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Reduced Stability and Accelerated Autoxidation of Tuna Myoglobin in Association with Freezing and Thawing

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The mechanisms involved in accelerated discoloration of frozen tuna meat after thawing were investigated. Bluefin tuna myoglobin (Mb) was dissolved in 50 mM phosphate buffer (pH 5.5–7.4) with or without 0.2 M NaCl and quickly frozen in dry ice–acetone, followed by a 2-h storage at −80 °C and subsequent thawing at 20 °C. The "frozen/thawed" Mb solution thus prepared, along with unfrozen (intact) Mb, was examined for the free energy for unfolding, helical content, and autoxidation rate. In 50 mM phosphate buffers, the free energy for unfolding (ΔG_D) was generally lower in frozen/thawed Mb than in unfrozen Mb, with the highest value (7.3 kcal/mol) at pH 6.5. Similar tendency was observed in 50 mM phosphate buffers containing 0.2 M NaCl, although $\Delta G_D^{H_2O}$ values were generally lower. Helical contents of frozen/thawed Mb were practically the same as those of unfrozen Mb, regardless of pH. Frozen/thawed Mb showed a higher autoxidation rate than unfrozen Mb. It was concluded that, during freezing and thawing, Mb suffered some conformational changes in the nonhelical region, resulting in a higher susceptibility to both unfolding and autoxidation.

It is empirically known that tuna meat, once frozen and thawed, discolors more quickly than unfrozen meat during subsequent storage, by an unexplained reason. Myoglobin (Mb), so far reported, shows a pH-dependent autoxidation, the rate increasing with decrease of pH (George and Stratmann, 1952; Matsuura et al., 1962; Brown and Mebine, 1969). As reported previously, however, we found a unique pH dependency of autoxidation in bluefin tuna Mb, associated with freezing and thawing: The formation of metmyoglobin (metMb) was minimum in the pH range 6.0–6.5, depending upon buffer species (Chow et al., 1985, 1987). On the other hand, freezing and thawing accelerated insolubilization of the Mb, with a minimum ratio in the same pH range. These findings suggested that some pH-dependent conformational changes of Mb might have occurred during freezing and thawing.

Free energy required to denature a protein completely is one of the best parameters to evaluate the degree of denaturation or "stability" against chemical denaturant (Ahmed and Bigelow, 1982). Guanidine hydrochloride (Gdn·HCl) and urea induce proteins into a randomly coiled denaturation state (Tanford, 1970). Gdn·HCl is a more potent denaturant, unfolding proteins at several times lower concentrations than does urea (Greene and Pace, 1974). In this connection, Fosmire and Brown (1976) found that yellowfin tuna Mb was much more susceptible to urea denaturation than sperm whale Mb. Balestrieri et al.

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(1978) also reported that bluefin tuna Mb showed a lower free energy for unfolding than mammalian Mb, when Gdn·HCl was used as a denaturant. On the other hand, circular dichroism (CD) spectrometry is a useful means to follow changes in the secondary structure of a protein, or to determine the stability. Fosmire and Brown (1976) found a lower α-helix content for yellowfin tuna Mb than sperm whale Mb and suggested that it could be associated with the lower stability of yellowfin tuna Mb.

These situations prompted us to examine structural changes of bluefin tuna Mb associated with freezing and thawing, using α-helix content and free energy for unfolding, as parameters. The effect of freezing and thawing on autoxidation rate of the Mb was also studied.

MATERIALS AND METHODS

Preparation of Mb. Mb was prepared from the dark muscle of bluefin tuna (*Thunnus thynnus*) that was stored at −80 °C for not longer than 2 months, by the method reported previously (Chow et al., 1985). It was almost homogeneous in isoelectric focusing on an LKB Ampholine PAG plate (pH 3.5–9.5). Bluefin tuna metMb thus isolated was stored in ice and used in the following experiments within a few days.

