Lipophilization of α_{s1}-Casein. 2. Conformational and Functional Effects

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Covalent incorporation of palmitoyl residues into α_{s1}-casein dramatically improved its ability to form and stabilize emulsions. Foam stability increased with an increase in incorporation and foam activity increased until the moles of palmitoyl residues attached per mole of the protein ($\bar{\nu}$) reached 6. Circular dichrosim studies revealed conformational change when the incorporation was the minimum ($\bar{\nu}$ = 0.3) whereas the secondary structure remained essentially unaltered in water at pH 6.8 and 9.0 in the rest of the palmitoyl proteins when $\bar{\nu}$ was 0.8, 2.5, 6.0, 8.8, 10.5, and 11.1. There was little change in the tertiary structure except when $\bar{\nu}$ = 0.3, and in the presence of glycerol the palmitoyl proteins seemed less structurally disturbed than the native protein. The increase of helical content at pH 1.5 indicated that hydrophobic ligands did not hinder organization through hydrogen bonding.

The hydrophilic shell of the protein has hydrophobic patches even in the native state. However, not only does the covalent attachment of a long-chain fatty acid create a new hydrophobic patch but also the taillike ligand, unless completely withdrawn within the protein core, should have a great deal more spacial flexibility to interact hydrophobically and could thereby geometrically enable the bulky fatty acyl protein molecules to associate by hydrophilic–liphophilic arrangement. The free energy of transfer of undissociated palmitic acid from a dilute aqueous buffer solution to liquid n-heptane at 23–25 °C is about 9 kcal/mol (Smith and Tanford, 1973). It is possible, however, that such incorporation may cause a drastic conformational change, thereby exposing parts of the hydrophobic core that could then act as new sites for hydrophobic interaction and this could contribute to increasing the amphipathic nature of the protein rather than the contribution made by the fatty acyl moiety per se. Tanford (1980) has noted that the native conformation of a protein molecule possesses only marginal stability because it is highly constrained.

In the preceding paper (Haque and Kito, 1983a) we have reported that palmitoyl residues can be covalently attached to α_{s1}-casein; stable isopeptide bonds are formed with the ϵ-amino groups of lysine residues that are predominantly the reactive species. The palmitoyl proteins showed an increased tendency to associate, and we have deduced that the palmitoyl-α_{s1}-casein molecules were able to form micelles. However, we are aware that α_{s1}-casein contains about 40% amino acids with hydrophobic side chains (Mercier et al., 1971), much of which could be exposed (Waugh et al., 1970). Incorporation of hydrophobic ligands would logically be expected to have little or even an adverse effect on the amphipathicity of the protein molecule. Furthermore, the α_{s1}-casein molecule, which is not stabilized by disulfide bonds, is unstable and easily unfolded by environmental changes (Ono et al., 1974a).

Our present paper deals with an attempt to observe the effect of the covalent attachment of palmitoyl residues to α_{s1}-casein on its conformational, surface, and interfacial characteristics.

EXPERIMENTAL SECTION

Materials. Soybean oil and androsterone were from Nakarai Chemicals, Ltd. The Millipore filter (0.45 μm) was from Millipore Corp., Bedford, MA. All other reagents were of analytical grade.

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Methods. *Lipophilization of α_{s1}-Casein.* Palmitoyl-α_{s1}-casein of various degrees of incorporation were obtained from bulk preparation experiments described in the preceding paper (Haque and Kito, 1983a), and the average number of moles of palmitic acid per mole of α_{s1}-casein has been denoted by $\bar{\nu}$. When $\bar{\nu}$ was 0.3, 0.8, 2.5, and 6.0, the palmitoyl proteins have been termed "less incorporated", and when $\bar{\nu}$ was 8.8, 10.5, and 11.1, the proteins have been termed "highly incorporated" for easy reference. The palmitoyl protein samples were stored only for short periods at 4 °C, and different concentrations were obtained for the following experiments by ultrafiltration as described in the preceding paper.

