

Disulphide bonds in casein micelle from milk

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Abstract

Mammary epithelial cells synthesised and secreted caseins, the major milk proteins in most mammals, as large aggregates called micelles into the alveolar lumen they surround. We investigated the implication of the highly conserved cysteine(s) of κ -casein in disulphide bond formation in casein micelles from several species. Dimers were found in all milks studied, confirming previous observation in ruminants. More importantly, the study of interchain disulphide bridges in mouse and rat casein micelles revealed that any casein possessing a cysteine is engaged in disulphide bond interchange; these species express four or five cysteine-containing caseins, respectively. We found that the main rodent caseins form both homo- and heterodimers. Additionally, disulphide bond formation among milk proteins was specific since the interaction of the caseins with cysteine-containing whey proteins was not observed in native casein micelles. © 2006 Elsevier Inc. All rights reserved.

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During lactation, mammary epithelial cells synthesise and secrete huge quantities of milk specific proteins and other components such as lipids and lactose. Except in primates, the main milk proteins synthesised by mammary epithelial cells are the caseins, a family of acidic phosphoproteins (α_{S1} , α_{S2} , β , and κ). Caseins are found in milk as aggregates, the casein micelles (for review, see [1]). The aggregation of the caseins into micelle is initiated in the trans cisternae of the Golgi apparatus and proceeds in secretory vesicles during transport to the apical surface [2]. However, the specific arrangement of the caseins in the micelle has not yet been established and much is still to be learned about the role of the individual casein in the aggregation process. Moreover, the possible role of casein aggregation in their transport in the secretory pathway has not directly been investigated. We have shown that α_{S1} -casein is required for the efficient transport of the other caseins from the endoplasmic reticulum to the Golgi apparatus [3]. We think that, by virtue of interacting with the

other caseins, α_{S1} -casein promotes their export to the Golgi. κ -Casein, which is O-glycosylated, is believed to be mainly located at the micelle surface where it might play a role in regulating their size and in maintaining them in suspension in milk [4]. In all species yet studied, this casein contains at least one cysteine close to its amino-terminus, whereas the other caseins often lack cysteine. Another cysteine in κ -casein is found in ruminants, close to the chymosin cleavage site. It is tempting to speculate that κ -casein multimerisation plays a role in casein micelle formation, since these cysteines are highly conserved between species and because disulphide bond formation occurs in the endoplasmic reticulum. However, results concerning the participation of cysteines in casein multimers have only been obtained from the milk of human and cow (for review, see [5]). Hence, the relevance of these studies for the aggregation of caseins and micelle formation in the secretory pathway remains to be established. We therefore wished to generalise these observations to κ -casein from other species and investigated whether other, if not all, caseins could be engaged in disulphide bond interchanges when they contain at least one cysteine.

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Fig. 1. Cysteine residues in the primary sequence of κ -caseins. The κ -casein sequences from the indicated species were aligned and domains containing the conserved cysteine residues are shown. Residues are numbered from the first of the mature chain. Sequence identities between series of species are marked with * underneath and residues which have similar or identical chemical features are labelled with . or :, respectively. The last lane below shows overall conservation through all species.

Table 1
Cysteine residues in caseins

Species	α_{S1} -Casein	α_{S2} -Casein	β -Casein	κ -Casein
Rabbit*				10
Pig		37, 41		11
Goat*		37, 41		10, 11, 88
Sheep		37, 41		10, 11, 88
Bovine		36, 40		11, 88
Camel		37, 41		10, 76
Human	60, 84, 89			10
Mouse*	106	80, 81 (γ)	68	9
Rat*	109	81 (γ); 46, 146 (δ)	66	9

We show species for which complete primary sequence of, at least, the four main caseins are known. The mouse ϵ -casein has no cysteine. Asterisks indicate species analysed in the present study.

complete primary sequence is known. This is exemplified in Fig. 1 for a limited number of κ -casein sequences of interest. In ruminants, horses, and camels, an additional cysteine residue is found more or less close to the chymosin cleavage site. There is no such conservation of cysteine residues throughout mammals for the other caseins. Except for α_{S2} -casein, the presence of cysteine in their sequence is a somewhat rare event (Table 1). These observations provided the basis to investigate whether disulphide bond between κ -casein molecules, a protein modification previously observed at least in bovine milk [12,13], is a general phenomenon taking place in most, if not all, species. With this aim we compared the electrophoretic mobilities of proteins from fresh, partially skimmed milk which

