

Reexamination of the Polymeric Distributions of κ -Casein Isolated from Bovine Milk

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κ -Casein the stabilizing protein of the colloidal milk protein complex was purified from bovine skim milk by the method of McKenzie and Wake (*Biochim. Biophys. Acta.* **47**, 240, 1961). The preparations were examined by sodium dodecyl sulfate gel electrophoresis in the presence and absence of a reducing agent. In the presence of a reducing agent, the κ -casein migrates as a single low molecular weight band. However, in the absence of a reducing agent, a characteristic pattern of aggregates of varying molecular weight was observed with components ranging from monomer to octamer in integer steps. Densitometry of the Coomassie blue stained gels showed an almost equal distribution of components in each band; carbohydrate staining showed preferential location of sugar residues in lower molecular weight components. Treatment with chymosin (rennin) caused a downward shift in apparent molecular weight for each band with no change in the relative intensity of the Coomassie blue stained bands. Similar gel patterns were observed in whole caseins and partially purified κ -caseins, indicating that this size distribution is a natural disulfide-linked reporter for the distribution of κ -casein in casein colloids (micelles).

KEY WORDS: Casein micelles; sulfhydryls; disulfides.

1. INTRODUCTION

At the heart of the skim milk system is a unique biocolloid, the casein micelle. The primary function of this complex is to transport calcium and phosphate to the neonate in a soluble form (Farrell and Thompson, 1988). This colloidal calcium transport complex is in dynamic equilibrium with its aqueous ionic environment. Changes in the state of the casein micelle system occur during milk secretion and processing (Farrell, 1988; Schmidt, 1982). Because of its innate importance to the milk system, the casein micelle and its four major protein components of bovine milk have been studied extensively.

κ -Casein differs from the other three caseins (α_{s1} -, α_{s2} -, and β -) in that it is soluble over a broad range of calcium ion concentrations (Waugh and Von Hippel,

1956). It was this calcium solubility which led the latter authors, upon discovering the κ -fraction, to assign to it the role of casein micelle stabilization. It is also the κ -casein fraction which is most readily cleaved by chymosin (rennin) (Jolles *et al.*, 1962). The resulting products are termed para- κ -casein and the macropeptide, and this protease-triggered reaction leads to milk coagulation during digestion. It would appear that κ -casein is the key to micelle structure in that it stabilizes the calcium insoluble α_{s1} - and β -caseins. κ -Casein is a glycoprotein displaying up to nine components based upon the degree of glycosylation (Takeuchi *et al.*, 1985); its micelle-stabilizing ability increases with increasing carbohydrate content. κ -Casein also contains two cysteine residues (Mercier *et al.*, 1973). Whether these can form intra- or intermolecular disulfide bonds and what the effects of such bonding are on micelle stabilization have not been clearly established. The occurrence of free sulfhydryl groups in the milk-protein complex has been reported by Beeby (1964), but not by others

(Swaigood *et al.*, 1964). Woychik *et al.* (1966) showed that completely reduced and alkylated κ -casein fractions had the ability to stabilize casein micelles as well as native κ -caseins. Swaigood *et al.* (1964) reported significant self-association as well as disulfide cross-linking in purified κ -casein. Parry and Carroll (1970) in an electron microscopy study found that purified κ -casein polymers were rather spherical and had a size range of 186 to 200 Å in diameter, in the absence of reducing and dissociating agents. Pepper and Farrell (1982) found that in whole casein, in the absence of Ca^{2+} , κ -casein occurs as a high molecular weight polydisperse complex with an average size of 94 Å Stokes radius. The nature of this polydispersity has not been investigated. This manuscript deals with a relatively simple method, polyacrylamide-gel electrophoresis (PAGE) in sodium dodecylsulfate (SDS), for studying and quantitating this polydispersity and its possible role in colloidal stability.

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). Some preparations were made fat free by ultracentrifugation at 4°C.

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 two sequential peaks designated κ -1 casein (91 mg) and κ -2 casein (42 mg); these peaks also correspond to fraction κ -I and κ -II of Vreeman *et al.* (1986).

Para- κ -casein (p- κ -) was prepared according to the method of Charles and Martin (1985), using 0.42 mM chymosin (rennin, NBC) at a κ -casein concentration of 2 mg/ml. After 20 min of digestion at 30°C, the solution became very turbid and the precipitated p- κ -casein formed a pellet upon centrifugation. The pellet was washed two times with water and recovered by lyophilization.