Circular Dichroism (CD) Measurements. Spectra were recorded with a Jasco J-5000C spectropolarimeter fitted to a Jasco Model DP-501 data processor and Jasco DP-104A data printer. The spectral bandwidth was 1 nm, the scanning rate was 5 nm/min, the sampling time interval was 10 ms, the sensitivity was 2 m^0/cm, the time constant was 4 s, the split width was adjusted automatically, and the number of repetitions per spectrum was four. The sample box was continuously flushed with nitrogen to prevent the formation of ozone and interference by oxygen at the far-UV range. The polarimeter was calibrated prior to each session with 0.05% (w/v) androsterone in dioxane as specified by the manufacturers. Quartz cells of 10-mm path length were used for the near-UV range (250–310 nm) and a cell of 1-mm pathlength was used for the far-UV range (190–250 nm). A 0.1 mm path length cell was used in the far-UV range only when perturbants were present in the protein solution. Solvent correction was made for all samples studied. Protein solutions were equilibrated to 22 °C and diluted to 0.01% for far-UV and 0.1% for near-UV studies and passed through a Millipore filter (0.45 μm) prior to CD analysis. In the presence of perturbants, the concentration was 0.2% for both far- and near-UV ranges. Sodium phosphate and sodium borate buffers (20 mM) were used for ellipticity study at pH 6.8 and 9.0, respectively, whereas 20 mM HCl was used for study at pH 1.5. The perturbants used were ethanol and glycerol. Ethanol (99.5%) was used in a 8:2 ratio with 20 mM imidazole (pH 6.8) or sodium borate buffer (pH 9.0) whereas glycerol was used in the ratios of 2:8 and 4:6 in 20 mM sodium phosphate buffer (pH 6.8).

The spectra obtained below 250 nm have been reported in terms of [Θ], the mean residual ellipticity with the dimensions of deg·cm^2·dmol^{-1}, which was calculated as

$$[\Theta] = 100d \cdot \mathrm{MRW} \cdot C^{-1} \quad (1)$$

where d is the rotation in degrees per 1.0-cm light path,

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C is the concentration of the protein solution in g/L, and MRW stands for mean residual weight of the protein, which was calculated to be 119 (Mercier et al., 1971). Spectra obtained above 250 nm are related more closely to the asymmetry of the aromatic tyrosine, phenylalanine, and tryptophan residues than to the protein peptide bonds (Jirgensons, 1973) and are better presented in terms of $\Delta\epsilon$, the molar circular dichroism (Strickland, 1974), which was calculated as

$$\Delta\epsilon = 0.0304 d \cdot M \cdot C^{-1} \quad (2)$$

where M is the molecular weight of the protein, which is 23613 (Mercier et al., 1971), and the rest of the values are as described above.

Estimation of Secondary Structure. The far-UV CD spectra were analyzed by the method proposed by Hennessey and Johnson (1981). The method is based on mathematical calculation of orthogonal basis CD spectra from the CD of proteins of known structure. Eight types of secondary structure were considered: helix; parallel and antiparallel β strand; type I, II, and III β turn; other β turns combined; "other" or random structures. β turns were classified into types as per the criteria of Chou and Fasman (1977). However, all β turns other than types I–III were grouped into a general catagory of turns (T).

A Facom AD-160 computer at the computer center of Kyoto University, Uji campus, was used for all calculations. A Hewlett-Packard plotter (7221T) connected to a Nippon minicomputer (Dasher TPI) was used subsequently to plot the different figures. The reliability index (RI) was calculated from, [CD(calculated) − CD(observed)]/CD(observed).

Emulsification. (1) Emulsification Activity (EA). The turbimetric end point determination method described by Pearce and Kinsella (1978) was adapted for our purpose. The protein solution (0.4% w/v) was kept constant at 3 mL (i.e., 12 mg of protein), and the oil to water ratio (o:w) was adjusted to 0.2, 0.5, 1.0, 2.0, and 3.0 by only increasing the amount of oil. The protein solution in 25 mM sodium phosphate buffer, pH 6.8, was added to a measured amount of soybean oil placed in a small beaker of 15-mL capacity and sonicated at 25 °C with a Branson sonifier (Cell Disrupter W-200P) fitted with a "stepped microtip" and operated at position 3 of a total amplitude of 10. Preliminary experiments were conducted to determine the time required to achieve a constant level of emulsification (60 s) and especial care was taken not to generate bubbles that easily form if the tip is not properly submerged. The exact degree of submersion that created a "whirlpool effect", which facilitated homogeneity, was fixed and kept constant for all samples. Three 10-μL portions were immediately pipetted off from different parts of the emulsion and diluted 2000-fold in 1% (w/v) SDS in water (20 mL) that had been previously measured out in test tubes, and the tube inverted 3 times to afford a homogeneous mixture following which the absorbance at 600 nm was recorded by using a Carl Zeiss (M4 0111) spectrophotometer. Light path was 1 cm in all cases. EA was the mean value of these three readings.

