

The multimeric structure and disulfide-bonding pattern of bovine κ -casein

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Bovine κ -casein was analyzed by SDS/PAGE, MS and amino acid sequence analysis in order to determine its multimeric composition and disulfide-bonding pattern. SDS/PAGE revealed that κ -casein in the native state can range in size from a monomer to a multimeric structure larger than a decamer. Three types of interchain disulfide linkage, Cys11–Cys11, Cys11–Cys88 and Cys88–Cys88, were all assigned in multimers purified from [¹⁴C]carboxymethylated and untreated bulk milk, as well as a milk sample from a κ -casein-variant-B homozygote Co20. These results indicate that multimerization occurs in a random or at present unpredictable disulfide-bonding pattern regardless of the size of the multimer or the genotype.

The casein micelle in milk is a unique structure capable of keeping a 30-mM concentration of calcium phosphate in a colloidal and bio-available state. The micelle provides the neonate with the necessary calcium for formation of hard tissue, and adults obtain up to 75% of its required calcium from milk products (McBean and Speckmann, 1988).

The caseins are among the most rapidly diverging protein families (Holt and Sawyer, 1988). In bovine milk, four caseins are present in the micelle (α_{s1} -, α_{s2} -, β - and κ -casein), while in human milk only two have been found (Kunz and Lönnerdal, 1990) corresponding to bovine β -casein and κ -casein (Mercier et al., 1973; Greenberg et al., 1984). The α_{s1} -, α_{s2} - and β -caseins are insoluble in the presence of calcium ions encountered in milk. In contrast, κ -casein is insensitive to calcium and plays a key role in maintaining the stability and solubility of the micelle structure (Linderstrøm-Lang, 1929; Waugh, 1971). Several studies, including ¹H-NMR spectroscopy, indicate that κ -casein is located mainly at the micelle surface (Griffin and Roberts, 1985; Rollema et al., 1988). It is believed that the micelles are composed of submicelles held together by calcium phosphate, although the exact quaternary structure of the casein micelle has remained controversial (Farrell, 1988).

One characteristic aspect of the caseins is their low content of cysteine. In the bovine sequences only α_{s2} -casein and κ -casein contain cysteines, with two residues in each polypeptide chain (Mercier et al., 1973; Brignon et al., 1977). α_{s2} -casein is found both as a monomer and a dimer (Annan and Manson, 1969). In the dimer, both cysteines form interchain disulfide bonds linking the two chains of α_{s2} -casein to each other, and both parallel and antiparallel configurations have been found (Rasmussen et al., 1992).

In contrast, κ -casein is present in a multimeric composition. Based on sedimentation-equilibrium studies it has been

reported that κ -casein exists naturally as disulfide-linked polymers, in a distribution of sizes (Swaigood et al., 1964; Talbot and Waugh, 1970). However, the localization of the disulfide bridges and the exact multimeric structure have not yet been identified. Proteins which are composed of identical subunits linked by disulfide bonds generally have a precise number of subunits (e.g. IgG, IgM and thrombospondin) with the exception of von Willebrand factor (vWF; Chopek et al., 1986) and multimerin (Hayward et al., 1991). These two blood platelet proteins are unique in that they exist in the native state as a series of disulfide-linked multimers ranging in size from a thousand kDa up to and over 10 MDa.

The clotting of milk and blood are well-known processes which share some common features; limited proteolysis is the triggering mechanism, and during both coagulation processes short soluble peptides are released (Jollès and Henschen, 1982). κ -casein and the γ chain of fibrinogen show sequence similarities at the amino acid (Jollès et al., 1978) and cDNA levels (Thompson et al., 1985) and the intron-exon organization of the two genes further supports an evolutionary relationship between the two proteins (Alexander et al., 1988).

The present paper describes the oligomeric nature and the disulfide-bonding pattern of κ -casein and discusses its structural resemblance to other multimeric proteins.

EXPERIMENTAL PROCEDURES

Materials

Pepsin and *N*-tosylphenylalaninechloromethane-treated trypsin were obtained from Worthington Biochemical Corp. Iodo[2-¹⁴C]acetic acid (54 Ci/mol) was from The Radiochemical Centre (Amersham, UK). Sepharose CL-6B, Sephadex G-25 and Mono S HR 5/5 were from Pharmacia (Uppsala, Sweden). Vydac C₁₈ was from The Separations Group (Hesperia, CA). Molecular-mass standards for SDS/PAGE were from Bio-Rad Laboratories (Richmond, CA). Reagents used for sequencing were purchased from Applied

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Abbreviations. PDMS, plasma-desorption-mass-spectrometric analysis; Pth, phenylthiohydantoin; vWF, von Willebrand factor.

Enzymes. Pepsin (EC 3.4.23.1); trypsin (EC 3.4.21.4).

Biosystems (Foster City, CA). All other chemicals were of analytical grade.