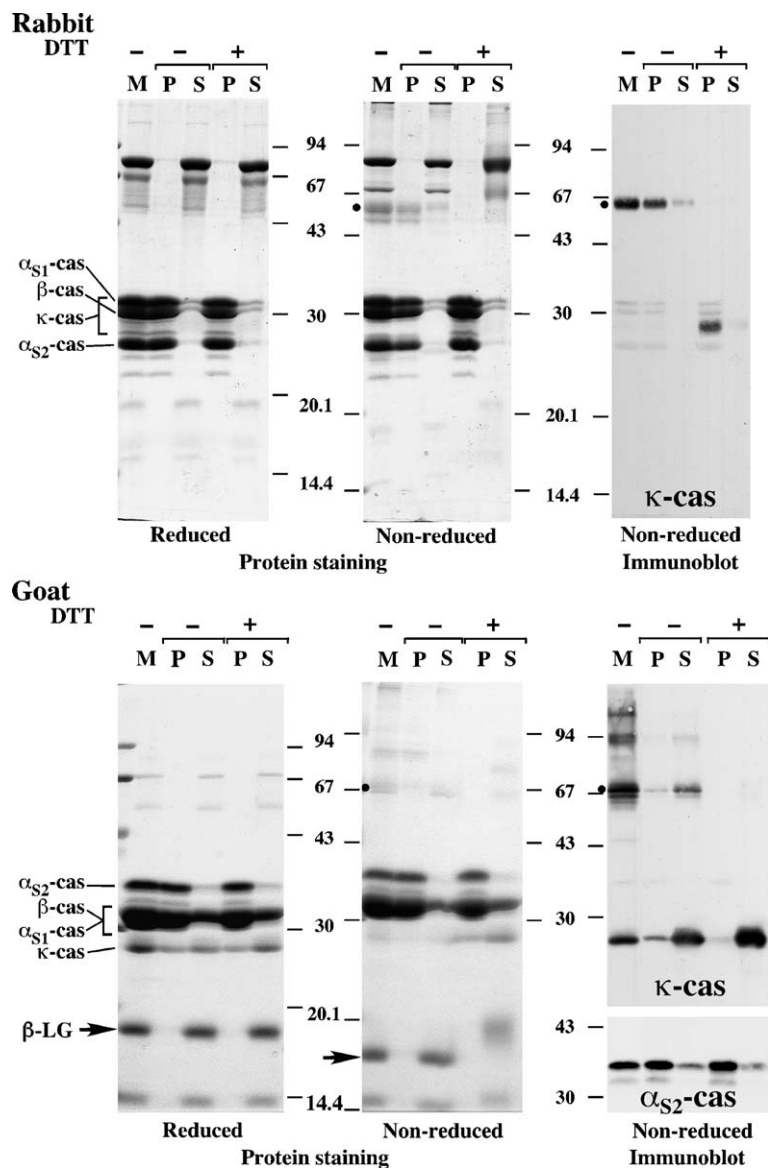


Fig. 2. Milk κ -casein is dimeric. Aliquots of the aqueous phase of rabbit or goat milk were either added to electrophoretic buffers or diluted in the absence (–) or the presence (+) of 5 mM DTT, incubated at room temperature, and centrifuged. Proteins from milk (M), milk pellet (P), and supernatant (S) were boiled in the absence or the presence of β -mercaptoethanol and analysed by SDS–PAGE, followed by protein staining or immunoblotting with either anti-rabbit κ -casein or anti-goat κ -casein (top panel in Goat) and then anti-goat α_{S2} -casein (bottom panel in Goat). κ -cas, κ -casein; α_{S2} -cas, α_{S2} -casein; β -LG, goat β -lactoglobulin. Dots, κ -casein dimer. For each species, the experiment was repeated at least three times.

were subjected or not to reduction. In order to analyse the disulphide bonds in casein micelles more specifically, milk proteins were diluted at physiological ionic strength, incubated (see below), and casein micelles were pelleted by centrifugation. The possible effect of thiol reducing agent on casein micelle structure was analysed by incubating the diluted milk proteins in the presence of dithiothreitol (DTT) at room temperature before centrifugation. This analytical procedure was applied to the milk of the species indicated by an asterisk in Table 1 and to bovine, sheep, and human milks (data not shown). Rabbit and goat casein assignment in SDS-PAGE was based on previously published works [14,15,3] and on our present immunoblotting data (see Fig. 2).