(2) Emulsion Stability (ES). The infuence of gravity on the stability of the emulsion was observed by placing the emulsion in graduated tubes (10-mL capacity) with conical bottoms, which facilitated fine graduation, and the "gravity-caused drainage", at different time intervals of 24, 48, and 96 h, was expressed as a percentage of the initial emulsion volume.

Whipping Property. When L^0 and L^1 are the volumes of the protein solution before and after shaking and F^1 and F^2 are the volumes of the foam after shaking and after

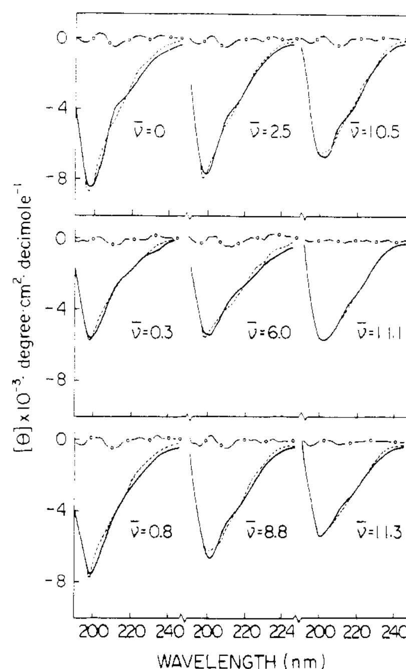

Figure 1. Far-UV CD spectra of native ($\bar{\nu}$ = 0) and palmitoyl proteins. (—) Observed spectra; (---) calculated spectra; (-o-) deviation of the observed spectra from the calculated spectra.

standing for 30 min, respectively, foam activity (FA) and foam stability (FS) may be expressed as

$$FA = \left(\frac{L^1 + F^1}{L^0} - 1\right) \times 100 \quad (3)$$

$$FS = \frac{100F^2}{F^1} \quad (4)$$

FA indicates whether a protein has the capacity to foam and, if so, to what extent when the increase in volume over L^0 is considered to be the activity. Different proteins may give foams of different gaseous content and therefore foam density (FD) has been taken into account and has been calculated as

$$FD = \frac{100(L^0 - L^1)}{F^1} \quad (5)$$

Experiments were carried out as described before (Haque et al., 1982) using 0.5% protein solution in water adjusted to pH 7.0 with 0.1 N HCl or NaOH.

RESULTS

Conformational Effects. The native α_{s1}-casein gave a spectrum in neutral solution (see Methods) that was similar to that obtained by Timasheff et al. (1967) but at a slightly reduced ellipticity with the trough at around 200 nm being appromximately 8500 deg·cm^2·dmol^{-1}. Even though we prepared α_{s1}-casein in the presence of urea, it has been reported that such a method does not cause irreversible denaturation (Ono et al., 1974b). The spectra of palmitoyl proteins of different degrees of incorporation were obtained at pH 6.8 and have been shown in Figure 1 along with the calculated values. The intensity of the negative trough seems to be reduced when $\bar{\nu}$ was 0.3, 6.0 and 11.3, being 5550, 7570, 7590, 5410, 6640, 6630, 5670, and 5430 deg·cm^2·dmol^{-1} when $\bar{\nu}$ was 0.3, 0.8, 2.5, 6.0, 8.8, 10.5, 11.1, and 11.3, respectively. The negative extreme that was initially at 198 nm seemed to shift to 200 nm after $\bar{\nu}$ reached 2.5. On the other hand, the intensity of the negative trough at pH 9.0 was 7400 deg·cm^2·dmol^{-1}, and