Amino acid analysis

Peptides were hydrolyzed in 6 M HCl at 100°C for 20 h in evacuated sealed tubes with a preceding performic-acid oxidation for the detection of cysteine/cystine as cysteic acid (Hirs, 1967). The amino acid composition was determined by precolumn derivatization with 2-phthalaldehyde in a Hewlett-Packard Amino Quant analyzer connected to a Hewlett-Packard 1046 A programmable fluorescence detector. Detection of cysteic acid was achieved by a modification of the procedure described by the manufacturer (Rasmussen et al., 1992).

Amino acid sequence analysis

Automated Edman degradation was carried out in an Applied Biosystems model 477A sequencer with on-line phenylthiohydantoin (Pth) analysis using an Applied Biosystems 120A HPLC. Pth₂—Cys was identified after its release in the corresponding cycle and eluted just before Pth—Tyr (Højrup and Magnusson, 1987; Marti et al., 1987).

Gel electrophoresis

SDS/PAGE was performed according to Laemmli (1970) on 10–20% gradient gels or 8% homogeneous gels. Reduction was carried out with dithioerythritol at a final concentration of 9 mM and the gels were stained with Coomassie blue R-250.

Plasma-desorption-mass-spectrometric analysis (PDMS)

MS was carried out in a BioIon 20K plasma-desorption time-of-flight instrument. The sample was dissolved in 0.1% trifluoroacetic acid and applied to a nitrocellulose-covered target, spin-dried and micro-rinsed. *In situ* reduction of disulfide bonds was performed after PDMS of a sample by incubation of the nitrocellulose-bound sample with a solution of dithiothreitol as described (Nielsen et al., 1988). Spectra were recorded at 15 kV for 10⁶ primary fission events.

Purification of [¹⁴C]carboxymethylated and untreated disulfide-linked κ -casein

Three kinds of milk were investigated: untreated bulk milk, [¹⁴C]carboxymethylated bulk milk and milk from a single Jersey cow typed κ^{BB} -casein. Skimmed milk was prepared from bulk milk and from the individual Jersey-milk sample by centrifugation at 2500 g for 10 min. 20 ml skimmed bulk milk was [¹⁴C]carboxymethylated as previously described (Rasmussen et al., 1992).

Whole casein was obtained from the milk samples by precipitation at pH 4.6 with acetic acid. The isoelectrically precipitated caseins were separated by centrifugation (2250 g, 5 min) and washed twice with 5 ml ammonium acetate (0.1 M, pH 4.6). Disulfide-linked κ -casein from the different milk samples was purified by gel chromatography on a Sepharose CL-6B column as described previously (Rasmussen and Petersen, 1991). Aliquots (20 μ l) from every second fraction from the chromatogram of [¹⁴C]carboxymethylated bulk milk were analysed in a scintillation counter (Beckman LS 6000TA). Disulfide-linked κ -casein from each of the various

preparations was separated into five pools (A1–5, Fig. 1) and each pool was desalted on a Sephadex G-25 column in 5% (by vol.) formic acid.

Whole casein obtained from untreated bulk milk (30 ml) was [¹⁴C]carboxymethylated in the presence of a denaturing agent. The casein was dissolved in 20 mM Tris/HCl, 8 M urea, pH 8, and 75 μ l iodo[2-¹⁴C]acetic acid was added. The mixture was incubated for 15 min in the dark at 20°C with stirring, after which iodoacetic acid (final concentration 3 mM) was added and the alkylation was allowed to proceed for a further 15 min. The solution was fractionated on a Sepharose CL-6B column under the same conditions as previously described (Rasmussen and Petersen, 1991). Aliquots (25 μ l) from every second fraction were analysed in a scintillation counter. Pool B and pool C (Fig. 1) were further separated on a Mono S HR 5/5 column equilibrated with 50 mM ammonium acetate, 8 M urea, pH 5, and eluted with a linear gradient to 1 M ammonium acetate, 8 M urea, pH 5, at a flow rate of 0.5 ml/min. The effluent was monitored at 280 nm, collected and analysed (100 μ l) in a scintillation counter.

Isolation of disulfide-linked peptides

In order to prevent disulfide-interchange reactions from taking place, all enzymic degradations and separations were performed at below pH 8. Purified disulfide-linked κ -casein pools A1–5 were digested with pepsin using an enzyme/substrate ratio of 1:100 (by mass) in 5% (by vol.) formic acid at 37°C for 24 h and the digests were freeze-dried. Some of the peptic peptides containing cystine were freeze-dried and further digested with trypsin at a concentration of 20 μ g/ml in 0.1 M NH₄HCO₃, pH 7.6, for 12 h and lyophilized. Separation of peptides was carried out by reverse-phase HPLC on a Pharmacia LKB HPLC system consisting of a 2249 LC gradient pump connected to a 2510 Uvicord SD detector equipped with a 226-nm or a 280-nm filter and a flow cell with a 2.5-mm path length.

RESULTS

The multimeric composition

The gel-filtration profile of whole casein from [¹⁴C]-carboxymethylated bulk milk is shown in Fig. 1 and is identical with the profile obtained from untreated bulk milk. Pools A1–5 correspond to disulfide-linked κ -casein, pool B mainly to α_{s2} -casein dimer and pool C is mainly a mixture of α_{s1} -, α_{s2} - and β -casein (Rasmussen and Petersen, 1991). Scintillation counting showed no incorporation of radioactivity in disulfide-linked κ -casein, indicating that no free thiol groups are present among the polymers. This was further verified by [¹⁴C]carboxymethylation of whole casein under denaturing conditions followed by gel-filtration as in Fig. 1. The elution and radioactivity profiles were similar to those shown in Fig. 1 (data not shown).

