Heat-Induced Interactions of Whey Proteins and Casein Micelles with Different Concentrations of α-Lactalbumin and β-Lactoglobulin

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The effect of the addition of individual whey proteins to skim milk on the interaction between casein micelles and whey proteins was studied, during heat treatment at 75, 80, and 90 °C. Not only temperature and time but also concentration affected the extent of the heat-induced reations of the whey proteins with casein micelles. In general, higher concentration of α-lactalbumin (α-la) in skim milk caused an increase in the amount of this protein associated with the micelles. On the other hand, a further addition of β-lactoglobulin (β-lg) hardly affected the maximum incorporation of this protein with the casein micellar fraction. To determine the effect of a lower concentration of whey protein than that present in natural skim milk, purified α-la and β-lg were added to separated casein micelles suspended in milk permeate from ultrafiltration, and the reconstituted mixture was heated at 80 °C. In the absence of β-lg, very little α-la was found associated with the micellar pellet after heating. When comparable amounts of whey proteins were present, the interaction behaviors of α-la and β-lg with casein micelles were very similar, resulting in an α-la/β-lg ratio close to 1. In general, the ratios of α-la/β-lg associated with the heated micelles were significantly affected by the amount of protein present, either in skim milk or in the presence of resuspended micelles. In milk, at temperatures <90 °C, α-la and β-lg may interact together in the serum phase, with a ratio depending on the original protein concentration, before interacting with casein micelles; this hypothesis could explain most of the observations made in the study.

Keywords: *Skim milk; heat treatment; α-lactalbumin; β-lactoglobulin; κ-casein; protein interactions*

INTRODUCTION

When milk is heated, whey proteins interact with casein micelles (Creamer and Matheson, 1980; Noh and Richardson, 1988). Knowledge of the precise mechanism of the reaction is still inadequate, and there is no clear picture that can aid in the understanding and the control of the heating processes. Most of the studies on heat treatment of milk have focused on the amount of native whey protein remaining in the serum phase at the end of the reaction (Manji and Kakuda, 1986; Dannenberg and Kessler, 1988a). Fewer studies have determined the products of the reaction, for example, the amount of whey protein present with caseins after separation of a micellar pellet (Noh and Richardson, 1988; Law *et al.*, 1994a; Corredig and Dalgleish, 1996a).

The two whey proteins β-lactoglobulin (β-lg) and α-lactalbumin (α-la) have been extensively characterized (Casal *et al.*, 1988; Papiz *et al.*, 1986; Brew and Grobler, 1992), as have the caseins and casein micelles (Holt, 1992; Holt and Horne, 1996). β-Lg contains two disulfide bridges and one free sulfhydryl group, which is important for the heat-induced interaction with κ-casein (Hill, 1989). On the other hand, α-la contains four disulfide bridges, which may contribute to the reversible changes of the protein upon denaturation in the presence of calcium (Relkin *et al.*, 1992). However, the apparent thermal stability caused by the reversible conformation of isolated α-la is much less pronounced when other whey proteins, such as β-lg or BSA, are present in the solution (de Wit and Klarenbeek, 1984). This additional instability is dependent on the concentration of free sulfhydryl groups present in the system (Calvo *et al.*, 1993).

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Thermal denaturation of β-lg and α-la has been studied in milk, in whey, and in isolated solutions (Parris *et al.*, 1991; Roefs and de Kruif, 1994; Qi *et al.*, 1995). It is well established that the rate and the extent of whey protein denaturation depend on environmental conditions (Dannenberg and Kessler, 1988b). Whey protein denaturation is the first step of a more complex process of protein aggregation in milk. Although other interactions such as intermolecular hydrogen bonds may play a part in complex formation (Parris *et al.*, 1991), it is believed that heat-denatured whey proteins bind to the micellar caseins mainly via disulfide bonds (Morr and Josephson, 1968). Only two caseins contain cysteine residues: α_{s2}- and κ-caseins, of which the latter is mainly present on the surface of casein micelles (Dalgleish *et al.*, 1989). The main reaction occurring during heat treatment has been reported to be the complex formation between β-lg and κ-casein (Hill, 1989; Singh and Fox, 1985), but the other cysteine-containing proteins may participate in the heat-induced interactions in skim milk (Dalgleish, 1990). The presence of both α-la and β-lg associated with the micelles isolated from heated milk has been demonstrated (Law *et al.*, 1994b; Corredig and Dalgleish, 1996a), and it is known that β-lg and α-la when heated together form complexes (Matsudomi *et al.*, 1992). However, the mechanism of reaction is not yet understood, and results on this matter are not in agreement. Smits and van Brouwershaven (1980) have shown that in the absence of β-lg, α-la hardly interacts with casein micelles in milk. Conversely, the observation that at pH 7.6 in phosphate buffer α-la binds to κ-casein (Doi *et al.*, 1983) suggests the possibility of an independent reaction of α-la with caseins, albeit under conditions not normally observed in milk. It is known that the degree of heat-induced aggregation of α-la is greater when the two whey proteins are present together than in pure α-la solution

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(Elfagm and Wheelock, 1977; de Wit and Klarenbeek, 1984) and that the behavior during heating is influenced by the presence of other proteins (in the case of α-la) (Calvo *et al.*, 1993).