J. Agric. Food Chem., Vol. 31, No. 6, 1983 1233

Table I. Secondary Structure of Native and Lipophilized α_{s1}-Casein at Neutral and Alkaline pH[a]

$\bar{\nu}$	pH	helix	β[b]		β turns				"others"	RI
			antipara	para	I	II	II	T		
0[c]	6.8	10.5	24.5	1.2	8.1	5.1	2.4	5.5	42.7	0.08
	9.0	8.1	27.2	3.8	8.4	4.6	2.4	5.5	40.1	0.10
0.3	6.8	10.6	26.4	0.1	8.4	5.3	2.5	5.5	41.3	0.07
	9.0	10.8	20.3	0.0	7.8	5.5	2.4	5.7	48.0	0.14
0.8	6.8	10.2	25.0	2.1	8.2	4.9	2.4	5.5	41.6	0.07
	9.0	9.1	26.1	2.2	8.3	4.9	2.3	5.5	41.5	0.09
2.5	6.8	10.2	25.3	2.5	8.2	4.8	2.4	5.5	41.2	0.06
	9.0	9.0	25.7	3.9	8.2	4.5	2.3	5.5	40.9	0.07
6.0	6.8	11.4	25.4	2.3	8.2	4.7	2.5	5.5	40.0	0.08
	9.0	10.7	25.4	2.6	8.2	4.7	2.5	5.5	40.4	0.07
8.8	6.8	10.7	26.3	3.1	8.3	4.6	2.5	5.5	39.0	0.07
	9.0	10.4	25.5	3.5	8.2	4.5	2.5	5.5	39.9	0.09
10.5	6.8	11.9	25.7	2.5	8.2	4.7	2.5	5.4	39.2	0.05
	9.0	10.2	24.2	4.4	8.0	4.4	2.4	5.4	41.1	0.08
11.1	6.8	11.4	27.2	2.8	8.4	4.6	2.6	5.4	37.6	0.04
	9.0	10.7	25.2	4.4	8.2	4.3	2.5	5.5	39.3	0.09

[a] See Methods. [b] β antiparallel (antipara) and parallel (para) pleated structure. [c] Native α_{s1}-casein.

Table II. Secondary Structure of Native and Lipophilized α_{s1}-Casein at pH 1.5

$\bar{\nu}$	helix	β[a]		β turns				"others"	RI
		antipara	para	I	II	III	T		
0[b]	15.4	28.2	0.3	8.7	4.9	3.1	5.5	33.9	0.11
0.8	15.0	30.6	0.2	8.4	4.3	3.0	5.7	32.8	0.12
2.5	18.0	27.3	0.0	8.7	4.9	3.3	5.5	32.6	0.07
6.0	19.3	24.2	0.3	8.2	4.7	3.2	5.4	34.6	0.07

[a] β antiparallel (antipara) and parallel (para) pleated structure. [b] Native α_{s1}-casein.

those of the palmitoyl proteins of the increasing order of incorporation as mentioned above were 1900, 6450, 6140, 5910, 6020, 5900, 5550, and 5530 deg·cm^2·dmol^{-1}, respectively. The amplitude of the least incorporated sample was unusually low. It may be mentioned here that when incorporation was being carried out at a active ester (16:0-Osu) to lysine mole ratio of 0.05 to obtain the least incorporated sample ($\bar{\nu}$ = 0.3), the protein solution (80% ethanol, v/v), which had been crystal clear before, became highly turbid and this turbidity disappeared following dialysis against water. Such a phenomenon was not observed with the other mole ratio experiments. However, when the reaction temperature was decreased to below 25 °C and the mole ratio was high (1.5), the reaction seemed to stop following the rapid development of turbidity as there was no further need to adjust the pH but this was immediately overcome by increasing the temperature to 30 °C.