Among the species for which we have entirely known the sequence of each of the caseins, rabbit is the sole animal possessing only one cysteine-containing casein, κ -casein, which has one cysteine residue (Fig. 1). When rabbit milk proteins were analysed by SDS-PAGE in non-reducing conditions, compared to samples heated in the presence of β -mercaptoethanol (Reduced in Fig. 2), an additional smeary band was clearly observed at M_r of ≈ 63 –65,000 (Fig. 2, Rabbit, Non-reduced, dots). This band was essentially present in the casein micelle fraction. When milk proteins were incubated with 5 mM DTT prior to centrifugation, the amount of casein recovered in the pellet was not affected (Fig. 2, Rabbit, Reduced, +). However, after SDS-PAGE analysis of DTT-treated samples in

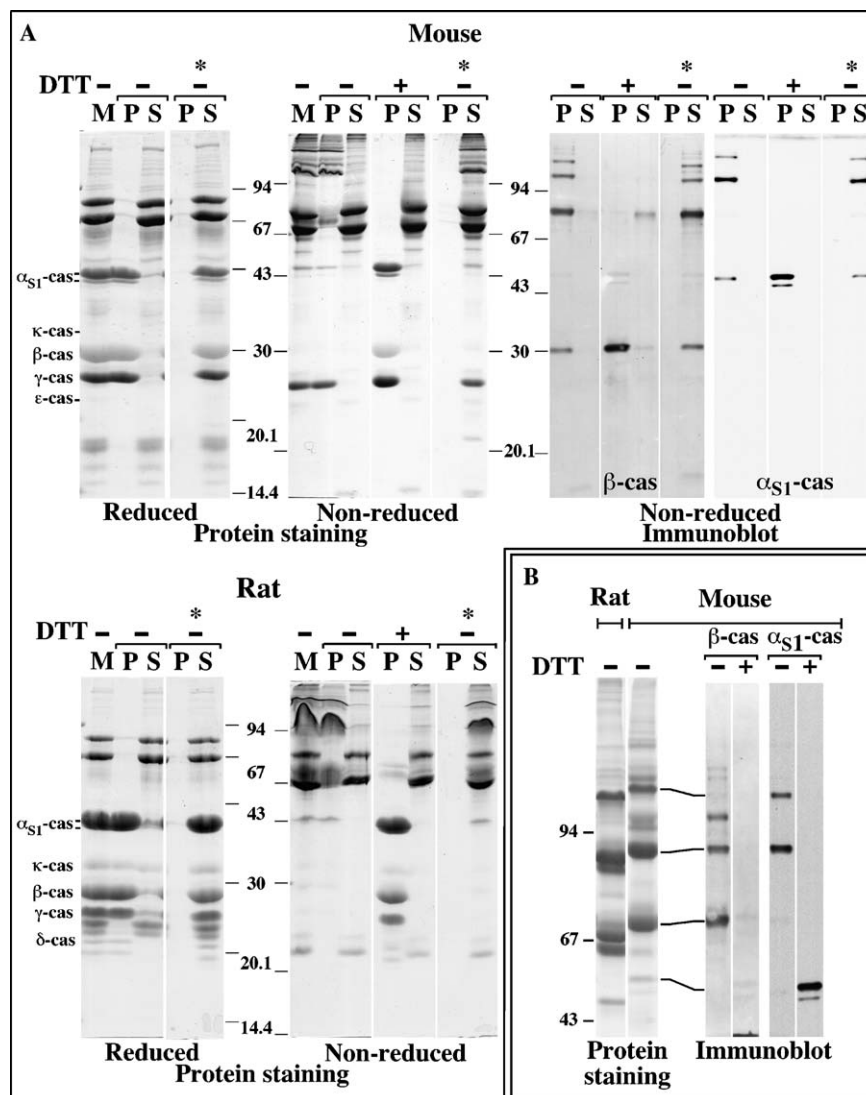


Fig. 3. All cysteine-containing caseins can form disulphide bonds. (A) Aliquots of the aqueous phase of mouse or rat milk were incubated and analysed as described in Fig. 2, except that milk proteins were also incubated in the presence of both EDTA and Tween 20 (*). Immunoblotting was done with antibodies directed against either mouse β -casein or mouse α_{S1} -casein. For each species, the experiment was repeated four times. The electrophoretic mobilities of mouse κ and ϵ -casein are indicated, although the relative proportion of these proteins in milk did not allow direct visualisation on the gels presented in this figure. (B) Material recovered in the casein pellet was analysed by SDS-PAGE in the absence of β -mercaptoethanol using 7.5% gels, followed by protein staining or immunoblotting. β -cas, β -casein; α_{S1} -cas, α_{S1} -casein.