In Fig. 2 the SDS/PAGE of pools A1–5 shows the different sizes of κ -casein multimers. In the absence of dithioerythritol (lanes 1–5), a number of bands forming a ladder of higher molecular mass are discerned. Upon reduction with dithioerythritol (lane 6) these bands are replaced by a single band of lower molecular mass and hence demonstrate that the native protein forms disulfide-linked homopolymers. Molecular mass of the various multimers was estimated by linear-regression analysis ($r = -0.998$, $P < 0.001$) using a 10–20% gradient gel. The bands migrated

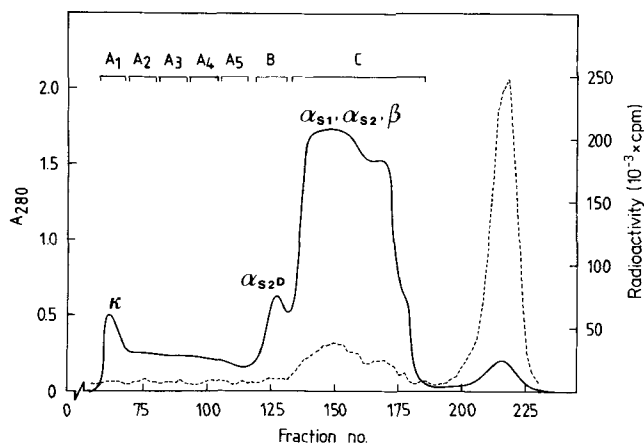


Fig. 1. Purification of large κ -casein multimers from [^{14}C]carboxymethylated whole casein. The proteins were separated on a Sepharose CL-6B column eluted with 50 mM ammonium acetate, 8 M urea, pH 5, at a flow rate of 35 ml/h. Fractions were pooled as indicated by vertical bars. A_{280} (—); radioactivity (---); κ , κ -casein; α_{s2} D, dimer of α_{s2} -casein; α_{s1} , α_{s1} -casein; β , β -casein.

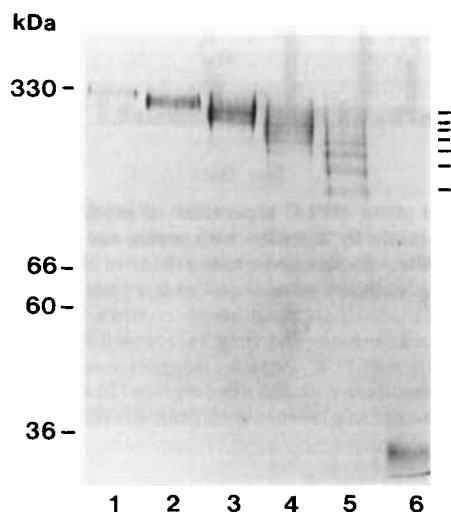


Fig. 2. SDS/PAGE analysis of large κ -casein multimers. Pools A1–5 in Fig. 1 were analyzed on a 8% polyacrylamide gel. The gel was stained with Coomassie blue R-250. Lanes 1–5 correspond to pools A1–5, respectively. Lane 6, pool A5 reduced with dithioerythritol. Markers (left) indicate the positions of molecular-mass standards: thyroglobulin, 330 kDa; bovine serum albumin, 66 kDa; catalase, 60 kDa; lactate dehydrogenase, 36 kDa. Markers (right) show the identified bands of κ -casein multimers.

with molecular masses of approximately 130, 162, 190, 214, 239, 265 and 283 kDa. The difference between the identified bands was on average 26 kDa, which approximately equals the estimated molecular mass (22 kDa) of monomeric κ -casein. The largest multimer eluting in pool A1 had a molecular mass of approximately 325 kDa. Based upon the 95% confidence limits of the average difference between the bands, the largest identified multimer was estimated to be composed of 11–15 subunits. We have counted up to eight bands on a 10–20% gradient gel and ten bands on the homogeneous gel. However, the number of bands may be higher since the larger