Bluefin tuna metMb was dissolved in 50 mM sodium phosphate buffers (pH 5.5–7.5) with or without 0.2 M NaCl. Aliquots of Mb solution were quickly frozen in dry ice–acetone, kept at −80 °C for 2 h, and thawed in a water bath at 20 °C. The solutions were filtered through a Millipore filter (0.45 μm) to eliminate insolubilized Mb and were used as "frozen/thawed" Mb solutions. The concentration of Mb was determined using a molar extinction coefficient of 11 300 for cyanmetMb at 540 nm (Drabkin, 1945).

Determination of Free Energy for Unfolding. To both unfrozen and frozen/thawed Mb solutions was added an equal volume of 0.5–6 M Gdn·HCl, which was previously adjusted to respective pH values (Puett et al., 1973). These solutions were allowed to stand at 4 °C for 20 h and subsequently incubated in a water bath at 20 °C for 2 h. Their Soret absorbances at 409 nm were recorded at due time intervals as a parameter of denaturation. Duplicate determinations were performed at respective pH, and the mean values were given.

The mechanism of denaturation of Mb has been reported to consist of one step in two transition states (Schechter and Epstein, 1968; Puett, 1973). Consequently, the free energy for unfolding or denaturation, ΔG_D, can be determined from the experimental data by

$$\Delta G_D = -RT \ln K_D \quad (1)$$

where R is the gas constant (1.986 cal/deg·mol), T the absolute temperature, and K_D the equilibrium constant of denaturation. The K_D value can be calculated from

$$K_D = \frac{f_D}{1 - f_D} = \frac{E_N - E_{obs}}{E_{obs} - E_D} \quad (2)$$

where f_D represents the fraction of denatured protein, E_N and E_D are the molar extinction coefficients of the protein in native and denatured states, respectively, and E_{obs} is the observed molar extinction coefficient of the protein between the native and denatured states.

The intrinsic value of ΔG_D of Mb can be obtained from the linear correlation between the value of ΔG_D and Gdn·HCl concentration, where the conformation of Mb is in a transitive state. Therefore, the conformational free energy of the protein in the absence of denaturant $\Delta G_D^{H_2O}$ can be calculated by

$$\Delta G_D^{H_2O} = \Delta G_D + mC \quad (3)$$

where C represents the molar concentration of the denaturant and m the slope of regression line. This method has been applied to estimate $\Delta G_D^{H_2O}$ for ribonuclease, lysozyme, α-chymotrypsin, β-lactoglobulin, α-lactalbumin, horse Mb, etc. (Greene and Pace, 1974; McLendon and Sandberg, 1978; Ahmed and Bigelow, 1982).

CD Spectrometry. CD spectra of Mb solutions were measured at 25 °C on a Jasco J-20 automatic recording spectropolarimeter equipped with a Jasco DP-501N data processor. Triplicate measurements were performed, and the data were given as mean values and standard deviations. Scanning was carried out in the range 260–200 nm. Base line was corrected by dispersion with the control buffer. The mean residue ellipticity at 222 nm ($[\theta]_{222}$, deg·cm^2/dmol) was calculated as

$$[\theta]_{222} = \theta^o/100Cl \quad (4)$$

where θ^o is observed ellipticity in degrees at 222 nm, C the molar concentration, and l the light path length in centimeters. In this study, a cell of 0.1-cm light path was used for measurement. The helical content was calculated according to Holzwarth and Doty (1965) as follows:

$$\% \text{ helix} = \frac{[\theta]_{222}}{-40000} \times 100 \quad (5)$$

Measurement of Autoxidation Rate. The autoxidation rate constant of frozen/thawed Mb was determined according to the method reported by Matsuura et al. (1962) with some modifications as follows. Bluefin tuna metMb was dissolved in a 50 mM phosphate buffers (with or without 0.2 M NaCl) of a given pH, and to the solution (15 mL) was added a trace of sodium hydrosulfite to convert metMb into deoxyMb. The deoxyMb was further converted into the oxy form by shaking the solution gently. The oxyMb solution was incubated at 20 or 30 °C under gentle shaking. A small portion of each Mb solution was taken out at 5–30-min intervals over a period up to 120 min, and the metMb to total Mb ratio (metMb %) was measured by the procedure as described below. Autoxidation rate (k) of Mb was calculated by a first-order reaction analysis of triplicate measurements (George and Stratmann, 1952; Matsuura et al., 1962). For comparison, the autoxidation rate of Mb isolated from unfrozen fresh dark muscle of bluefin tuna was similarly examined.

MetMb % was determined essentially according to the procedure reported by Sano and Hashimoto (1958). Briefly, a sample solution was mixed with an equal volume of 1 M phosphate buffer (pH 7.0) and divided into three portions. A trace of sodium hydrosulfite was added to the first portion, and to the mixture was gently bubbled through CO gas, in order to completely convert the whole Mb into carbon monoxide Mb. Absorbance at 568 nm was recorded (A_1). The second portion was treated similarly except that sodium hydrosulfite was not added and the absorbance determined (A_2). The whole Mb in the third portion was completely converted into metMb by adding a trace of ferricyanide, and the absorbance was determined (A_3). MetMb % was calculated by

$$\text{MetMb } \% = \frac{A_1 - A_2}{A_1 - A_3} \times 100 \quad (6)$$

RESULTS

Changes in the Soret band of bluefin tuna Mb as a function of Gdn·HCl concentration disclosed that higher concentrations of Gdn·HCl were required to unfold completely the unfrozen Mb and frozen/thawed Mb at neutral pH than at acidic pH (data not shown). Both Mb's were unfolded in a one-step process with two transition states, reminding us of the data reported for sperm whale and horse Mb (Schechter and Epstein, 1968; Puett, 1973). Therefore, the equations for calculating ΔG_D were judged to be applicable to unfrozen and frozen/thawed bluefin tuna Mb.

ΔG_D values calculated by eq 1 and 2 were dependent on pH and Gdn·HCl concentration for both unfrozen and frozen/thawed Mb. pH dependency of these values in 50 mM sodium phosphate buffer is shown in Figure 1. ΔG_D values decreased with increasing Gdn·HCl concentration linearly, irrespective of pH, and its slope against Gdn·HCl concentration became steeper with decreasing pH. ΔG_D values calculated for unfrozen Mb were very similar to those for frozen/thawed Mb at the same Gdn·HCl concentration. Similar results were obtained in the presence of 0.2 M NaCl (data not shown).

The $\Delta G_D^{H_2O}$ value was calculated by eq 3, and its pH dependency for unfrozen and frozen/thawed Mb in 50 mM

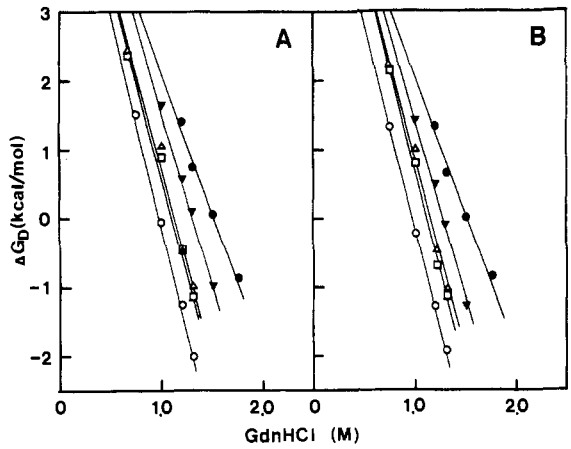

Figure 1. Influence of Gdn·HCl concentration on the free energy (ΔG_D) for unfolding the unfrozen (A) and frozen/thawed (B) bluefin tuna Mb. A frozen/thawed sample was prepared by freezing Mb solution in dry ice–acetone, followed by 2-h storage at −80 °C and subsequent thawing at 20 °C. Solvent: 50 mM sodium phosphate buffer (pH 5.5–6.9). Symbols used: ○, pH 5.55; △, pH 5.85; □, pH 6.04; ▼, pH 6.50; ●, pH 6.90.