Polyacrylamide gel electrophoresis was 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; protein concentration was 0.2 mg or less per gel.

Carbohydrate was determined in gels after electrophoresis by a modification of the method of Kapitany and Zebrowski (1973) as follows:

1. After electrophoresis, gels were placed in tubes (1 × 12.3 cm) with 12.5% TCA 1 hr and changed to fresh TCA and stored at 4°C overnight.
2. Next gels were washed with water and an oxidizing agent (1% periodic acid in water) added. The gels were kept in the dark for all subsequent steps. After 1 hr at 25°C, fresh oxidizing agent was added and the gels stored for another hour.
3. The gels were then washed with 15% acetic acid, which was changed at 10 min intervals for 1 hr.
4. Schiff's Reagent was added and gels stored in the dark at 25°C; after 30 min, fresh Schiff's Reagent was added and gels incubated 25°C for 3.5 hr.
5. Gels were then washed with 7% acetic acid and stored in the dark at 25°C overnight, until lightly pink. Washing gels with 7% acetic acid was continued until pink bands of glycoproteins became visible.

For amino acid composition, protein samples were hydrolyzed at 110°C in a forced-air oven for 24 hr, with 4.7 N HCl containing phenol (0.05%) in sealed, evacuated tubes. Analyses were performed in triplicate using a Beckman 119 CL amino acid analyzer. Cysteine was identified as 1/2 cystine by comparison of its retention time with a known standard. Reduction and alkylation was carried out according to Shechter *et al.* (1973). Data are reported as molar ratios with arginine fixed at 5 residues/molecule. Cystine/cysteine was determined by performic acid oxidation using the method of Hirs (1967).

Densitometry of the gels was carried out on a BioRad Model 222 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.

Hexose was measured by the orcinol-sulfuric acid method of Winzler (1955).

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.

The amino acid compositions of κ -casein and para- κ -casein are shown in Table I, together with their composition based on the amino acid sequence composition (Mercier *et al.*, 1973). The sequence data shown is for κ -casein A; the composition data of the sample is consistent with that of κ -casein A, showing apparently equivalent aspartic, alanine, and isoleucine contents. This confirms the alkaline urea PAGE phenotype. Evaluation of the cystine/cysteine content will be given below.

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 8) of orderly increasing size were observed. Polymerization appears to be driven by

Table I. Amino Acid Composition of κ - and Para- κ -Casein—Comparison with Sequence Data

Amino acid	Residues/mol			
	Purified κ -casein ^a	Sequence κ -A ^b	Purified para- κ -	Sequence para- κ -
Asp	12.1	12 (11)	8.3	7
Thr	13.3	15 (14)	4.1	3
Ser	13.0	13	6.8	7
Glu	25.6	27	18.1	17
Pro	19.3	20	11.3	12
Gly	3.1	2	3.1	1
Ala	12.6	14 (15)	9.4	9
Val	9.5	11	5.9	5
Met	2.4	2	1.1	1
Ile	12.1	12 (13)	6.3	6
Leu	9.6	8	8.3	7
Tyr	8.8	9	8.8	9
Phe	5.1	4	4.1	4
Lys	8.8	9	6.7	6
His	2.6	3	2.9	3
Arg	5.0	5	5.0	5

^a Twenty-four hour hydrolysis molar ratio, Arg = 5 average of three determinations.

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

either one or both of the two cysteines in κ -casein. 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).

κ -Casein (carbohydrate free) has a molecular weight of 19 kD, and its para- κ -casein peptide gives a value of 12 kD based on sequence data (Mercier, 1973). In this study, monomeric κ -casein shows a molecular weight of 30 kD on SDS-PAGE with 7.5% acrylamide; no bands are found in the 19 kD range but caseins do display somewhat anomalous SDS-PAGE patterns (Doi *et al.*, 1979). Doi *et al.* (1979) also showed that the carbohydrate-free protein has the same mobility as the glycosylated κ -casein on SDS-PAGE. Recent apparent molecular weight determinations on isolated κ -casein reveal values of 30 kD (Cernina-Beroard and Zevaxo, 1984) for porcine and