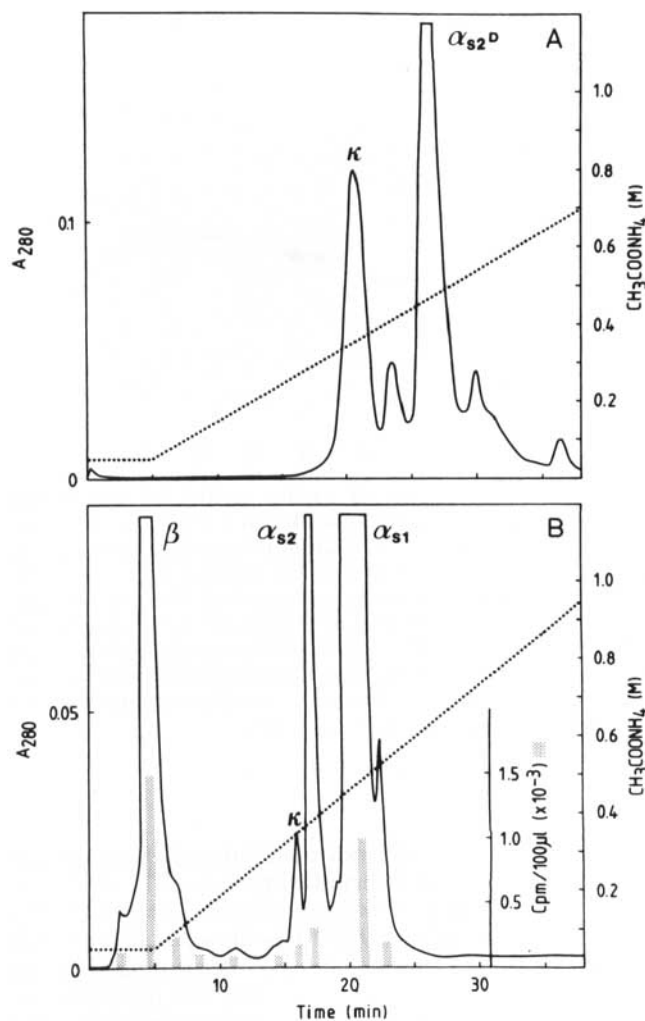


Fig. 3. Purification of small κ -casein multimers from [^{14}C]carboxymethylated whole casein. Pool B (A) and pool C (B) were separated on a Mono S column equilibrated with 50 mM ammonium acetate, 8 M urea, pH 5, and eluted with a linear gradient of 1 M ammonium acetate, 8 M urea, pH 5 (·····), at a flow rate of 0.5 ml/min. The effluent was monitored at 280 nm (—) and collected manually. Hatched bars indicate radioactivity.

multimers are difficult to distinguish due to the resolution of the gel.

To investigate the occurrence of smaller κ -casein multimers, pools B and C from the gel filtration of [^{14}C]carboxymethylated whole casein were separated on a Mono S column (Fig. 3A and B, respectively). Chromatography of pool B resulted in the separation of smaller κ -casein multimers from α_{s2} -casein dimer as shown by SDS/PAGE analysis (Fig. 4). Chromatography of pool C followed by SDS/PAGE analysis of the resulting fractions revealed that this pool not only contained α_{s1} -, α_{s2} - and β -casein but also κ -casein in a monomeric form (Fig. 4). The identity of this fraction corresponding to the monomeric form of κ -casein was further verified by amino acid analysis. Scintillation counting showed practically no incorporation of radioactivity in monomeric κ -casein and α_{s2} -casein indicating that no free thiol groups are present in these two proteins (Fig. 3B). It was shown by autoradiography on the SDS/polyacrylamide gel that the radioactive bands did not correspond to β -casein and α_{s1} -

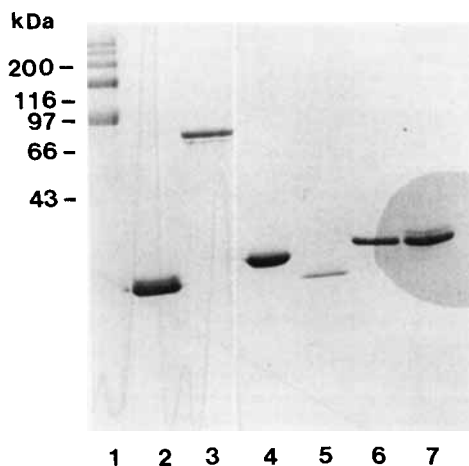


Fig. 4. SDS/PAGE analysis of caseins purified by ion-exchange chromatography. Separation of pool B and C (Fig. 3A and B) was analyzed on a 10–20% linear gradient polyacrylamide gel. The gel was stained with Coomassie blue R-250. Markers (left) indicate the positions of molecular-mass standards: myosin, 200 kDa; β -galactosidase, 116 kDa; phosphorylase *b*, 97 kDa; bovine serum albumin, 66 kDa; ovalbumin, 43 kDa. Lane 1, small κ -casein multimers; lane 2, small κ -casein multimers reduced with dithioerythritol; lane 3, α_{s2} -casein dimer; lane 4, β -casein; lane 5, monomeric non-reduced κ -casein; lane 6, α_{s1} -casein; lane 7, α_{s2} -casein.

casein, suggesting that the measured radioactivity originated from small amounts of unidentified proteins. The elution order of β -, κ -, α_{s2} - and α_{s1} -casein is consistent with that presented by St-Martin and Paquin (1990) for the separation of whole casein on a Mono S column using a non-denaturing detergent. The present findings demonstrate that the multimers are composed of monomeric κ -casein chains and can range from a monomer to larger than a decamer.