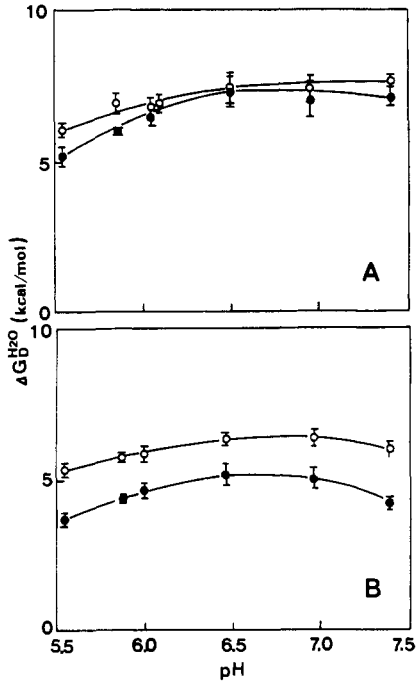

Figure 2. pH dependency of the conformational free energy in the absence of Gdn·HCl ($\Delta G_D^{H_2O}$) of unfrozen (○) and frozen/thawed (●) bluefin tuna Mb, as calculated by extrapolation from Figure 1 using eq 3 in the text. Solvent: 50 mM sodium phosphate buffer (A); 50 mM sodium phosphate buffer containing 0.2 M NaCl (B). Each point represents the mean ± SD of three determinations.

sodium phosphate buffer is shown in Figure 2A. The $\Delta G_D^{H_2O}$ of unfrozen Mb decreased with decreasing pH from 6.5 to 5.5 and remained almost constant above pH 6.5 with a value around 7.5 kcal/mol. For frozen/thawed Mb, the $\Delta G_D^{H_2O}$ values were lower than those of unfrozen Mb by 0.1–0.9 kcal/mol. It was noted that the difference was smallest at around pH 6.5.

When bluefin tuna Mb was dissolved in 50 mM sodium phosphate buffer containing 0.2 M NaCl, the highest value of $\Delta G_D^{H_2O}$ was obtained at around pH 6.5 again: i.e., 6.4 and 5.2 kcal/mol for unfrozen and frozen/thawed Mb, respectively (Figure 2B). In addition, the values obtained in this buffer were generally lower than those in 50 mM phosphate buffer.

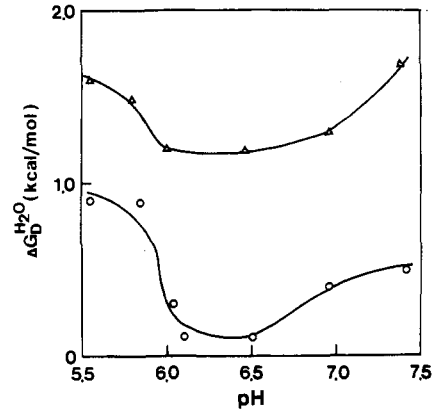

Figure 3. pH dependency of the difference in $\Delta G_D^{H_2O}$ between unfrozen and frozen/thawed bluefin tuna Mb, in 50 mM sodium phosphate buffer (○) and the same buffer containing 0.2 M NaCl (△).

Table I. α-Helical Content of Unfrozen and Frozen/Thawed Bluefin Tuna Mb in 50 mM Sodium Phosphate Buffer

	α-helical content, %	
pH	unfrozen Mb	frozen/thawed Mb[a]
5.55	65	63
5.85	67	65
6.04	67	65
6.40	67	67
6.95	67	67
7.41	67	67

[a] The frozen/thawed Mb solution was prepared by freezing metMb solution at −80 °C for 2 h, subsequent thawing at 20 °C, and filtering through a Millipore filter (pore size 0.45 μm).