It is not clear whether heat-denatured α-la and *β*-lg have similar affinities for casein micelles and what, if any, is the stoichiometry of the complex formed during heat treatment. To observe how the two whey proteins react or how they affect each other, we used a model system as close as possible to that of skim milk, but with reduced whey protein concentration. The mechanisms and extent of the interactions of α-la and *β*-lg with caseins were also studied when their concentrations were higher than in natural skim milk. The types and amounts of milk proteins that form the micellar pellet were investigated when milk was heated for periods of time, up to 60 min, at different temperatures in the range 75−90 °C. The mutual effect of the presence of whey proteins with casein micelles on their incorporation into a complex was determined. The particular temperature range was selected to ensure that the mechanism of the reaction was similar throughout; there appears to be a change in the behavior of whey proteins at ∼90 °C (Dannenberg and Kessler, 1988a,b; Hillier and Lyster, 1979), and it has been shown that covalent, non-disulfide, intermolecular bonds are formed at high temperature (Singh and Latham, 1993).

MATERIALS AND METHODS

Heating Conditions and Sample Preparation. Milk and commercial whey protein fractions were obtained as described previously (Corredig and Dalgleish, 1996a). Individual highly purified α-la and *β*-lg were prepared by preparative ion exchange chromatography on Sepharose Fast-Flow Q (Pharmacia Biotech, Baie d'Urfé, Quebec, Canada), with a buffer containing 20 mM Tris, pH 7.0, and a gradient of 0−1 M NaCl in a total volume of 4 L. The purified fractions were dialyzed exhaustively against deionized water and lyophilized.

Three different additions of whey protein to skim milk were made: (i) α-la (2 g L^{-1}), (ii) *β*-lg (2 g L^{-1}), or (iii) a mixture of equal weights of α-la and *β*-lg (1 g L^{-1} each). None of these additions changed the pH of the milk from 6.8.

Samples (15 mL) of milk, with or without added whey proteins, were heated for different lengths of time from 0 to 40 min in glass test tubes in a water bath at 75, 80, or 90 °C. After heating, the samples were cooled rapidly below room temperature (15 °C) by immersion in ice.

In a second approach, a model system was reconstituted from skim milk. Casein micelles were isolated by centrifuging untreated skim milk at 60000*g* for 40 min in a Beckman preparative ultracentrifuge (L8-70M, Ti-70 rotor) (Beckman, Palo Alto, CA). After centrifugation, the supernatant was decanted, and the centrifuge tubes were allowed to drain in an inverted position. The micellar pellet was collected and further drained by placing it on No. 1 Whatman filter paper (Whatman, Maidstone, U.K.). Milk ultrafiltrate was prepared using a laboratory ultrafiltration unit (thin-channel UF unit, Model TCF 10) with a 10 000 Da cutoff membrane (Amicon Inc., Mississauga, ON, Canada). The drained casein micelles were resuspended in the ultrafiltrate to their initial concentration in skim milk, so as to preserve as much as possible the original environment. The mixture was left overnight at 5 °C to equilibrate. SDS−PAGE was performed to confirm that this procedure had reduced the total whey protein to <5% of the total protein (compared with its original 20%). Purified α-la and *β*-lg were added to the resuspended micelles following the scheme in Table 1. A concentration of α-la lower than that present in skim milk was employed in treatments 1−3, while treatments 4 and 5 used an amount of α-la within the range naturally present in milk but in the presence of smaller than normal amounts of *β*-lg. Treatment 6 aimed to provide whey protein concentrations similar to those in original milk, with *β*-lg constituting 70% of the total whey protein. Mixtures lacking *β*-lg (treatments 2 and 5) or α-la (treatments 1 and 8) were also prepared. In treatment 7 more α-la than *β*-lg was present in the resuspended micellar sample (70% α-la and 30% *β*-lg), in a ratio reversed to that present in skim milk. The mixtures were heated as described above, but only at 80 °C.

Table 1. Summary of the Different Concentrations of α-La and *β*-Lg Present in the Reconstituted Mixtures with Resuspended Casein Micelles

treatment	α-la (g L^{-1})	*β*-lg (g L^{-1})
1	0	0.8
2	0.8	0
3	0.8	0.8
4	1.2	1.2
5	1.2	0
6	1.2	3.2
7	3.2	1.2
8	0	3.2

Determination of the Amount of Whey Protein Associated with the Micelles. In each of the experiments, the heated milk or reconstituted milk after cooling was centrifuged at 60000*g* for 40 min. The serum was decanted from the micellar pellet, which was collected and washed by resuspension in a buffer at pH 7.0, containing 20 mM imidazole, 5 mM $CaCl_2$, and 50 mM NaCl. After the ultracentrifugation was repeated under the same conditions, the pelleted fraction was collected and drained on No. 1 Whatman filter paper.