The initial attachment of the hydrophobic ligand (at Lys-34 and/or -36) (Haque and Kito, 1983a) through the formation of a covalent bond, which has a heat of formation (30–100 kcal/mol) that is much higher than that of the hydrogen bond (1–5 kcal/mol), electrostatic bond (10–20 kcal/mol), or van der Waals attractive forces (1–3 kcal/mol) (Whitaker, 1977), causes a drastic change in general conformation whereby the protein became insoluble in a relatively hydrophobic system but was soluble in aqueous system. Using the secondary structure prediction method of Chou and Fasman (1974) and using 29 reference proteins (Chous and Fasman, 1978; Haque and Kito, 1983b) we saw that Lys-34 and Lys-36 are in a site on the polypeptide chain where helical structure is possible. The clear aqueous solution of the least incorporated protein ($\bar{\nu}$ = 0.3), however, gave a lower ellipticity at the neutral and much lower ellipticity at the alkaline pH in which, as will be seen later, the ellipticity at the near-UV range was also almost nil. Even though palmitoyl proteins were soluble in the reaction mixture when $\bar{\nu}$ was 0.8, 2.5, and 6.0, like the least incorporated sample these too were insoluble in the reaction solvent following dialysis in water. Table I, however, shows that only when $\bar{\nu}$ was 0.3 was there some noticeable change in the observed secondary structure, whereas those of the rest of the above palmitoyl proteins apparently remained essentially unaltered. It is probable that the "other" structure changed from one form to another within the same "subset" and an occult change had actually occurred. When $\bar{\nu}$ was 8.8, 10.5, and 11.1, the proteins displayed the ability to give a clear solution in both neutral and alkaline buffer and in 80% ethanol but was insoluble at pH 1.5. Table I shows that the secondary structure of these "highly incorporated" samples was similar except for a slight tendency for helical and parallel structure to increase with a concomitant decrease in the "other" structure.

Table II the secondary structure of native and incorporated α_{s1}-casein when $\bar{\nu}$ was 0.8, 2.5, and 6.0 at pH 1.5. As mentioned earlier, palmitoyl proteins with a higher degree of incorporation were insoluble at this pH. When $\bar{\nu}$ was 0.3, the computed data repeatedly showed a high RI value (see Methods) under this condition and hence was excluded. The helical content shows an inclination to increase even more than it does in the native protein under this condition except when $\bar{\nu}$ was 0.8 when the helical content was similar to the native protein. The antiparallel β pleated structure increases initially but this decreases as the helical content increases with incorporation.

Figure 2 shows the near-UV CD spectra of the native and palmitoyl proteins at pH 1.5, 6.8, and 9.0 and in the presence of perturbants. Figure 2A shows the spectra at pH 6.8 where the native protein displays the characteristic tyrosyl bands at 273 and 280 nm and the phenylalanyl (^1L_b) fine structure at 257, 263, and 269 nm. The intensity of the dichroism increases toward the shorter wavelength as $\bar{\nu}$ increases to 6.0 and then decreases with a further increase, which tends to deepen and slightly red shift the negative trough between 275 and 282 nm. Two low-intensity bands are seen at around 294 and 301 nm. Since the protein under study does not have any disulfide, the