The disulfide-bonding pattern

To assign the interchain disulfide bridges in κ -casein multimers, pool A1 isolated from [^{14}C]carboxymethylated bulk milk was digested with pepsin and the resulting peptides were separated by HPLC (Fig. 5A). Amino acid analysis of performic-acid-oxidized samples from each peak showed that cysteic acid was primarily present in five major peaks, P1–5 (Fig. 5B). These cross-linked peptides were, after further purification by reversed-phase HPLC, analyzed by MS and the combined results are shown in Table 1. Identical HPLC elution and cysteic-acid profiles were obtained from peptic degradation of pools A1–5 isolated from untreated bulk milk as well as from the individual milk sample (data not shown).

Based on PDMS before and after reduction, P1 appeared to contain a dimer of residues 80–95 in κ -casein linked by a disulfide bond between the two Cys88 residues. To verify the assignment of the sequence, a portion of P1 was digested with trypsin, and the resulting peptides were separated by HPLC. Amino acid analysis showed that only one peak, P1T1, contained cysteic acid (data not shown). Sequence analysis of P1T1 with the intact disulfide bridge revealed two identical disulfide-linked peptides, corresponding to residues 87–95 resulting from a cleavage after Lys86, as would be expected (Fig. 6).

PDMS of P2 (Fig. 7A) showed a single-charged (MH^+ 3821.4 Da) and a double-charged (MH_2^{2+} 1911.6 Da) molec-

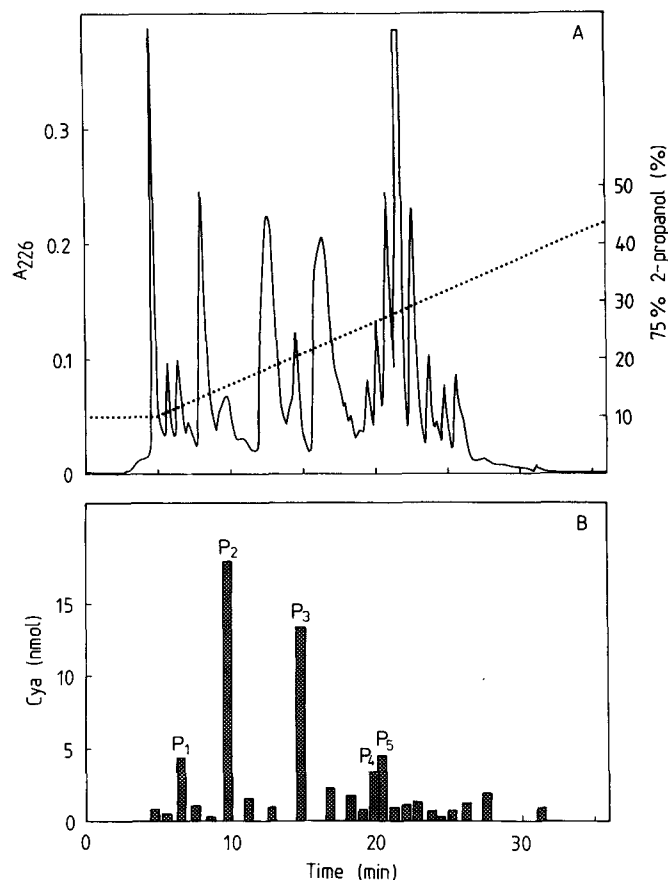
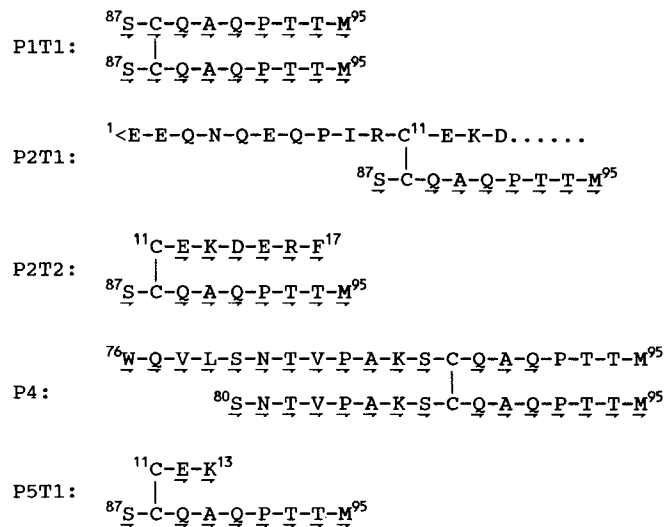


Fig. 5. Reversed-phase HPLC separation of peptides obtained from multimers of κ -casein by digestion with pepsin and the corresponding cysteic acid profile. (A) Reversed-phase HPLC of the peptic digest of pool A1 (Fig. 1). Peptides were eluted with a linear gradient of 75% 2-propanol in 0.1% trifluoroacetic acid (·····) on a Vydac C_{18} (10 μm) column (4 mm $\times$ 250 mm). The flow rate was 0.85 ml/min and the oven temperature was 40°C. Peptides were detected in the effluent by recording the absorbance at 226 nm (—). (B) Cysteic acid (Cya) profile. Total amount of cystine in each peak was determined as cysteic acid.