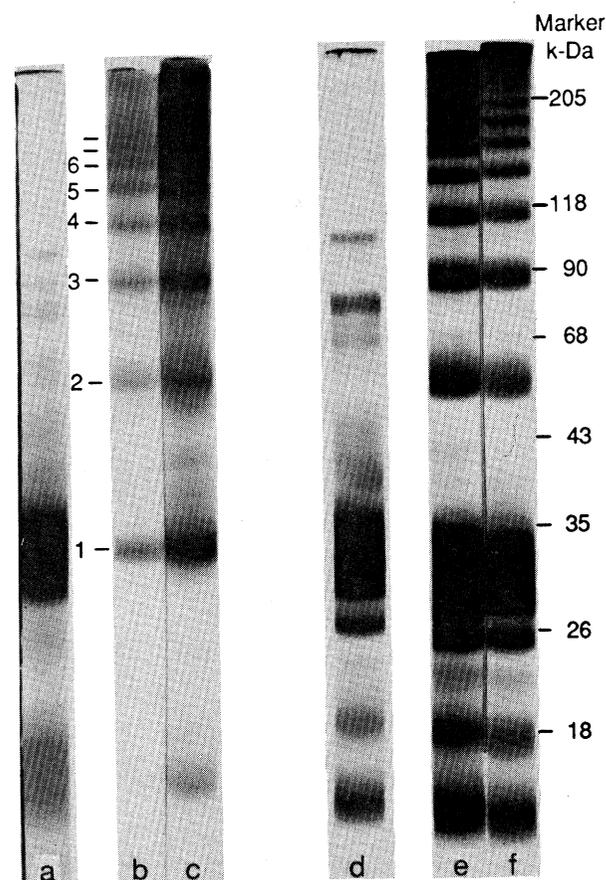


Fig. 1. SDS gel electrophoretic patterns: κ -casein reduced (a) and unreduced (b, c); crude κ -casein reduced (d) and unreduced (e, f). All gels stained with Coomassie blue.

33 (Yamauchi *et al.*, 1981) for human, the latter contains about 40% carbohydrate which is greater than that of bovine κ -casein.

In Fig. 1, the protein bands labeled 1 in gels a, b, and c represent the κ -casein monomer. Variable amounts of slower-moving components were found; these could represent unreduced κ -casein. A faster-moving component, which might represent some para- κ -casein, is also observed. The gel in Fig. 1a is somewhat "overloaded." Fraction III, the final byproduct in the isolation procedures of McKenzie and Wake (1961), is shown in Fig. 1. For fraction III, the monomer band 1, in Fig. 1 gels d, e, and f, is masked by other casein impurities. Bovine κ -casein under nonreducing conditions apparently forms polymers by adding monomers to form the dimer, trimer, tetramer, and pentamer (i.e., bands 2–5 in Fig. 1). Bands 6, 7, and 8 though not precisely measured probably represent up to an octomer of κ -casein.

Densitometer traces of the Coomassie blue gels were made, and an estimate of the percent of total area given for each band. Six scans of three different preparations are averaged in Table II. The results show good agreement for the lower molecular weight bands, but there is considerable variation in the estimates of higher molecular weight bands. Bearing in mind that the dye-binding constants may not be identical for all polymers, the results suggest a relatively even distribution of area among the different bands and that less than 12% of whole κ -casein is monomeric. This is in accord with earlier data by Swaisgood *et al.* (1964), who observed polydispersity by ultracentrifugation in urea, and with gel chromatographic

data (Pepper and Farrell, 1982), which indicated that reducing agents were needed to produce monomeric κ -casein.

Attempts were made using amino acid analysis to determine whether or not the 10% κ -casein present as a monomer is disulfide linked. Accurate determination of cystine in protein is difficult, and results for five determinations yields a value of 0.913 ± 0.113 mol %. Based upon the recovery of all amino acids (column 1, Table I) the expected mol % would be 1.28 if all the cysteine is present as cystine, a 10% difference would not be detectable within the error of the analysis. Performic acid oxidation of the κ -casein yielded an average value for cysteic acid of 1.24 mol %; reduction and alkylation with iodoacetic acid gave a comparable value (1.22). Since both of these analyses have errors of 8%, it was not possible to use them in combination to find a difference of 10% presumably due to 10% of κ -casein present as monomer. Attempts at quantitating the "free-SH" of putative κ -casein monomers, using previously tested methods (Beeby, 1964; Swaisgood *et al.*, 1964) were also inconclusive. One explanation for the occurrence of monomeric κ -casein could be that the free-SH groups, if they indeed occur, are bound to metal ions, and metal sulfides have high association constants. Results from atomic absorption analysis of these preparations gave values of 3.5 ± 0.5 and 2.3 ± 0.3 mol of iron and calcium per mol of κ -casein, respectively. Thus, the possibility exists that a metal sulfide could mask free-SH if it exists either in the monomer or in oligomers. Whether this metal is actually bound to the cysteine is not