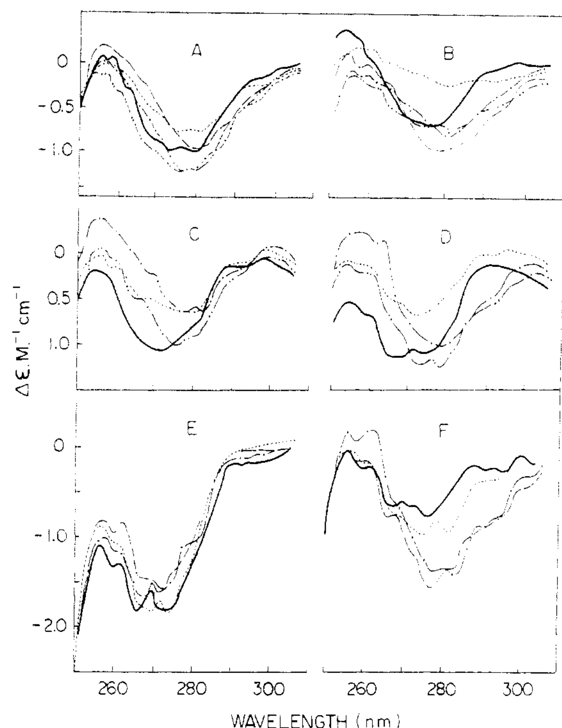

Figure 2. Near-UV CD spectra of palmitoyl proteins under different conditions (see Methods). (A) pH 6.8: (—) native (similar to $\bar{\nu}$ = 0.8); (---) $\bar{\nu}$ = 0.3; (– –) $\bar{\nu}$ = 2.5 (similar to $\bar{\nu}$ = 6.0); (-●-) $\bar{\nu}$ = 8.8 (similar to $\bar{\nu}$ = 10.5); (-●●-) $\bar{\nu}$ = 11.1. (B) pH 9.0: same as (A). (C) 20% glycerol, pH 7.0: (—) native; (---) $\bar{\nu}$ = 0.8 (similar to $\bar{\nu}$ = 2.5 and 6.0); (-●-) $\bar{\nu}$ = 8.8 (similar to $\bar{\nu}$ = 10.5); (-●●-) $\bar{\nu}$ = 11.1. (D) 40% glycerol, pH 7.0; same as (C) except that $\bar{\nu}$ = 6.0 was similar to $\bar{\nu}$ = 8.8. (E) 80% ethanol, pH 9.0: (—) native; (---) $\bar{\nu}$ = 8.8; (– –) $\bar{\nu}$ = 10.5; (-●-) $\bar{\nu}$ = 11.1. (F) pH 1.5: (—) native; (---) $\bar{\nu}$ = 0.8; (– –) $\bar{\nu}$ = 2.5; (-●-) $\bar{\nu}$ = 6.0.

only other fine structure that occurs in this region is that of the 0 p 850^{-1} $^{1}L_b$ and O–O bands of tryptophanyls. The spectrum of palmitoyl protein having a $\bar{\nu}$ value of 0.8 has not been shown since it was similar to that of the $\bar{\nu}$ = 2.5 sample at both pH 6.8 and pH 9.0. The least incorporated sample ($\bar{\nu}$ = 0.3) shows a shallower trough in the midregion and hardly any diichroism at pH 9.0 (Figure 2B), indicating a large conformational change, and has not been studied any further since it could not be compared with the other palmitoyl proteins.

In the presence of 20% glycerol (pH 7.0) (Figure 2C), the native protein showed the most prominant broad negative trough, the tyrosyl being prominant at 273 nm and its O–O band was red shifted to 282 nm. Palmitoyl proteins when $\bar{\nu}$ was 6.0 and 2.5 have not been shown since their spectra were similar to that of the $\bar{\nu}$ = 0.8 sample. In the presence of 40% glycerol (Figure 2D) the smaller wavelength dichroism of the native sample became much more negative and the fine structures of phenylalanyl and tyrosyl were disturbed. Only an intense 288-nm band was seen at the longer wavelength. In the case of the palmitoyl proteins, however, the overall spectra were closer to those seen in the presence of 20% glycerol and the tyrosyl-contributed trough at the midregion was more intense for the highly incorporated samples. The spectrum of the $\bar{\nu}$ = 2.5 sample was similar to that of the $\bar{\nu}$ = 0.8 sample, and those of $\bar{\nu}$ = 10.5, 11.1, and 11.3 samples were similar to the $\bar{\nu}$ = 8.8 sample and have not been shown for reasons of propinquity. The $\bar{\nu}$ = 6.0 sample gave a spectra that was similar to $\bar{\nu}$ = 0.8 and 2.5 except that the negative trough (275–282 nm) was about 1.2 times more intense than that of the $\bar{\nu}$ = 0.8 sample and was similar to the spectra of the highly incorporated samples as far as band positions were