ular mass, which indicated that P2 consisted of residues 1–17 and residues 80–95. The spectrum, in addition to the MH^+ and MH_2^{2+} ions of the linked peptides, also contained a fragment ion (MH^+ 2160.5 Da) corresponding to one of the peptides (residues 1–17). Fragment ions in PDMS of disulfide-linked peptides are often present due to cleavage of the disulfide bonds and support the identity of the peptides (Sørensen et al., 1990). *In situ* reduction of P2 with dithiothreitol (Fig. 7B) resulted in two MH^+ ions at m/z 1666.5 Da and m/z 2161.6 Da, corresponding to residues 80–95 and 1–17, respectively, confirming the presence of a disulfide bridge connecting the two peptides. Tryptic degradation of P2 followed by amino acid analysis of the separated peptides showed two cystine-containing peptides, P2T1 and P2T2. Sequence analysis of P2T1 yielded only one sequence, corresponding to residues 87–95 (Fig. 6). P2T2 contained the same peptide but joined to residues 11–17. Since the N-terminal residue in κ -casein has been found to be pyroglutamate (Mercier et al., 1973), consistent with the present MS data, it was concluded that P2T1 was a cleavage variant of P2T2 resulting from an incomplete cleavage at Arg10.

Table 1. Localization of interchain disulfide bridges in κ -casein multimers by MS analysis. The peak numbering is as presented in Fig. 5B. n.d., not determined.

Peak	Expected molecular mass Da	Observed molecular mass (MH ⁺)		Disulfide-linked peptides
		native	reduced	
P1	1661.9 + 1661.9 = 3323.8	3327.6	1663.9	80–95/80–95
P2	2157.3 + 1661.9 = 3819.2	3821.4	2161.6 1666.5	1–17/80–95
P3	2157.3 + 2157.3 = 4314.6	4322.7	2159.7	1–17/1–17
P4	1661.9 + 2188.5 = 3850.4	3853.4	n.d.	76–95/80–95
P5	2157.3 + 2188.5 = 4345.8	4350.5	2190.6	1–17/76–95

**Fig. 6. Amino acid sequence analysis.** The tryptic peptides P1T1, P2T1/P2T2 and P5T1 were obtained from P1, P2 and P5, respectively. The N-terminal residue in P2T1 is a pyroglutamate (<E). (→) Positive identification of the amino acids in the respective steps.

PDMS of P3 before and after reduction with dithiothreitol (Table 1) indicated that P3 contained two identical peptides corresponding to residues 1–17 in κ -casein linked by one disulfide bond. The MS data showed that P4 was a cleavage variant of P1, since it contained the Cys88–Cys88 bond. Sequence analysis (Fig. 6) confirmed the assigned sequence and showed that residue 81 was an asparagine instead of an aspartate (Mercier et al., 1973) in agreement with the sequence deduced from cDNA (Stewart et al., 1984) and the genomic DNA sequence (Alexander et al., 1988). Likewise, P5 was a cleavage variant of P2 consisting of residues 1–17 joined to residues 76–95 resulting from an incomplete cleavage at Leu79 in one of the chains. Due to suppression effects (Roepstorff et al., 1988) only one molecular mass (MH⁺ 2190.6 Da) corresponding to residues 76–95 was observed in the mass spectrum of P5 after reduction with dithiothreitol. The identities of the cross-linked peptides were further verified by tryptic degradation of P5 followed by sequence analysis of one of the resulting cystine-containing peptides, P5T1 (Fig. 6).

Assuming an identical recovery from the HPLC column, the mean percentage ($\pm$ SD) of the three linkages obtained from peptic degradation of pool A1 from [¹⁴C]carboxymethylated and untreated bulk milk was $19.8 \pm 2.9\%$ of Cys11–Cys11, $48.8 \pm 3.8\%$ of Cys11–Cys88 and $31.5 \pm 0.9\%$ of Cys88–Cys88. This demonstrates that all three types of linkage are commonly involved in the assembly of monomers to form large multimers.

DISCUSSION

The present data demonstrates that κ -casein in the native state is composed of monomeric κ -casein chains and can range from a monomer to multimers larger than a decamer. Analysis of κ -casein by means of SDS/PAGE showed a number of bands forming a ladder of higher molecular mass, which upon reduction were replaced by a single band of lower molecular mass. As these bands are evenly spaced and have the same relative intensity, they fit a pattern of a series of multimers composed of a regularly increasing number of identical subunits. This is in general agreement with early sedimentation-equilibrium studies (Swaigood et al., 1964) where κ -casein was shown to occur as a disulfide-linked polymer in a wide distribution of sizes, occasionally including monomers (Talbot and Waugh, 1970).

The two common genetic variants of κ -casein, A and B, have two cysteine residues located at positions 11 and 88 (Mercier et al., 1973). Hence, three possible disulfide-bonding patterns exist, as shown in Fig. 8. By a combination of MS measurements and amino acid sequence analysis, three types of interchain disulfide linkages were assigned: Cys11–Cys11, Cys11–Cys88 and Cys88–Cys88. All three disulfide bridges were found in the different sizes of κ -casein multimers (pools A1–5) isolated from [¹⁴C]carboxymethylated and untreated bulk milk. Since the milk probably contained κ -casein of both genotype A and B, a milk sample from a Jersey cow typed κ^{BB} was also investigated. Among the different sizes of multimers all three types of disulfide bonds were found.