non-reducing conditions, the M_r 63–65,000 band disappeared, demonstrating an effect of the thiol reducing agent on this molecular form (Fig. 2, Rabbit, Non-reduced, +). The M_r 63–65,000 molecular form of micellar protein reacted with an antiserum raised against rabbit κ -casein (Fig. 2, Rabbit, Immunoblot, dot). Indeed, after reduction for SDS–PAGE analysis (data not shown) or by DTT treatment, the signal was found at the level of the M_r 28,000 monomeric forms of κ -casein. The presence of κ -casein in the M_r 63–65,000 band was confirmed by mass spectrometry (MS) analysis (data not shown). We concluded that in rabbit milk, κ -casein is dimeric.

The same experimental conditions were applied to goat milk which contains two cysteine-containing caseins, α_{S2} - and κ -casein (Table 1). In contrast to our finding with rabbit milk, a large proportion of certain goat casein, notably of κ -casein, was found in supernatants after incubation and centrifugation of the diluted milk. In contrast, almost all α_{S2} -casein was recovered in the pellets (Fig. 2, Goat). This reflected the well-known fact that there is a substantial amount of non-micellar casein in goat milk [16]. One additional explanation for this result is that dilution of milk with NaCl-containing buffer induces some dissociation of the casein micelles. However, we noted that this was not the case for rabbit casein micelles and, as will be shown below (see Fig. 3), for the milk of rodents. On the other hand, analysis in non-reducing conditions revealed at least two additional smeary bands of $M_r \approx 67,000$ and 82,000, the amount of which largely decreased upon DTT treatment. Concomitantly, the M_r 28,000 band corresponding to the monomeric κ -casein increased significantly in DTT treated samples. Immunoblotting also showed that the bands of $M_r \approx 67,000$ indeed contained κ -casein (Fig. 2, Goat, Immunoblot, dot) and, most likely, consisted of κ -casein dimers. Moreover, the antibody also reacted with a M_r 94,000 band which might be homotrimeric κ -casein, as previously envisioned (see Table 1 in [5]). The M_r 82,000 band most likely corresponded to some α_{S2} -casein dimers, a molecular form of the protein already observed in the milk of cow [17]. The presence of κ -casein in the M_r 67,000 band and of α_{S2} -casein in the M_r 82,000 band from goat samples (as well as of α_{S2} -casein in an equivalent of the later band obtained with cow milk) was confirmed by MS analysis (data not shown). However, the relative proportion of multimeric caseins that we found in goat milk with this approach was clearly quite low. This observation was in striking contrast to that made with rabbit and rodents (see below).

As to human milk, our preliminary experiments were in agreement with previous observations of the presence of disulphide bridges between κ - and α_{S1} -casein [18]. Notably, monomeric α_{S1} -casein was only observed after disulphide bridges' reduction (data not shown).

Since any of the cysteine-containing caseins studied so far was demonstrated to be engaged in homo- or heteropolymers formation using disulphide bridges, it was important to determine whether or not all caseins can be

cross-linked via disulphide bonds, as they possess a cysteine. To investigate this possibility, we studied the milks of mouse and rat which contain four and five cysteine-containing caseins, respectively (Table 1).

As previously observed in other species, rodent caseins behave anomalously upon SDS–PAGE. Their electrophoretic mobilities are markedly higher than expected from their real molecular weights (compare M_r and M_w in Table 2). For this reason, and also because we may cope with the high heterogeneity of the caseins due to genetic variants or other events such as post-translational modifications, we decided to identify precisely each of the casein bands obtained in our experimental conditions. With this aim, casein micelle pellets were prepared from the rodent milks as described above, i.e., after dilution and incubation followed by centrifugation, and analysed by SDS–PAGE in reducing conditions. The identification of the protein contained in the indicated portion of the gels (see Fig. 3, Reduced, lanes P. Note that in the analysis presented here for mouse milk, κ -casein, and ϵ -casein are not visible) was based on MALDI-MS peptide mass fingerprinting. Mass spectra analysis produced the results displayed in Table 2. These data were used for the characterisation of the casein multimers (see below).