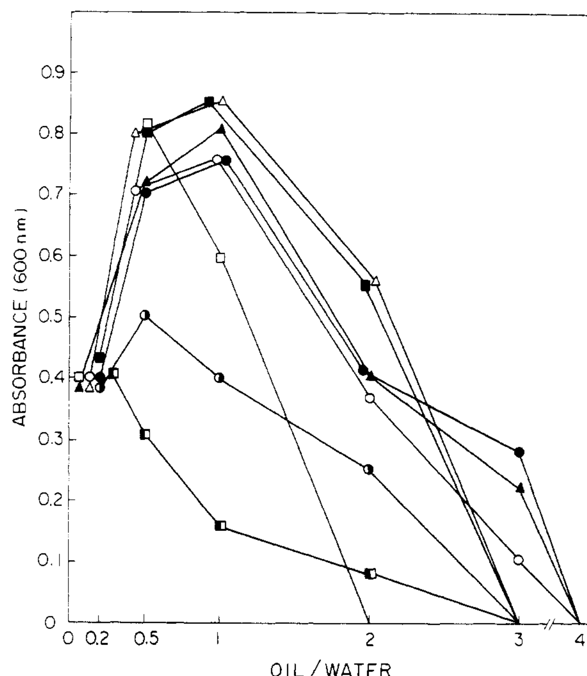

Figure 3. Comparative EA at different oil:water ratios and protein concentrations. Protein concentrations at each oil:water ratio (o:w) were as follows: o:w 0.2, 0.33%; o:w 0.5, 0.26%; o:w 1, 0.20%; o:w 2, 0.10%; o:w 3, 0.1%; o:w 4, 0.08%. (◧) Native; (◑) control; (□) $\bar{\nu}$ = 0.3; (■) $\bar{\nu}$ = 0.8; (△) $\bar{\nu}$ = 6.0 (similar to $\bar{\nu}$ = 2.5); (▲) $\bar{\nu}$ = 8.8; (●) $\bar{\nu}$ = 10.5; (○) $\bar{\nu}$ = 11.1.

concerned. It is apparent that glycerol perturbs the native state but the perturbation decreases as $\bar{\nu}$ increases.

Parts E and F of Figure 2 show the spectra of the highly incorporated samples in 80% ethanol (pH 9.0) and of the less incorporated samples at pH 1.5. Both conditions brought about drastic spectral disturbance that was similar in the native and the palmitoyl proteins.

Functional Effects. *Emulsification Activity (EA).* Figure 3 shows the EA of the different palmitoyl proteins at different oil:water ratios. We see that at a ratio of 0.2, native, control, and all the palmitoyl proteins show similar EA but when the ratio is increased to 0.5 the palmitoyl proteins fare much better, a higher EA being shown by the less incorporated than the highly incorporated samples. EA of palmitoyl protein with a $\bar{\nu}$ value of 2.5 has not been shown since it was similar to that of palmitoyl protein when $\bar{\nu}$ was 6.0 at all oil:water ratios. When the ratio was increased to 1, the least incorporated protein ($\bar{\nu}$ = 0.3) showed a significantly low EA whereas the rest of the less incorporated palmitoyl proteins still showed a higher EA than the highly incorporated samples. As the ratio was increased further to 2, the least incorporated protein ($\bar{\nu}$ = 0.3) failed to show EA even though the control and native samples still did, the higher EA still being shown by the less incorporated. It is noteworthy that the two groups of palmitoyl proteins showed intragroup similarity in EA except when the oil:water ratio was increased to 3, when the less incorporated failed to show any EA, but among the palmitoyl proteins of the highly incorporated group, the palmitoyl protein with a $\bar{\nu}$ value of 10.5 showed the highest EA whereas the highest incorporated sample ($\bar{\nu}$ = 11.1) showed the lowest. The corollary is that the less incorporated proteins showed higher EA up to an oil:water ratio of 2 but only the highly incorporated proteins showed EA at a ratio of 3.

Emulsion Stability (ES). Table III shows the "gravity-caused drainage" as a cumulative percentage (CP) of the initial emulsion volume at an oil:water ratio of 0.5.