Portions of the drained micelles (0.0150 g) were analyzed by SDS−PAGE using the methods described previously (Corredig and Dalgleish, 1996a).

The amounts of α-la and *β*-lg associated with the micelles were quantified by relating the protein bands to the amount of *κ*-casein present in the micellar sample. These quantity ratios were used as an index of the interaction of whey proteins with caseins (Corredig and Dalgleish, 1996a).

In the experiments with the reconstituted mixtures of casein micelles and whey protein, it was also possible to determine the amounts of α-la and *β*-lg associated with the micelles by using the original mixture, where the amounts of whey protein were known, as the internal standard in each electrophoretic analysis.

Principles of the Interpretation of the Results. It is assumed in this work that the whey proteins which are present in the sedimented micellar pellets from the heated preparations are bound to the micelles, although it is in principle possible that some or all of this whey protein consists of aggregates (precipitates) of whey proteins alone, which cosediment with the casein micelles and give an appearance of having reacted with them (Davies *et al.*, 1978). There is no method available, either chromatographic or centrifugal, for separating these complexes from the casein micelles.

We believe that the whey proteins are indeed bound to the casein micelles, for a number of reasons. It has been assumed in previous studies of heated milks, in which centrifugation or gel permeation chromatography has been used to separate the reacted products, that there were true complexes formed between whey proteins and casein micelles (Smits and van Brouwershaven, 1980; Manji and Kakuda, 1986; Morr, 1992; Law, 1996). In milk without added whey proteins this is certainly the case, since the casein micelle/whey protein complexes can be dispersed in urea to give only small casein/whey protein aggregates with no traces of independently aggregated whey proteins (Dalgleish, 1990), and the disulfide linkage between denatured *β*-lg and *κ*-casein is well-known (Jang and Swaisgood, 1990). Recently, we have shown (Dalgleish *et al.*, 1997a) that micelle sizes in heated milks do not increase, except when large amounts of *β*-lg are added, precluding the formation of large complexes bound to the micelles. Also, in studies of ultrahigh-temperature-treated concentrated milks, electron microscopy has suggested that the whey proteins do not aggregate independently of the casein micelles (Singh and Creamer, 1992). Furthermore, in our experiments, described below, some of the added whey proteins remained in the serum, even after our most severe heat

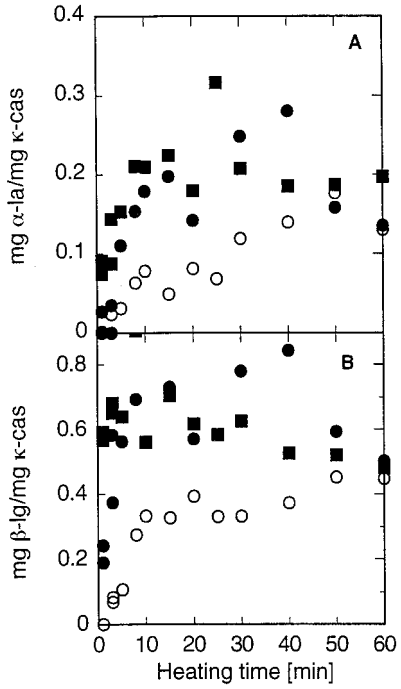

Figure 1. Weight ratio of α-la/κ-casein (A) and β-lg/κ-casein (B) in the micellar pellet of skim milk as a function of time. Heat treatment was carried out at 75 (○), 80 (●), and 90 °C (■). Results are the averages of four independent experiments.

treatments, once the amounts of whey proteins associated with the casein micelles had reached a plateau; evidently, at least part of the denatured whey proteins did not form precipitates or bind to the micelles under the conditions of the experiments. This is also confirmed by the studies of Law (1996). The fact that we found it difficult to increase the amount of β-lg bound to the micelles simply by adding more of the protein to the milk (see below) also suggests that nonspecific complexes are not formed.

In the discussion which follows, it is assumed that all of the whey protein which we find associated with the micellar fraction is indeed linked to the micelles themselves.

Statistical Analysis. The results presented are the average of at least four experiments. Results were analyzed statistically using the SAS package (SAS Institute, 1991). The general linear model procedure was employed to determine the variance of the factors that affected the response variables (the ratios of α-la/κ-casein, β-lg/κ-casein, and α-la/β-lg). The independent variables were time, temperature, and whey protein concentration. Differences were considered significant at $p \leq 0.05$. The least-squares means of the ratios of α-la/β-lg were also calculated, and a *t*-test for differences was performed ($p \leq 0.05$).