Differences in $\Delta G_D^{H_2O}$ between unfrozen and frozen/thawed Mb, which correspond to the degree of denaturation during freezing and thawing, were plotted as a function of pH and are shown in Figure 3. In 50 mM sodium phosphate buffer, the difference was minimum (0.1 kcal/mol) at around pH 6.5, and increased up to 0.5–0.9 kcal/mol at lower or higher pH values. In the presence of 0.2 M NaCl, the differences in $\Delta G_D^{H_2O}$ were clearly enhanced and the minimum value (1.2 kcal/mol) appeared between pH 6.0–6.5.

In 50 mM sodium phosphate buffer, the $[\theta]_{222}$ values for unfrozen Mb were in a range from 25 000 to 27 000 deg·cm^2/dmol. The values of frozen/thawed Mb were clearly lower than those of unfrozen Mb, especially below pH 6.5. When Mb was dissolved in 50 mM phosphate buffer containing 0.2 M NaCl, the values fell in a narrower range of 26 000–27 000 deg·cm^2/dmol, and the values for unfrozen Mb were higher by ~1000 deg·cm^2/dmol than those for frozen/thawed Mb, over the pH range examined. These values were somewhat higher than those of Mb obtained in the absence of NaCl for both unfrozen and frozen/thawed Mb.

In 50 mM phosphate buffer, α-helical contents calculated by eq 5 were in ranges of 65–67% and 63–67% for unfrozen and frozen/thawed Mb (Table I). In the presence of 0.2 M NaCl, the values were 66–68% and 65–67% for unfrozen and frozen/thawed Mb, respectively (data not shown). Smaller values were obtained at lower pH region, although the decrease associated with freezing and thawing was 2% at the largest. Thus, the α-helical content seems not to provide any direct evidence for the conformational change.

Autoxidation rates of frozen/thawed Mb in 50 mM phosphate buffers with or without 0.2 M NaCl are shown in Figures 4 and 5, along with the previous data for un-

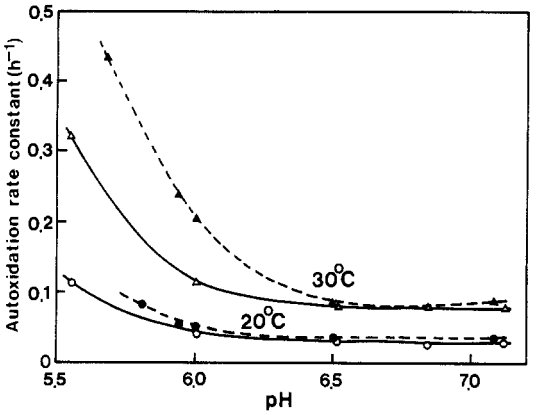

Figure 4. Autoxidation of frozen/thawed bluefin tuna Mb in 50 mM sodium phosphate buffer at 20 (●) and 30 °C (▲), in comparison with that of unfrozen Mb at 20 (○) and 30 °C (△).

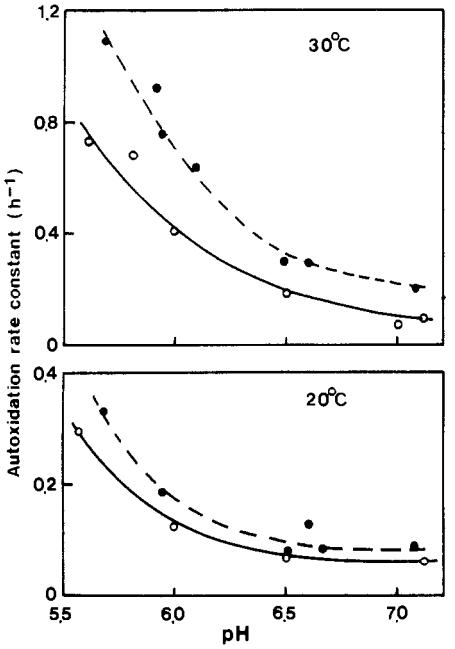

Figure 5. Autoxidation of frozen/thawed bluefin tuna Mb in 50 mM sodium phosphate buffer containing 0.2 M NaCl at 20 (●) and 30 °C (▲), in comparison with that of unfrozen Mb at 20 (○) and 30 °C (△).