These findings eliminate both the disulfide-bonding patterns in Fig. 8A and B as the only disulfide-bonding pattern in large multimers. It is concluded that the monomers are assembled into multimers of different sizes and that the multimerization occurs in a random or at present unpredictable disulfide-bonding pattern, regardless of the size of the multimer or the genotype (Fig. 8C). Although it is unlikely, the co-existence of the disulfide-bonding patterns shown in

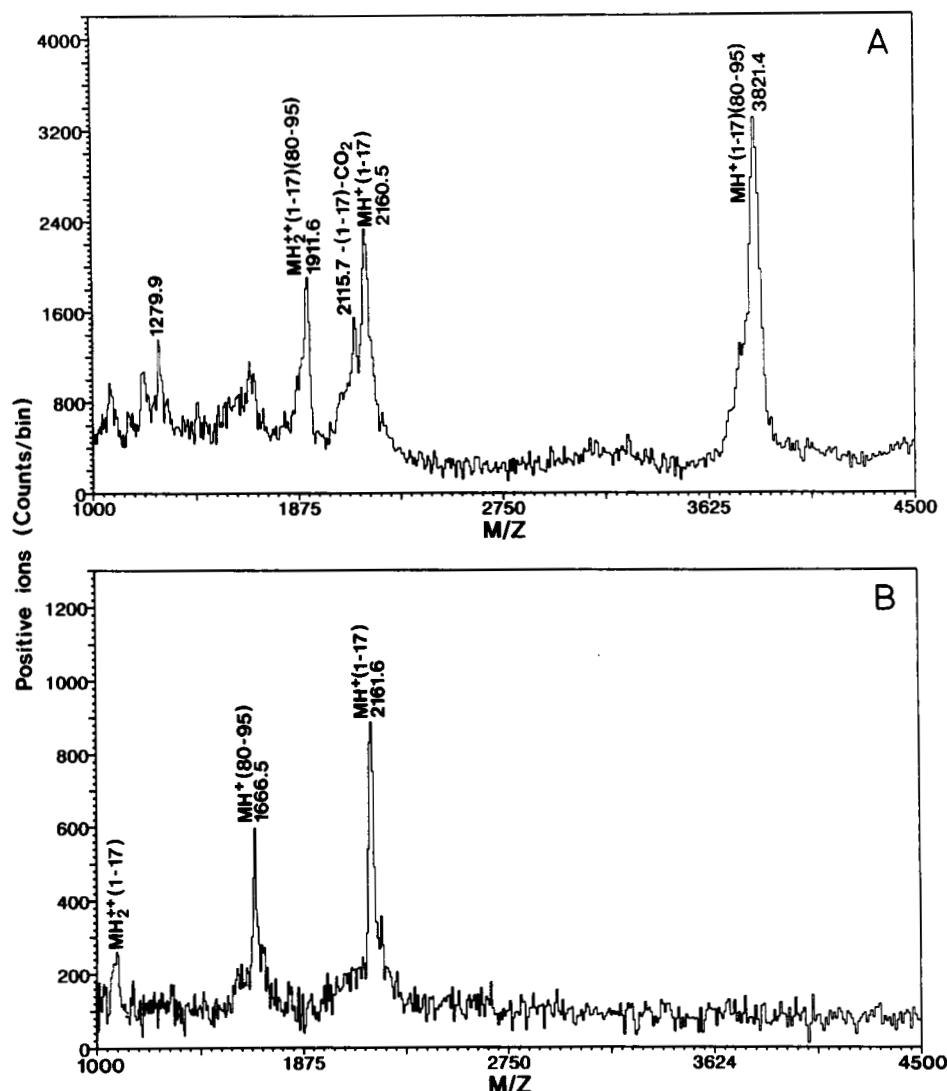


Fig. 7. PDMS spectra of P2. (A) The MH^+ at m/z 3821.4 Da corresponds to residues 1–17 joined to residues 80–95 in κ -casein. The peak at m/z 2160.5 Da is a fragment ion and corresponds to residues 1–17. The peaks at m/z 1279.9 Da and 2115.7 Da arise from an unidentified impurity and loss of CO_2 from residues 1–17, respectively. (B) *In situ* reduction of P2 by dithiothreitol shows two MH^+ , 2161.6 Da and 1666.5 Da, which correspond to residues 1–17 and 80–95, respectively.

Fig. 8A and B is a theoretical possibility. The shape of the multimer may exist as an unbranched chain or a closed loop whereas a branched chain is not possible due to the presence of only two cysteine residues in the protein. No free thiol groups are present among the various sizes of κ -casein multimers as judged by [^{14}C]carboxymethylation of bulk milk as well as whole casein under denaturing conditions. This suggests that the two cysteine residues in the terminal subunits either form an interchain disulfide linkage or that small thiol-containing compounds are attached to them. Recently, a three-dimensional molecular modeling of bovine κ -casein (Kumosinski et al., 1991) has predicted that the two cysteine residues are located near the surface of the molecule directed away from each other. This could account for the presence of all three types of interchain disulfide linkages in multimeric κ -casein.