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

Band no. ^c	Percentage of total area \pm SD					
	Whole κ -casein ^a		κ -casein 1 ^b		κ -casein 2 ^b	
	Coomassie stain ^d	Schiff stain ^e	Coomassie stain ^d	Schiff stain ^e	Coomassie stain ^d	Schiff stain ^e
1	11.5 \pm 1.2	17.7	31.0 \pm 1.8	59.5	42.6 \pm 1.5	61.0
2	11.1 \pm 2.3	15.3	15.6 \pm 0.5	17.1	15.5 \pm 1.2	16.1
3	15.0 \pm 2.4	17.8	10.3 \pm 0.8	10.0	9.4 \pm 0.7	10.6
4	12.1 \pm 1.9	12.2	6.6 \pm 1.0	8.0	7.4 \pm 1.8	5.2
5	14.0 \pm 0.9	12.7	5.5 \pm 0.6	5.4	4.6 \pm 0.8	4.5
6	11.9 \pm 1.3	10.7	4.5 \pm 0.4	—	2.8 \pm 0.6	2.5
7	12.8 \pm 3.3	6.8	2.6 \pm 0.5	—	2.3 \pm 0.8	—
8	11.6 \pm 4.2	6.5	3.0 \pm 1.0	—	1.6 \pm 0.5	—

^a McKenzie-Wake whole κ -casein.

^b κ -I and κ -II as designated by Vreeman *et al.* (1986); these fractions are reduced for DEAE chromatography, but apparently reoxidize during dialysis and lyophilization.

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

^d Average values for four scans of three preparations.

^e Six scans of one preparation.

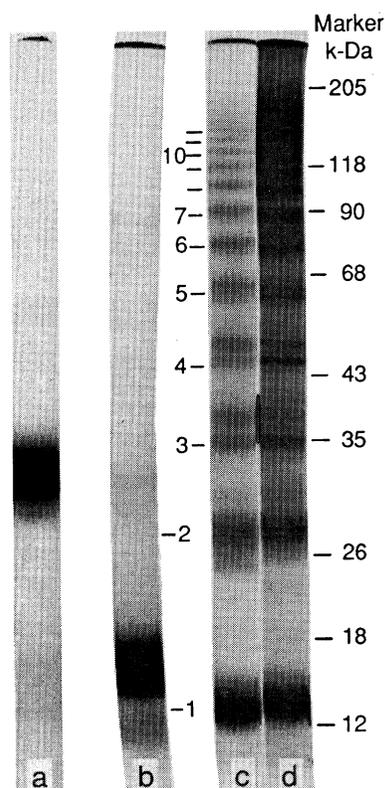


Fig. 2. SDS gel electrophoretic patterns: κ -casein reduced (a); p- κ -casein reduced (b); and p- κ -casein not reduced (c, d); Coomassie blue.

known, nor is it known if it is an artifact of the isolation procedure.

On SDS-PAGE of κ -casein in the absence of 2-mercaptoethanol (Fig. 1b, c, e, and f) there is a

diffuse staining area in the upper portion of the gels. It appears to be an aggregate of κ -casein which is not fully broken down and does not form discrete bands. On electrophoresis under reducing conditions, this material disappears with the polymer bands and apparently migrates as κ -casein monomer.

3.3. Degree of Polymerization of Para- κ -Casein

When κ -casein is treated with chymosin, the specific bond between phenylalanine and methionine (residues 105–106) is cleaved leaving carbohydrate free para- κ -casein (p- κ), residues 1–105, with a molecular weight of 12 kD, and the macropeptide, residues 106–169. The two cysteines of κ -casein are found in the p- κ -casein, one near the N-terminal (residue 11) and the other near the center portion (residue 88) of the p- κ -molecule. In order to learn whether p- κ -casein, like κ -casein occurs as discrete polymers, p- κ -casein was subjected to SDS-PAGE under reduced and nonreduced conditions. The results (Fig. 2) are similar to those of the parent κ -casein. Some bands in this preparation appear as doublets, but these are much reduced in other protease-generated preparations and may represent secondary cleavage of κ -casein.