RESULTS

Reactions in Skim Milk. A series of trials on skim milk with no addition of whey protein was carried out first. Figure 1 illustrates the weight ratio of α-la and β-lg to κ-casein in the micellar pellet as a function of heating time in these control experiments.

At 75 °C a slow increase with heating time in the ratios of whey protein to κ-casein was measured in the micellar pellet for both α-la and β-lg (Figure 1). In general, at 80 and 90 °C the incorporation of whey proteins with the casein micellar fraction was more rapid than at 75 °C. The amount of β-lg associated with the micelles was lower at 75 °C than at 80 and 90 °C (Figure 1B). At the higher temperatures the amount of β-lg found with the casein micelles leveled off within a very short heating time; at 90 °C the time for skim milk to reach the desired temperature was around 100 s, and this was all that was necessary for the reaction to reach its maximum extent.

After the maximum, there was a tendency for the amount of α-la and β-lg associated with the micelles to decrease somewhat; these effects of long-term heating have not been fully explained but may be caused by partial dissociation of the κ-casein/whey protein complex from the micelles. Singh and Fox (1985) showed that κ-casein/β-lg complexes dissociated from heated micelles, especially when the pH was altered from its natural value, although at higher temperatures than those used here. Law (1996) also found that dissociation of κ-casein occurred after heating at 85 °C for 10 min. We checked for micellar dissociation by measuring the amounts of κ-casein liberated into the serum but found that only slight dissociation of κ-casein occurred in the early part of the reaction: the amounts dissociating were too small to be quantified. At longer heating times and higher temperatures the extent of dissociation increased, but this was in all cases at times after the reaction of whey proteins was virtually complete.

It was possible that the method of preparing the micellar pellet may bias our results toward larger casein micelles; in particular, it is probable that our washing procedure may remove the κ-casein-rich opalescent layer which is formed when the micelles are centrifuged from milk (Morr, 1973). However, in our procedure such a layer would be amalgamated with the decanted serum, and we found little evidence for the presence of any caseins in this decanted liquid.

We should note also that in experiments involving skim milk, the micellar fraction after heating contained bovine serum albumin (BSA) associated with the micelles. This protein is in considerably smaller amount than the β-lg or α-la present in the milk, but it evidently associates strongly with the casein micelles. BSA is also known to interact with α-la during heating (Matsudomi *et al.*, 1993). However, we do not consider this protein further in this paper, because of its low concentration in milk and its absence from the micellar preparations, which were also studied.

Effect of the Addition of Whey Proteins to Skim Milk. When purified α-la and β-lg were added to skim milk, the amount of α-la or β-lg associated with the casein micelles was affected by the protein concentration. The analysis of variance demonstrated that not only time and temperature of heating but also the amount of whey protein present in skim milk significantly affected the amounts of α-la/κ-casein and β-lg/κ-casein associated with the micellar pellet. Figure 2 shows the ratios of α-la and β-lg to κ-casein when α-la at 2 g L^{-1} was added to skim milk and heat treatment performed at 75 °C. The amount of α-la increased from about 0.15 to 0.3 mg of α-la/mg of κ-casein, and the rate of incorporation of α-la at this concentration seemed different from that in the control milk (Figures 1A and 2). Figure 2 shows clearly the similarity of the rate of incorporation of α-la and β-lg into the casein micellar fraction during the heat treatment of the α-la-enhanced milk at 75 °C. This result agrees with previous reports in milk (Hillier and Lyster, 1979; Dannenberg and Kessler, 1988b) and in homogenized milk (Sharma and Dalgleish, 1993). The addition of α-la at 2 g L^{-1} to the milk caused the two major whey proteins to be present in approximately equal amounts before heating, and the quantities of α-la and β-lg relative to the amount of κ-casein present in the micellar pellet were also the same (Figure 2). The addition of α-la at 2 g L^{-1} to skim

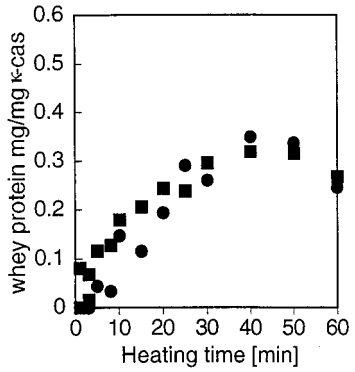

Figure 2. Weight ratio of α-la/κ-casein (●) and β-lg/κ-casein (■) in the micellar pellet of skim milk heated at 75 °C as a function of time. The analyses were carried out on casein micelles isolated from skim milk to which α-la (2 g L^{-1}) had been added before heating. Results are the averages of four independent experiments.