Fig. 3A, top panel, shows that only γ -casein was found, at least in part, as a monomer when mouse milk caseins were not reduced. As a result, the top of the gel contained many more proteins than that observed in reducing conditions. As expected, monomeric forms of the other caseins were recovered after DTT treatment and the amount of γ -casein was also higher, as compared to samples not treated with the reducing agent (Fig. 3A, top panel, compare P– and P+). The specific behaviour of α_{S1} - and β -casein was investigated by immunoblotting. The antibodies raised against mouse β -casein recognised multiple bands of high

Table 2
Rodent casein identification in SDS–PAGE protein pattern

Species	M_r (Da) ^a	Protein identity	Accession number ^b	M_w (Da) ^c
Mouse	42,000	α_{S1} -Casein	P19228	34,042
	41,000	α_{S1} -Casein	P19228	34,042
	33,000	κ -Casein	P06796	17,701
	30,000	β -Casein	P10598	23,779
	27,000	γ -Casein	Q02862	19,446
	25,000	ϵ -Casein	P02664	15,309
Rat	39,000	α_{S1} -Casein	P02661	30,276
	38,000	α_{S1} -Casein	P02661	30,276
	32,000	κ -Casein	P04468	17,187
	28,000	β -Casein	P02665	23,811
	25,000	γ -Casein	P02667	18,649
	21,000	δ -Casein	Q8CGR3	18,244

A casein fraction was prepared from rodent milks and analysed by SDS–PAGE in reducing conditions. Identification of the bands was based on MALDI-MS peptide mass fingerprinting of tryptic digests.

^a See reduced gels in Fig. 3.

^b Swiss-Prot/TrEMBL accession number.

^c On the basis of the amino acid sequence (mature chain) of the most frequent variant.

M_r in addition to the monomeric form of the protein (Fig. 3, Mouse, Immunoblot). One possible explanation for the latter finding is that, in addition to the potential β -casein homodimers, heterodimers of β -casein with other cysteine-containing caseins would form. In line with this, the antibodies directed against mouse α_{S1} -casein recognised two high M_r bands, one co-migrating with a molecular form that contained β -casein. Finally, when milk proteins were incubated in the presence of EDTA and the non-ionic

detergent Tween 20, the caseins were recovered in the supernatant after centrifugation (Fig. 3A, Mouse, *). This indicated that the high degree of casein cross-linking in mouse casein micelle does not impair casein micelle destruction by classical means, such as calcium chelation. In line with the above results, analysis of rat caseins revealed that, consistent with their cysteine content, all caseins were in homo- or heterodimeric forms in native casein micelles and migrated at the top of the gel, consistent with

Table 3
Identification of casein multimers in rodent casein micelle

Species	M_r (Da) ^a	Peptides found/expected (% chain coverage) ^b	Protein identified as (SWISS-PROT Accession number)	Protein identity by SDS-PAGE ^c	Likely dimers
Mouse	136,000	6/21 (27) 3/11 (14)	α_{S1} -Casein (P19228) β -Casein (P10598)	ND	—
	122,000	6/21 (27) 3/11 (14) 3/17 (15)	α_{S1} -Casein (P19228) β -Casein (P10598) γ -Casein (Q02862)	α_{S1} -Casein β -Casein γ -Casein	
	114,000	5/21 (20)	α_{S1} -Casein (P19228)	α_{S1}-Casein (κ -Casein)	α_{S1} – α_{S1} or α_{S1} – κ
	98,000	5/21 (20) 3/11 (14)	α_{S1} -Casein (P19228) β -Casein (P10598)	β-Casein (γ -Casein)	
	94,000	5/21 (20) 3/9 (12) 2/11 (13) 3/17 (15)	α_{S1} -Casein (P19228) κ -Casein (P06796) β -Casein (P10598) γ -Casein (Q02862)	β -Casein γ -Casein	
	88,000	5/21 (20) 3/11 (15)	α_{S1} -Casein (P19228) β -Casein (P10598)	α_{S1}-Casein β-Casein	α_{S1} – β
	71,000	3/11 (14) 4/17 (22)	β -Casein (P10598) γ -Casein (Q02862)	β-Casein γ -Casein	β – γ
	57,000	6/21 (27)	α_{S1} -Casein (P19228)	α_{S1} -Casein	—
Rat	112,000	7/21 (25)	α_{S1} -Casein (P02661)	α_{S1} -Casein	α_{S1} – α_{S1}
	87,000	6/21 (22) 3/10 (25) 3/13 (12)	α_{S1} -Casein (P02661) κ -Casein (P04468) β -Casein (P02665)	α_{S1} -Casein β -Casein	α_{S1} – β
	84,000	6/21 (22) 2/13 (12)	α_{S1} -Casein (P02661) β -Casein (P02665)	α_{S1} -Casein β -Casein (γ -Casein)	α_{S1} – β + α_{S1} – γ
	81,000	5/21 (17) 5/10 (29)	α_{S1} -Casein (P02661) κ -Casein (P04468)	κ -Casein	κ – κ
	70,000	4/21 (17) 5/10 (29) 4/13 (14)	α_{S1} -Casein (P02661) κ -Casein (P04468) β -Casein (P02665)	κ -Casein β -Casein	κ – κ + β – β or β – κ
	67,000	4/21 (17) 4/13 (14)	α_{S1} -Casein (P02661) β -Casein (P02665)	(γ -Casein) β -Casein	β – β
	62,000	4/13 (14)	β -Casein (P02665)	β -Casein (γ -Casein)	β – γ
	50,000	6/21 (22)	α_{S1} -Casein (P02661)	α_{S1} -Casein	—