Table III lists the approximate molecular weights of the polymers of κ - and p- κ -casein as determined by SDS-PAGE. κ -Casein aggregates increase stepwise apparently by adding one monomer to form each higher molecular weight band. p- κ -Casein behaves in a similar fashion; taking the monomer as 13 kD, the

Table III. Molecular Weights of κ - and p- κ -Casein Polymers

κ -Casein			p- κ -Casein		
Band no.	By SDS gels		Band no.	By SDS gels	
	kD	Theory ^a kD		kD	Theory ^b kD
1	30	30	1	13	13
2	57	60	2	25	26
3	85	90	3	35	39
4	120	120	4	45	52
5	145	150	5	60	65
6	160	180	6	70	78
7	185	210	7	90	91
8	205	240	8	115	104
			9	120	117
			10	135	130
			11	140	143
			12	150	156

^a $30 \times N$, where N = no. of monomers.

^b $13 \times N$, where N = no. of monomers.

p- κ -polymer also grows by adding a monomer to form each higher molecular weight band.

Molecular models for κ -casein (Kumosinski *et al.*, 1991) suggest that the two cysteine residues of κ -casein are more than 33 Å apart, and thus not capable of forming an intramolecular disulfide. The amino acid analysis suggests that the majority (>73%) of the cysteine is disulfide-linked in the purified κ -casein, but it is not possible to determine if the polymers are closed or open ended.

3.4. Carbohydrate and Degree of Polymerization of κ -Casein

In early studies of κ -casein in which starch gel and alkaline urea PAGE were used to distinguish charge heterogeneity, κ -casein was fractionated into several components which were related to variations in degree of glycosylation (Woychik *et al.*, 1966). Doi *et al.* (1979) found with DEAE-cellulose chromatography using urea, under reducing conditions, that κ -fractions eluted with increasing salt concentration had increasing amounts of carbohydrate and that one κ -casein peak, eluted early, had no carbohydrate. They also showed that on SDS-PAGE all of the reduced κ -casein fractions isolated from DEAE-cellulose gave single bands with the same mobility and the same amino acid composition. Vreeman *et al.* (1986) also recovered κ -casein in several fractions following a similar method. They found that about 37% of κ -casein is not glycosylated and that this compound is the slowest-moving band on alkaline urea PAGE.

Fractionation of crude κ -casein (Fig. 1d, e, and f) by DEAE-cellulose as described in Materials and Methods following Doi *et al.* (1979) gave two major κ -casein fractions designated κ -1 and κ -2. The SDS-PAGE of these fractions (Fig. 3a and b) indicate that the proteins display polydispersity. With reducing agent added, the SDS-PAGE gels of κ -1 and κ -2 showed them to be monomeric. Furthermore, the amino acid composition (not shown) was in good agreement between κ -1 and κ -2 caseins and also with whole κ -casein isolated by the method of McKenzie and Wake (1961).

In the present study, the distribution of polymers in the whole κ -casein was also examined by staining the SDS-PAGE for carbohydrate. Bearing in mind that the degree of the reaction with Schiff stain may be different for differing degrees of glycosylation, there appeared to be a concentration of carbohydrate stain in the lower molecular weight polymers. Bands 1 through 8 contained carbohydrate; bands 7 and 8 were diffuse, but most Coomassie blue bands also

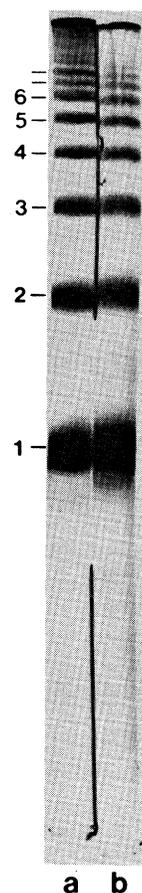


Fig. 3. SDS electrophoretic patterns of DEAE purified κ -casein (not reduced): κ -1 casein (a); κ -2 casein (b); Coomassie blue stain.