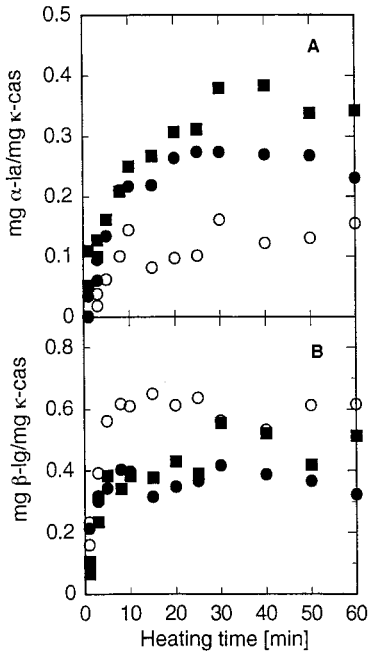

Figure 3. Weight ratio of α-la/κ-casein (A) and β-lg/κ-casein (B) in the micellar pellet of skim milk heated at 80 °C as a function of time. The analyses were carried out on casein micelles isolated from skim milk with added β-lg (2 g L^{-1}) (○), α-la and β-lg each at 1 g L^{-1} (●), and α-la (2 g L^{-1}) (■). Results are the averages of four independent experiments.

milk did not seem to greatly affect the extent of incorporation of β-lg into the micellar fraction; if anything, the maximum amount of β-lg was slightly decreased.

Figure 3 shows the results obtained after skim milk containing different concentrations of whey proteins had been heated at 80 °C. When α-la was added, either in combination with β-lg (α-la at 1 g L^{-1} and β-lg at 1 g L^{-1}) or alone as α-la at 2 g L^{-1}, the incorporation reached higher values of α-la/κ-casein than the control (Figure 3A). The addition of β-lg at 2 g L^{-1} did not significantly affect the incorporation of α-la compared with the skim milk control (compare Figures 3A and 1A). The addition of β-lg at 2 g L^{-1} to skim milk only slightly, if at all, increased the extent of incorporation of β-lg into the casein micellar fraction. The plateau value (~0.6 mg of β-lg/mg of κ-casein) was not significantly different from that of control milk. Thus, even when the concentration of β-lg was higher than that

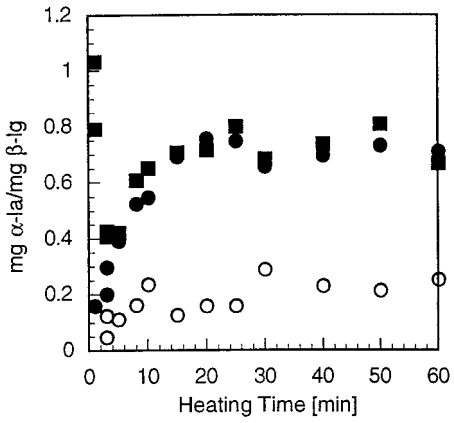

Figure 4. Weight ratio of α-la/β-lg in the micellar pellet of skim milk heated at 80 °C as a function of time. The analyses were carried out on casein micelles isolated from skim milk with added β-lg (2 g L^{-1}) (○), α-la and β-lg each at 1 g L^{-1} (●), and α-la (2 g L^{-1}) (■). Results are the averages of four independent experiments.

usually present in milk, the maximum ratio of β-lg/κ-casein in the micelles was comparable to that found in the micellar pellet of control milk. This suggests that only a discrete number of sites might be available on casein micelles for the interaction with β-lg and that the noninteracted β-lg does not precipitate from the milk on its own. Furthermore, when more α-la was added to the skim milk, it appeared that even less β-lg interacted with the micelles (Figure 3B), since the plateau value was significantly lower than in the control.

The ratio of α-la/β-lg associated with the micelles, as illustrated in Figure 4, suggested even more clearly that the interaction behavior of the two whey proteins changed as a function of their concentration in skim milk. When comparable amounts of the two proteins were present in skim milk, the ratio of α-la to β-lg associated with the micellar pellet after heating reached values slightly lower than 1. A similar ratio was found also when α-la was added to skim milk at 1 g L^{-1} together with β-lg at 1 g L^{-1}. If β-lg had a limited number of sites available for the interaction, its amount would not increase with the addition of more β-lg. On the other hand, as shown in Figure 4, the amount of α-la associated increased, and the ratio of α-la/β-lg reached values comparable to the one calculated when only more α-la was added to skim milk. Only when β-lg was in higher concentration than α-la was the α-la/β-lg ratio significantly lower, around 0.3 mg/mg. As already suggested by Law *et al.* (1994b), the variation of the ratio between the two whey proteins could be used as a means of estimating the extent of heat treatment in skim milk. Different ratios of β-lg/α-la can be quantified in the serum phase after heating of milk, depending on the temperature of heating and holding time. It is also important to note that the amount of α-la associated with the micelles also comes to a limit. We saw little or no difference between the effects of addition of 1 and 2 g L^{-1} of α-la (Figures 3 and 4). This suggests that, as for β-lg, there may be a limited number of sites by which α-la can interact with casein micelles.