Casein fractions were prepared from rodent milks and analysed by SDS-PAGE in non-reducing conditions. Identification of the proteins contained in the indicated bands was based on MALDI-MS peptide mass fingerprinting of tryptic digests.

^a See non-reduced 7.5% gels in Fig. 4.

^b Calculated for the mature chain.

^c In parentheses, protein not found by MS analysis; in bold, protein also detected by immunoblotting. ND, not determined.

their cysteine content, whereas, indeed, monomers were obtained by DTT treatment (Fig. 3A, Rat, Non-reduced). As observed above in mouse, EDTA treatment in the presence of detergent also solubilised the rat micelle edifice (Fig. 3, Rat, *). Rat and mouse casein dimers were resolved further by SDS–PAGE using 7.5% gels (Fig. 3B). Under these conditions, the main bands migrated at lower M_r , but still much higher than could be calculated from their monomeric form M_r , as estimated on 13% gels. However, it should be noted that monomeric mouse α_{S1} -casein had a M_r of $\approx 57,000$ in these electrophoretic conditions

(Fig. 3B, Immunoblot). This analysis also confirmed the strict co-migration of α_{S1} - and β -casein in a band of $M_r \approx 88,000$.

Having established that κ -casein from all the species studied here (and by others) forms multimers which are cross-linked via disulphide bonds, and that all cysteine-containing caseins do so, we wished to determine whether those multimers are preferentially homo- or heteropolymers. We attempted to study this issue by peptide mass fingerprinting. However, while mass spectrometric analysis of gel eluted tryptic peptides allowed to identify proteins in all treated bands, certain caseins were poorly represented, e.g., κ -casein in mouse, or not found at all, like γ -casein in rat (Table 3). Moreover, α_{S1} -casein peptides were recovered from almost all bands. It was therefore necessary to identify further the content of the bands by using an alternative approach. With this aim, the proteins contained in the polyacrylamide gel bands obtained after SDS–PAGE analysis of the casein fraction in non-reducing conditions (Fig. 4, 7.5% gels) were reduced, extracted from the gel pieces by passive elution, and resolved by SDS–PAGE analysis in reducing conditions (Fig. 4, 13% gel). As expected, most of the bands were found to contain two caseins. Three caseins were occasionally observed. This was the case with the material prepared from the M_r 112,000 and 84,000 bands from mouse and rat milk, respectively. The results obtained with this approach were, however, consistent with the data obtained by MS or by immunoblotting. Moreover, it allowed the identification of the γ -casein-containing bands in rat. On the other hand, we cannot explain why α_{S1} -casein derived peptides were almost systematically recovered in the peptide mass fingerprinting analysis. Finally, it is worth noting that we never detected whey proteins in those multimeric molecules prepared from the native casein micelles, whatever the method used.

Altogether, these results suggest that, in native casein micelles, most of the molecular forms consist of dimers that contain essentially two different caseins. In Table 3, we attempted to define the most likely homo- and heterodimers contained in rodent milk casein micelles.

Discussion

Previous work has established that bovine κ -casein molecules have the capability to cross-link covalently via cysteine since disulphide-bonded κ -casein polymers were found in milk [12,13]. In the present study, we have investigated this type of bonding between caseins in several other species. From all these results, one can conclude that intermolecular disulphide bonds between κ -casein might form in most, if not all, species. However, in contrast to previous reports, we found that the majority of κ -casein from ruminants, notably from goat, formed homodimers. Only few amounts of potential trimers and tetramers were found in this species, when non-reduced proteins were analysed by immunoblot. One should note that the relative proportion of κ -casein engaged in interchain disulphide