frozen Mb (Chow et al., 1985). The rate was not largely affected at 20 °C in 50 mM sodium phosphate buffer, irrespective of pH. At 30 °C, however, the rate of frozen/thawed Mb was generally higher than that of unfrozen Mb below pH 6.5 (Figure 4). The lower the pH was, the more remarkably the autoxidation was accelerated. In 50 mM phosphate buffer containing 0.2 M NaCl, the rate of frozen/thawed Mb was clearly higher than that of unfrozen Mb, regardless of pH and incubation temperatures tested (Figure 5). In this connection, it should be noted that the autoxidation rate of bluefin tuna Mb isolated from unfrozen fresh dark muscle did not differ from that of the Mb which was isolated from frozen dark muscle (data not shown).

DISCUSSION

Frozen/thawed bluefin tuna Mb was more susceptible to unfolding than unfrozen Mb, especially in the presence of 0.2 M NaCl (Figure 2). A pH dependency of $\Delta G_D^{H_2O}$ which decreased with decreasing pH from 7 to 4 was reported for sperm whale and horse Mb (Puett, 1973). In the present study, a similar pH dependency was obtained in unfrozen bluefin tuna Mb in 50 mM sodium phosphate. However, it was noted that the Mb remained most stable at around pH 6.5 during freezing and thawing. The differences of $\Delta G_D^{H_2O}$ between unfrozen and frozen/thawed bluefin tuna Mb (Figure 3) gave a pH dependency reminiscent of the pH dependency in autoxidation of this Mb associated with freezing and thawing: MetMb % and insolubilization ratio were minimum at around pH 6.5 (Chow et al., 1985).

In the presence of NaCl, the highest values $\Delta G_D^{H_2O}$ were found at pH 6.5 with both unfrozen and frozen/thawed Mb and were lower than those in the absence of NaCl. These results suggested that NaCl affected the stability of unfrozen and frozen/thawed Mb. In addition, the differences in $\Delta G_D^{H_2O}$ between the unfrozen and frozen/thawed Mb were enhanced in phosphate buffer containing 0.2 M NaCl, and its minimum appeared between pH 6.0–6.5. In this connection, the minimum value of metMb % also increased and shifted from pH 6.5 to about 6.0, when 0.2 M NaCl was added to 50 mM phosphate buffer (Chow et al., 1987).

$\Delta G_D^{H_2O}$ values obtained in the present study (3.8–7.7 kcal/mol) were smaller than those reported previously for bluefin tuna Mb (9.1 kcal/mol) in 50 mM phosphate buffer (pH 7.0) containing 0.1 M KCl (Balestrieri et al., 1978). The $\Delta G_D^{H_2O}$ value of horse Mb was reported to be 11.0 kcal/mol in 10 mM phosphate buffer (pH 8.0) containing 0.1 M KCl (Schechter and Epstein, 1968) and 7.9 kcal/mol in 0.1 M phosphate buffer (pH 6.6) containing 0.1 M KCl (Ahmed and Bigelow, 1982). These discrepancies may partly be attributed to different calculation methods adopted (Pace and Vanderburg, 1979).

Garcia-Moreno et al. (1985) found that both helical and interhelical portions of sperm whale Mb showed the maximum electrostatic free energy at around pH 6.5. The present results, along with those findings, suggested that the environmental pH affects the conformation of globin.