Results obtained from heating at 90 °C confirmed those reported above. The incorporation of whey proteins into the micellar fraction of the control milk and of skim milk with added whey proteins was very rapid. Similarly to the observations at lower temperature, neither the rate nor the extent of the incorporation of

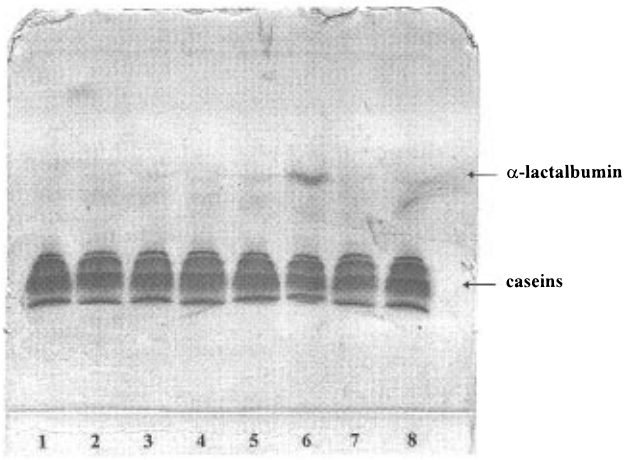

Figure 5. SDS−PAGE electrophoresis of micellar pellet isolated by ultracentrifugation after heat treatment at 80 °C, as a function of time, from reconstituted micelles with added α-la at 1.2 g L^{-1}. Treatment time is indicated as follows, lanes from left to right: lane 1, 4 min; lane 2, 10 min; lane 3, 0 min; lane 4, 20 min; lane 5, 30 min; lane 6, original mixture of casein micelles with added α-la (internal standard); lane 7, 45 min; lane 8, 60 min. Analysis was performed under reducing conditions.

α-la was affected by the addition of β-lg alone. During heating at 90 °C, β-lg associated with the casein micellar fraction very rapidly. No significant difference in the plateau value of β-lg associated with the micelles was found after β-lg had been added to the skim milk, and the presence of α-la appeared to depress this level, as found during heating at 80 °C. A trend toward lower ratios of β-lg relative to the amount of κ-casein in the micellar pellet as a function of time seemed to occur, although the analysis of the data did not show this to be significant at the 5% level.

Addition of Whey Proteins to Resuspended Casein Micelles. The study of the effect of different concentrations and whey protein ratios in skim milk suggests that a specific stoichiometry governs the mechanism of interaction between whey proteins and casein micelles. We sought to broaden the range of the observations, by measuring the reactions occurring between isolated casein micelles and smaller amounts of whey protein than those present in skim milk, or when different ratios of the whey proteins were present. To allow comparison with previous results, 80 °C was chosen as the temperature of treatment of the whey protein/casein mixtures. In a resuspended mixture containing casein micelles and α-la only, no incorporation of α-la occurred (Figure 5). The importance of the presence of β-lg for the incorporation of α-la into the micellar pellet is clearly demonstrated in Figure 6. This is in agreement with the findings of Calvo *et al.* (1993), who found that the presence of β-lg seemed necessary for the incorporation of α-la in the heat-induced aggregates. When β-lg at 0.8 g L^{-1} was added along with α-la at 0.8 g L^{-1}, the incorporation into the micellar fraction was slow, but significantly more α-la was present with the micelles than in the unheated control or when micelles were heated with α-la alone. When α-la and β-lg each at 1.2 g L^{-1} were present, the amounts of α-la/κ-casein in the micellar fraction increased as a function of heating time. The weight ratios determined by SDS−PAGE and scanning densitometry were 0.62 ± 0.19 α-la/κ-casein when α-la and β-lg were added each at 1.2 g L^{-1} (treatment 4) and 0.33 ± 0.08 when α-la and β-lg each at 0.8 g L^{-1} (treatment 3) were

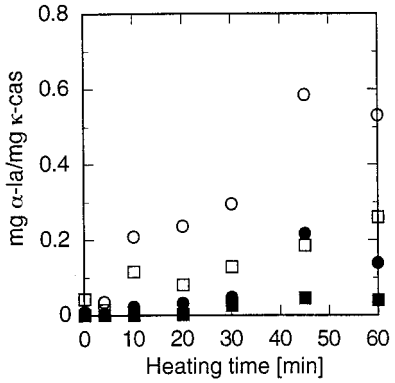

Figure 6. Weight ratio of α-la/κ-casein in the micellar pellet isolated after heating at 80 °C as a function of time. The analyses were performed on reconstituted casein micelles with the following whey protein concentrations: α-la (0.8 g L^{-1}) (■); α-la (1.2 g L^{-1}) (●); α-la and β-lg each at 0.8 g L^{-1} (□); α-la and β-lg each at 1.2 g L^{-1} (○). Results are the averages of four independent experiments.