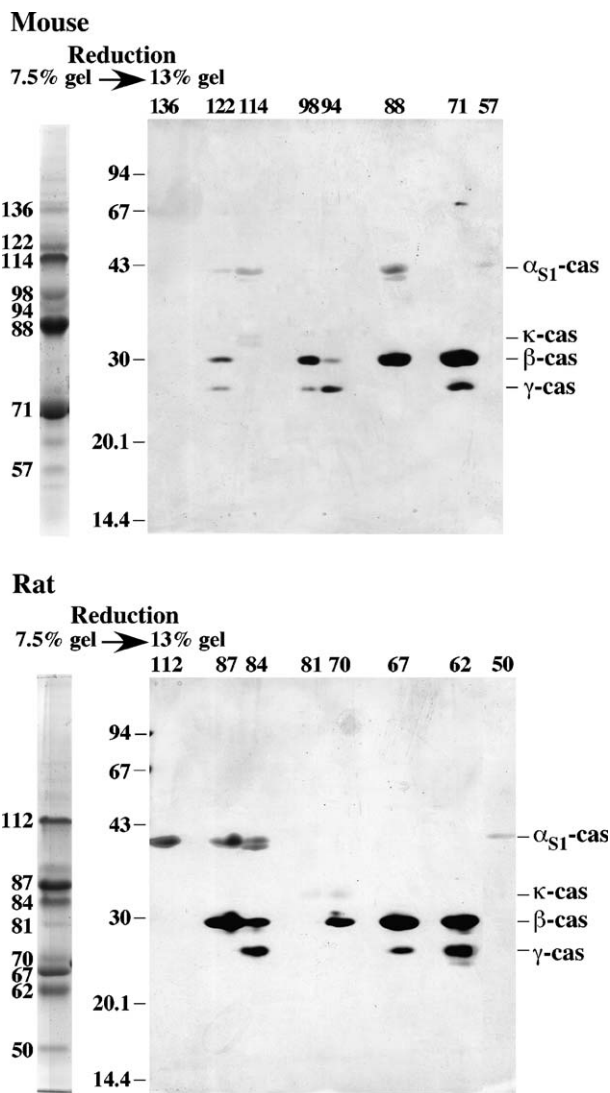


Fig. 4. Cysteine-containing caseins form homo- and heterodimers. Aliquots of the aqueous phase of milk from the indicated species were diluted, incubated, and centrifuged as described in Fig. 2. Proteins contained in the milk pellet were analysed by SDS–PAGE in non-reducing condition using 7.5% gel, followed by Coomassie blue staining. Proteins in the bands identified by their relative molecular mass (kDa) were reduced and subjected to a second SDS–PAGE analysis in reducing conditions (13% gel), followed by silver staining. Representative gels from four independent experiments are shown. Proteins from the bands obtained after SDS–PAGE analysis in non-reducing conditions were also analysed by peptide mass fingerprinting and the results are shown in Table 3. α_{S1} -cas, α_{S1} -casein; κ -cas, κ -casein; β -cas, β -casein; γ -cas, γ -casein.

bridges was quite low compared to that we observed with the other species studied. Correlatively, the amount of non-sedimentable caseins was high in goat milk, except for α_S -caseins. κ -casein contains a single cysteine in most species, except in ruminants, horses, and camels, and can only form homo- or heterodimers. Indeed, κ -casein in rabbit milk was apparently exclusively dimeric. As will be further discussed below, we also demonstrated the presence of other types of casein heterodimers containing κ -casein in rodent milk casein micelles.

It is believed that to fulfil its function, κ -casein is present at the interface between the hydrophobic core of the micelle, mostly made of the calcium-sensitive caseins, and the aqueous environment (for review see [19,4]). Because κ -casein is poorly phosphorylated when compared to the other caseins, phosphoserine residues and colloidal calcium phosphate are not very likely to participate in its binding with the other caseins. Therefore, hydrophobic interaction is most likely the main force involved in the interaction of κ -casein with the calcium-sensitive caseins. Since the C-terminal macropeptide is highly charged, as it contains all the post-translational modifications such as the sites for O-glycosylation [20] and phosphorylation, and the para- κ -casein domain is predominantly hydrophobic with most of the β -sheet structure of the protein (for review see [21]), κ -casein might interact with the other caseins via its N-terminal domain. For these reasons, one might also speculate that the κ -casein molecules are in parallel configuration in the dimer, the para- κ -casein part of the dimer being in interaction with the other caseins. Dimerisation in such configuration would present the advantage of concentrating all the hydrophilic features at one end of the multimer, greatly amplifying the solvation properties of the molecule, as compared to the monomer. This would provide a high efficiency hydrophilic coating of the hydrophobic core of the micelle, keeping the macromolecular structure in suspension.