stained for carbohydrate. Table II gives the estimated percent area for the carbohydrate-stained bands. Diffuse slow-moving material noted earlier for Coomassie stained gels makes it difficult to determine the presence or absence of carbohydrate in bands 7 and 8. Based on the results of Doi *et al.* (1979), the first κ -casein eluted (κ -1) should be free of carbohydrate. The purity of this fraction was confirmed on alkaline urea PAGE (not shown). However, it did show some reaction with the carbohydrate stain, but in a much reduced amount compared to κ -2 casein. Quantitative analysis for hexose gave values of 0.31 and 1.05% for κ -1 and κ -2, respectively. Hexose represents on the average one third of the total carbohydrate in κ -casein (Eigel *et al.*, 1984), thus the total carbohydrate content would be 0.93 and 3.15% for κ -1 and κ -2. Since the starting material for the κ -1 and κ -2 casein fractionation was not purified κ -casein, which Doi *et al.* (1979) used but a crude fraction of the McKenzie and Wake κ -casein (Fig. 1d, e,

and f), some difference in carbohydrate distribution could be expected.

Densitometric analyses of κ -1 and κ -2 stained for carbohydrate and for protein are given in Table II. The carbohydrate stain is concentrated primarily in polymers 1 through 5 and 6 for these fractions. The diffuse slow-moving material makes the quantitation of the carbohydrate in higher molecular weight polymers (those >8) difficult for these fractions. However, this is in part due to the fact that SDS-PAGE patterns from Coomassie blue stain for κ -1 and κ -2 caseins display about a threefold increase in monomers compared to that of κ -caseins, which apparently results from the reduction for DEAE-chromatography followed by reoxidation of κ -1 and κ -2 during dialysis and lyophilization.

Since purified κ -casein displays numerous polymers in the presence of SDS, it is not unreasonable to assume that these polymers also occur in the absence of SDS. Polymers can grow or terminate at the two cysteine sites on κ -casein. These polymers of κ -casein might be reflective of the different-sized colloidal micelles, where they are present in the calcium caseinate complex. The data of Slattery (1978) indicate that larger micelles contain less total κ -casein, greater amounts of glycosylated κ -casein, and yield purified κ -casein with a higher degree of aggregation as judged by sedimentation velocity studies. In addition, Carroll and Farrell (1983) showed κ -casein to occur primarily on the surface of large micelles. Although κ -casein represents only 13% of total casein, the SDS-PAGE data in this study suggest more than 90% of the κ -molecules have one or more other κ -casein molecules as their nearest neighbors in order to form these disulfide-linked polymers. The patterns observed in Fig. 1 thus represent a natural reporter group for the κ -casein. Sulfhydryl oxidation into disulfides could be the final structural touch that holds the κ -casein polymers together because: (1) on SDS-PAGE with urea polymers are resolved that are like those obtained on SDS-PAGE without urea; and (2) on reduction in urea, the polymers are converted to monomers and subsequently can be reoxidized to polymers in agreement with previous data (Pepper and Farrell, 1982). These reoxidized polymers obtained from κ -casein, however, have a very different size distribution than those obtained from the original κ -casein preparation (Table II).

One theory of casein micelle structure holds that spherical casein micelles (with molecular weights $\sim 10^{10}$) are formed from spherical submicellar complexes through Ca^{2+} -driven aggregation in Golgi vesicles of lactating mammary gland (Farrell and

Thompson, 1988). The molecular weight of a putative casein submicelle is 285,000 (Farrell *et al.*, 1990). Comparison of Tables II and III suggests that about 10% of purified κ -casein has a molecular weight such that if submicelles do occur on the surface of the casein micelle, then some of these could represent nearly pure κ -casein (band 8 has an apparent molecular weight of at least 240,000). Alternatively, disulfide bonds can be formed between adjacent submicelles once micelle formation has occurred. Small angle X-ray scattering data of caseins *in vitro* appear to indicate that interdigitation of submicelles must occur as calcium addition causes some swelling of the outer hydrophilic region of the submicelles (Pessen *et al.*, 1991). This interdigitation which causes an overlap of over 20 Å is sufficient to bring κ -casein from neighboring submicelles into close enough proximity to allow disulfide bond formation. Once oxidation of the sulfhydryls to disulfide occurs a certain amount of structural integrity is imparted to the casein micelle. Thus, κ -casein may play the role of a protective surface colloid in the casein micelle by covalently linking adjacent submicelles. It is of interest to note that human κ -casein (Brignon *et al.*, 1985) has only one cysteine, while goat κ -casein has three (Mercier *et al.*, 1976). Thus, the human κ -casein could form only dimers, while goat might produce different patterns than cow. It may be speculated that these disulfide linked κ -casein patterns contribute to known species differences in micelle size and structure (Farrell and Thompson, 1988).

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