Differences in helical content between unfrozen and frozen/thawed bluefin tuna Mb were not as pH dependent as those in free energy nor did they provide any clue to elucidate the mechanisms involved in pH-dependent metMb formation associated with freezing and thawing, because the presence of 0.2 M NaCl caused no noticeable change. However, the results obtained suggested that conformation of frozen/thawed Mb was perturbed more in nonhelical rather than helical regions.

Autoxidation rate of frozen/thawed bluefin tuna Mb was much higher than that of unfrozen Mb (Figures 4 and 5). These results demonstrated that the conformation of Mb was affected by freezing and thawing. Bito (1973) reported that the tuna meat, once frozen and thawed, seemed to discolor quickly during subsequent iced storage. The mechanism involved may be the same as that with Mb per se, as described above. However, autoxidation rate constants were very similar between Mb preparations isolated from unfrozen fresh and frozen/thawed dark muscle. There could be two possible reasons for these rather unexpected results. One is the presence of some protective substances in the muscle, which prevent Mb from conformational changes or denaturation during freezing and thawing. In this connection, Brown and Dolev (1963) observed that autoxidation rate of beef or yellowfin tuna Mb during freezing and thawing was clearly slower in crude extracts than in pure solution. An alternative reason is that only native or intact Mb might have been isolated from the muscle by the procedure adopted here.

In conclusion, the stability of bluefin tuna Mb was decreased by freezing and thawing especially at acidic pH, accompanying the acceleration of autoxidation rate. The

results obtained suggested that Mb suffered conformational changes at nonhelical region during freezing and thawing.

ABBREVIATIONS USED

CD, circular dichroism; ΔG_D, free energy for unfolding of a protein; $\Delta G_D^{H_2O}$, free energy for unfolding a protein in the absence of denaturant; Mb, myoglobin; metMb, metmyoglobin; Gdn·HCl, guanidine hydrochloride; $[\theta]_{222}$, mean residue ellipticity at 222 nm.

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Thermally Induced Complex Formation in an Artificial Milk System

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Artificial casein micelles were prepared from their component caseins in a milk salts buffer and mixed with whey proteins prior to heating at 95 °C for 30 s or 20 min. Trace amounts of radiolabeled κ-casein, β-lactoglobulin, α-lactalbumin, or α_{s2}-casein, in various combinations, were included in the mixtures. The heated artificial milk samples were chromatographed on Sephacryl S-300 in a dissociating buffer of 6 M guanidine hydrochloride, and the proportion of the labeled proteins that had formed high molecular weight complexes eluted at the void volume of the column. Analysis of these heat-induced complexes by gel filtration on Sephacryl S-1000 or controlled-pore glass in 6 M guanidine hydrochloride showed that β-lactoglobulin complexed most readily and that α_{s2}-casein was relatively unreactive. When a mixture of α_{s2}-casein and β-lactoglobulin was heated, complexes formed more readily, suggesting that the positioning of α_{s2}-casein within the casein micelle inhibited its reaction with the denaturing β-lactoglobulin or α-lactalbumin. The structure of α_{s2}-casein is likely to be such that its disulfide bond(s) may be less available for reaction than those of κ-casein.

Milk is heat-treated for a number of technologically important effects. This heat treatment brings about a series of protein–protein interactions in the milk. One that was identified in early studies was the formation of a complex between β-lactoglobulin, the major whey protein, and κ-casein, a cysteine-containing protein that generally lies on the surface of the casein micelle. This interaction has been studied extensively, particularly in model systems. While many studies have involved complex formation between β-lactoglobulin and κ-casein, or the loss, by thermal denaturation, of the whey proteins from the serum phase, relatively few have focused on the formation of the large protein complexes in milk. No doubt this is partly because of the complexity of the system. Creamer et al. (1978) isolated a heat-induced protein complex from heated milk that had been dispersed in citrate buffer and showed that its amino acid composition was consistent

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