Interestingly, κ -casein is not the sole casein able to multimerise via disulphide bonds. It has been shown that bovine α_{S2} -casein [17] and human α_{S1} -casein [18] can also form dimers. In the present study, we further demonstrated that this is the case for any casein, seeing it has a cysteine residue. The best models to investigate this issue are mouse and rat which have four and five cysteine-containing caseins, respectively.

For the reasons described in the result section, we decided to identify precisely where each of the caseins from both mouse and rat migrates in our gels. The identification of the protein contained in the main bands showed that the M_r of the various caseins were ranked in the order of their real M_w , except for the two κ -caseins with M_w which were almost two times less than their apparent M_r . In the latter case, this is most likely due to the considerable post-translational modifications of that peculiar casein, notably glycosylation. The fact that the actual polypeptide lengths of the other caseins are considerably less than their M_r is not only due to post-translational modifications but also

reflects inherent properties of the polypeptide backbone, presumably the abundance of acidic amino acid residues and a rather unfolded conformation. This has been observed for other acidic proteins such as neurofilament polypeptides [22] and proteins of the chromogranin/secrctogranin family [23,24]. Holt and Sawyer [25] have used the term rheomorph to qualify such proteins. The fact that highly acidic proteins give too high M_r is probably due to restricted SDS-binding. Apparently, high degree of phosphorylation of the caseins also contributes to an overestimate of the M_r . In line with this, enzymatic removal of phosphate leads to a substantial increase in electrophoretic mobility (see Fig. 6 in [3]).

It is important to note that caseins can only form dimers in rodents. We established that the calcium-sensitive caseins were mostly in the form of heterodimers, with apparently no preferential partner. We found κ -casein under both homo- and heterodimeric forms. We concluded from these results that multimerisation of the casein mostly occurred at random. It should be noted, however, that our results also suggest that not all theoretical heterodimers were formed. Disulphide bonds between κ -casein and α_{S1} -casein or γ -casein appeared to be less probable. Finally, while a certain proportion of caseins were occasionally found in the supernatant for some species, whey proteins were indeed exclusively recovered in the supernatant, excluding the possibility of substantial disulphide bond formation between caseins and cysteine-containing whey proteins such as WAP or β -lactoglobulin in native milk. One can therefore conclude that, despite the fact that disulphide bond formation within the casein family of protein seems to be at random, self-association of the caseins is quite specific.

Surprisingly, the relative proportion of dimeric γ -casein in mouse was low. In striking contrast, all rat γ -casein was under dimeric form. It is tempting to speculate that the much lower propensity of mouse γ -casein to be involved in dimers is due to the presence of two contiguous cysteines in its sequence (see Table 1) since the primary sequence of the two caseins is well conserved around their cysteine residues. In this context, it is worth noting that goat κ -casein, that presented a poor level of dimerisation, has also two adjacent cysteines in its N-terminal domain. Instead of being in favour of the establishment of a disulphide bridge, the presence of contiguous cysteines may partially impair their formation.

In striking contrast to the milk of goat and cow, rodent milks appeared to contain very few amount of non-micellar casein as all of them were pelleted from the diluted milks, even with the low centrifugal force used here. One possible explanation for the later finding is that multimerisation of the caseins via disulphide bonds reinforces their aggregative properties. It may also stabilise the micellar structure. Whatever the explanation, disulphide bonds obviously contain a high energy force which, in addition to the classical attractive interaction between the hydrophobic regions of the caseins, must play an important role in cementing the

caseins in the micelle. In conclusion, our results suggest that in rodents, whose milks are casein-rich, disulphide bonds help the selective aggregation of the caseins in the secretory pathway of mammary epithelial cells and contribute to the stability of the association of each of these proteins within the casein micelle.

The present comparison of disulphide bond patterns in casein micelle aggregates indicated a large variation between species, from single κ -casein dimers in rabbit milk to a mix of homo- and heterodimers including all the caseins in rat milk. It is therefore difficult to conclude on the potential role of disulphide bond formation in casein aggregation in the secretory pathway of mammary epithelial cells, or on its impact on micelle structure and/or stabilisation. Clearly, disulphide bonds are not an obligatory requirement for the aggregation of every casein. However, the question arises whether disulphide bonds have a general role for the function of κ -casein in casein micelle. Our future goal is to study the role of disulphide bonds in casein trafficking and casein micelle formation in the secretory pathway of mammary epithelial cells.

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