α_{s2} -casein, which like κ -casein contains two cysteine residues, exists both as monomers and dimers (Annan and

Manson, 1969). The assembly of the polypeptide chains in α_{s2} -casein dimer occurs more or less at random since both parallel and antiparallel orientation of the chains have been found (Rasmussen et al., 1992). However, interchain disulfide bridges between α_{s2} -casein and κ -casein have not been found, which suggests that the organisation of disulfide bonds in the casein micelle are not totally random.

The oligomeric nature of κ -casein described here is remarkable. Among other well-known multimeric proteins, nearly all are composed of a fixed number of identical subunits, e.g. thrombospondin and immunoglobulins. However, two proteins from blood platelets, multimerin (Hayward et al., 1991) and vWF (Wagner et al., 1987; Dent et al., 1991), and a recently described protein from skeletal-muscle triadic vesicles, triadin (Caswell et al., 1991), are unique in that like κ -casein, they exist in the native state as disulfide-linked multimers in various sizes. In triadin, the multimers, ranging up to 680 kDa, appear to be composed of monomers of molec-

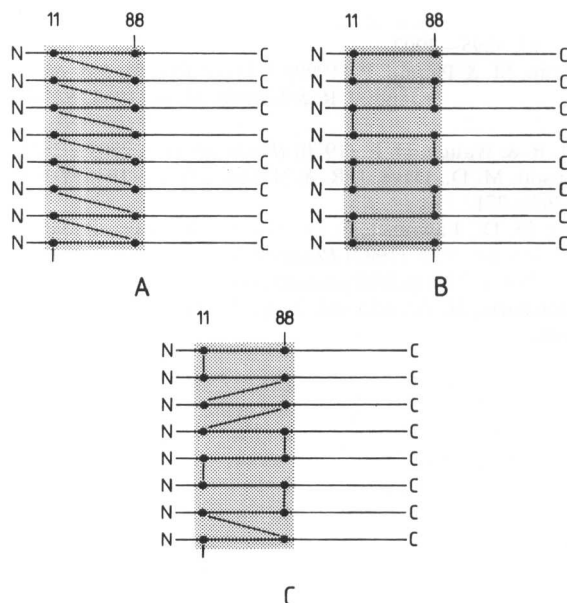


Fig. 8. Schematic representation of possible disulfide-bonding patterns in multimers of κ -casein. (A) Regular pattern involving one type of interchain disulfide linkage (Cys11–Cys88). (B) Regular pattern involving two types of interchain disulfide linkage (Cys11–Cys11 and Cys88–Cys88). (C) Random or unpredictable pattern involving three types of interchain disulfide linkage. Since three types of interchain disulfide bridges (Cys11–Cys11, Cys11–Cys88 and Cys88–Cys88) are found in the multimers, the disulfide-bonding pattern of κ -casein is best represented by C. (●) Cysteine.

ular mass 95 kDa and thus it resembles κ -casein in its multimeric composition. The protomers of vWF are dimers which can be assembled in head-to-head and tail-to-tail fashion forming extremely large multimers with molecular mass exceeding 10 MDa. Multimerin, another recently described blood platelet protein also forms very large multimers where the smallest multimer has been proposed to be a trimer composed of three 155-kDa subunits (Hayward et al., 1991). The vWF participates in hemostasis by mediating the adhesion of platelets to exposed subendothelium leading to the formation of the platelet plug during vascular damage. Several functional domains of vWF, which include binding domains for heparin, collagen, factor VIII and platelets, have been identified (Ruggeri and Zimmerman, 1987). It has been reported that disassembly of the multimers produces essentially monovalent protein with platelet-binding properties similar to that of the smallest multimer (Chopek et al., 1986). Hence, the physiological significance of the multimeric nature of vWF, and probably multimerin, is to provide multiple functional sites for binding to the platelet surface and for binding to other ligands (Chopek et al., 1986; Ruggeri and Zimmerman, 1987; Hayward et al., 1991).

A functional aspect of κ -casein is to stabilize the micelle structure in milk. Several studies including ^1H -NMR spectroscopy (Griffin and Roberts, 1985; Rollema et al., 1988) and electron microscopy (Carroll and Farrell, 1983) indicate that κ -casein is located mainly at the micelle surface. Early observations suggest that monomeric κ -casein is able to interact with α_s -casein and β -casein although it has decreased stabilizing properties. Thus, interchain disulfide bonds do not seem to be required in interactions between κ -casein and the

other caseins (Talbot and Waugh, 1970). The present findings support these considerations, since it seems unlikely that a random disulfide-bonding pattern would exist if interchain disulfide bridges were essential for interaction specificity. In addition, a functionally important role for the cysteine residues is not indicated from comparative sequence studies. In sheep and goat three cysteines are present in positions 10, 11 and 88 (Jollès et al., 1974; Mercier and Chobert, 1976) whereas only one cysteine is present in mouse, rat, and human (Thompson et al., 1985; Nakhasi et al., 1984; Brignon et al., 1985). Thus, the multimeric nature of bovine κ -casein seems to facilitate its role in covering the micelle surface, thereby stabilizing the micelle structure.

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