Table 2. Analysis of Variance of the Ratio α-La/β-Lg

	DF	SS	mean square	*F* value	Pr > *F*
time	4	0.6596	0.1649	0.90	0.4682
[α-la]	2	16.7383	8.3691	45.81	0.0001
[β-lg]	1	1.4340	1.4340	7.85	0.0068
interaction	12	1.0952	0.0912	0.50	0.9068

treatment	mean α-la/β-lg
α-la (0.8 g L^{-1}) and β-lg (0.8 g L^{-1}) [3]	0.606 [a]
α-la (1.2 g L^{-1}) and β-lg (1.2 g L^{-1}) [4]	0.722 [a]
α-la (1.2 g L^{-1}) and β-lg (3.2 g L^{-1}) [6]	0.347 [b]
α-la (3.2 g L^{-1}) and β-lg (1.2 g L^{-1}) [7]	1.614 [c]

[a] Factors considered in the model were time of heating, concentration of α-la and β-lg added to the resuspended micelles, and time × [α-la] × [β-lg] (interaction). The treatments considered in the analysis were treatments 3, 4, 6, and 7 (Table 1). The values of α-la/β-lg in the first 10 min of heating were omitted from the analysis. The lower part of the table shows the least-squares means for each treatment as calculated by the general linear model procedure; values indicated with the same letter are not significantly different ($p < 0.05$).

added to the resuspended micelles. It should be noted, however, that the coaggregation appeared to be slower than in the control skim milk used in the experiments described above.

As already shown for the heating of skim milk, in the experiments with resuspended micelles the whey protein concentration significantly affected the interaction of α-la and β-lg with casein micelles. The analysis of variance showed a significant effect of time and concentration on the α-la/κ-casein and β-lg/κ-casein ratios present in the heated micellar pellet. Table 2 illustrates the results of the statistical analysis for time and protein concentration on the ratio of α-la/β-lg associated with the micellar pellet after heating. The variable of time was not significant (if we excluded the first 10 min of heating from this analysis); that is, the ratio α-la/β-lg did not change with time after 10 min of heating at 80 °C, as shown already in the experiments with skim milk, where the plateau in the ratio of the two proteins is reached at an early stage (Figure 4). On the other hand, the concentrations of protein present in the resuspended micelles significantly affected the values of α-la/β-lg found in the pellet after heating. Table 2 also illustrates the average ratio of α-la/β-lg for treatments 3, 4, 6, and 7. The amount of α-la to β-lg associated with casein micelles when the whey protein

concentration was comparable to the one in skim milk (treatment 6) was significantly lower than in the other three treatments considered (treatments 3, 4, and 7) but was comparable to the value found for skim milk with no added whey protein (Figure 1). When α-la and β-lg were added in similar amounts in the reconstituted micelles (α-la at 0.8 g L^{-1}, β-lg at 0.8 g L^{-1} and α-la at 1.2 g L^{-1}, β-lg at 1.2 g L^{-1}), their plateau value was significantly higher than that of treatment 6 and significantly lower than that of treatment 7. On the other hand, the ratio of α-la to β-lg was higher than 1 mg/mg when α-la was added at 3.2 g L^{-1} and only 1.2 g L^{-1} of β-lg was present in the mixture. This was the only case when much higher amounts of α-la than β-lg were recorded. It is possible to conclude than the interaction of α-la with the micelles occurs via an intermediate with β-lg and that the amount of α-la associated with the micelles depends on the concentration present in the mixture.

DISCUSSION

We may conclude from the results the following:

β-Lg Interacts with Casein Micelles Only to a Limited Extent in the Temperature Range 75−90 °C. This is evident from the results shown in Figures 1 and 3, where the quantity of β-lg associated with the micelles rises to a plateau with a value for β-lg/κ-casein of ∼0.6, independently of whether or not β-lg has been added to the milk. This implies a molar ratio of <1, since the two proteins have similar molecular masses. The implication is that there is sufficient β-lg present in milk to saturate the available sites for disulfide formation between β-lg and casein micelles (with κ- and α_{s2}-caseins) and that not all of the κ-casein is available for this reaction. Alternative interactions (non-disulfide) are also possible (Parris *et al.*, 1991), but there is strong evidence for the final interaction being via disulfides (Jang and Swaisgood, 1990). The low ratio of β-lg to κ-casein in the heated micelles also carries the implication that the β-lg is not binding to the micelles in polymeric or oligomeric form. The low molar ratio is in agreement with the knowledge that κ-casein is itself likely to be in the form of disulfide-linked oligomers on the micellar surface (Rasmussen *et al.*, 1992) and, therefore, may not be in a position to react stoichiometrically with the β-lg. An alternative explanation is that some of the κ-casein is buried within the micelle and is unavailable for reaction, although current thinking on the casein micelle is that this protein is mainly on the surface of the particles (Holt and Horne, 1996), whereas α_{s2}-casein is located on the surface and in the interior of the micelle (Dalgleish *et al.*, 1989).

