With respect to reduction, κ -casein polymers are fairly readily reduced even in the absence of denaturants. This could mean either that the disulfides are close to the surfaces or that the polymeric aggregates are highly porous. The SANS study (Thurn *et al.*, 1987) would argue for the latter case. On the other hand, the proteolysis observed for the samples held at 37°C indicates that residues 21 and 24 (lysine) are near the surface of the polymer, indicating that Cys₁₁ should be somewhat close to the surface. With respect to reoxidation, native like polymers (partially buried S-S) apparently form only when the protein is highly aggregated (pH 5.2). Perhaps the combination of other caseins and the acidic pH present in Golgi apparatus during micelle assembly help to dictate the disulfide patterns achieved in native κ -casein (Farrell and Thompson, 1988). Thus, κ -casein disulfide patterns report upon κ -casein within the casein polymeric complex as a whole.

In summary, κ -casein 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 -casein.

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