If β-Lg Is Present at Less than Its Normal Concentration in Milk, It Binds Efficiently to the Micelles. This is evident from the results of the reactions between separated micelles and whey proteins. The lower concentrations of β-lg used all bound to the micelles, but not to saturating levels (i.e., the β-lg/κ-casein ratios were <0.6). This confirms the possibility that there are a certain number of sites only on the micellar surface by which interaction with whey proteins can be achieved.

α-La Does Not, on Its Own, Interact with Casein Micelles. This confirms the results of Smits and van Brouwershaven (1980), who found that there is little or no interaction between even heat-denatured α-la and the caseins in the micelle. This presumably arises because there is a need for the presence of proteins with free sulfhydryl groups to interact with the α-la and in turn render it reactive (Calvo *et al.*, 1993). This function is of course normally performed by β-lg when milk is heated, and the absence of reaction between α-la and micellar κ-casein may perhaps be taken as an indication that the sulfhydryl groups of the latter are bound up in the formation of the oligomeric complexes.

If α-La and β-Lg Are Present in Similar Amounts, They Bind to Casein Micelles in a Weight Ratio of Somewhat Less than 1. This is shown by the results in Figures 2 and 4 and by the ratios shown in Table 2. This weight ratio (α-la/β-lg) is slightly smaller than a molar ratio of 1:1, taking monomer molecular masses for β-lg and α-la of 18277 and 14178, respectively. This in turn suggests that under the conditions of relatively low concentration of whey proteins used in these experiments, for which the total concentration is always <1%, a major interaction is that between heat-denatured β-lg and α-la on a 1:1 molar basis; this complex then interacts with micellar κ-casein to give a complex containing one of each type of molecule. Because such complexes need occupy only one of the sulfhydryl groups of the κ-casein, it is possible for a variety of complexes to be formed, linked by disulfide formation between adjacent molecules of κ-casein. There is also some evidence from Figure 4 that the ratio α-la/β-lg associated with the micelles increases to its final value as the heating proceeds; it appears from this that the β-lg must be the first protein to interact with the micelles and that the binding of α-la is slower.

In one of our experiments there was an indication that excess α-la bound extensively to the micelle; this was in the case when the separated micelles were mixed with larger amounts of α-la than β-lg, and the latter was in a concentration less than that required for saturation. We have as yet no explanation for this behavior, except to suggest that the reaction of κ-casein with β-lg also caused liberation of some sulfhydryl groups by which the α-la could be attached.

This leads to a mechanism for the reaction at normal pH of milk with whey proteins in concentrations around their normal values, where they are too dilute to form gels during heating (Matsudomi *et al.*, 1992; Gezimati *et al.*, 1997; Dalgleish *et al.*, 1997b). As heating progresses, the β-lg denatures, first by dissociating and then by unfolding, as described by other authors (Roefs and de Kruif, 1994). At this stage, a β-lg molecule may potentially interact with another lactoglobulin molecule, a molecule of denatured α-la, or a molecule of κ- or α_{s2}-casein. On the basis of our results (Figure 3), it appears that the last of these is more likely than the others, so that a reaction sequence may be defined whereby sites capable of binding α-la are created by the interaction of denatured β-lg and κ-casein. This will result in the limited binding of both of the whey proteins to the casein micelles.

This mechanism may be operative over only a relatively small range of temperature. It is known from numerous experiments that the mechanism of the reaction changes at ∼90 °C, with effects on the composition and the functional properties of the heated milk. In these reactions, it appears that the binding of β-lg is favored over that of α-la, so that the stoichiometry of the complexes is biased toward the former protein (Corredig and Dalgleish, 1996b) and, therefore, that further studies of milks heated at temperatures >90 °C are required to elucidate the mechanism in that region of temperature.

CONCLUSION

We conclude from the results shown above that in milk in which the whey proteins are in relatively low concentration (~0.7%), they interact with the casein micelles to form rather simple molecular complexes when the milk is heated in the temperature range 75−90 °C. Both of the major whey proteins interact with the casein micelles, but it is likely that the β-lg is the first to interact with the micellar κ-casein and that the α-la interacts once this complex is formed. The number of sites on the casein micelles whereby such interactions can take place is limited, and the addition of sufficient whey protein can easily saturate them. Although this mechanism is capable of explaining the results under specific conditions, it is likely that increasing the temperature of heating or the whey protein/casein ratio in the milk may lead to the formation of different products, because the relative rates of interaction of whey proteins and caseins will change.

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