Table III. Drainage as Cumulative Percentage (CP) of Initial Emulsion Volume (Oil:Water Ratio of 0.5)[a]

$\bar{\nu}$	24 h	48 h	96 h	7 days[b]
0 (native)	20.0	24.0	28.0	III
0 (control)	22.2	*22.2*	24.1	III
0.3	14.8	*14.8*	*14.8*	II
0.8[c]	*18.5*	*18.5*	*18.5*	II
2.5[c]	*15.4*	*15.4*	*15.4*	I
6.0[c]	*15.4*	*15.4*	*15.4*	*15.4*
8.8[c]	*16.9*	*16.9*	*16.9*	*16.9*
10.5[c]	*28.8*	*28.8*	*28.8*	*28.8*
11.1[c]	*30.7*	*30.7*	*30.7*	*30.7*

[a] Italics indicate no increase in CP. Protein concentration in the system has been shown in the figure legend of Figure 4. [b] Stored at 4 °C after 96 h at 25 °C. I, II, and III indicate an increasing degree of disintegration (flocculation and coalescence). [c] Palmitoyl proteins that showed negligible drainage at higher oil:water ratios.

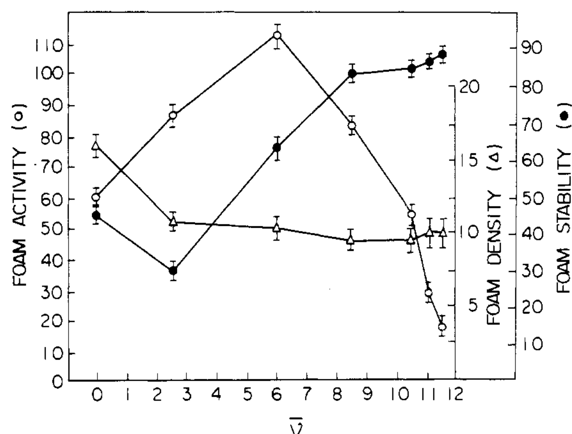

Figure 4. Foam activity (FA), stability (FS), and density (FD) of palmitoyl-α_{s1}-casein.

The drainage appeared macroscopically to entirely consist of the aqueous continuous phase. Both the native and control samples showed a high CP of drainage and the emulsion was completely disintegrated within 7 days. The aqueous drainage of emulsions staiblized by the palmitoyl proteins increased as $\bar{\nu}$ increased. However, the highly incorporated proteins including that when $\bar{\nu}$ was 6.0 gave emulsions that were highly stable following the initial drainage. The drainage at an oil:water ratio of 1 has not been shown since the drainage was almost negligible for the palmitoyl proteins except when $\bar{\nu}$ was 0.3 it was 9.3% (24 h), which did not increase any further up to 96 h but showed distinct signs of coalescence (large oil droplets) after 7 days. The native and control samples showed a similar tendency. The highly incorporated samples showed excellent stability; there was no drainage or visible signs of flocculation or coalescence even affter 7 days, and this was specially noted at an oil:water ratio of 3 when hard emulsions were obtained.

Whipping Property. Figure 4 shows FA, FS, and FD of the different palmitoyl proteins at pH 7.0. The FD was higher in the native and control protein but was about the same for all the palmitoyl proteins shown. The control and palmitoyl proteins having a $\bar{\nu}$ value of 0.3 and 0.8 have not been shown since the FS for the control was less than 10 and that for the palmitoyl proteins was almost nil. FA was the highest when $\bar{\nu}$ was 6.0, following which it decreased as $\bar{\nu}$ increased, whereas the FS increased and appeared similar for all the highly incorporated proteins.

DISCUSSION

Figure 1 shows that after the initial imbalance, the molecule seemed to readjust to the environment, and when $\bar{\nu}$ was ≥0.8, the dichroism was very close to that of the native protein at both the neutral and alkaline pH. About 40% of the amino acids composing α_{s1}-casein are hydrophobic. Mercier et al. (1971) and Waugh et al. (1970) have noted that it should be impossible for the protein to pack all the apolar residues internally. It is therefore highly feasible that the incorporated hydrophobic ligands could not be withdrawn within the core. The palmitoyl protein molecules have a pronounced tendency to associate even in the presence of sucrose the molecules associate when $\bar{\nu} \geq 2.5$ (Haque and Kito, 1983a). Perhaps in the absence of sucrose even when $\bar{\nu}$ was 0.8, the palmitoyl protein associates and the hydrophobic ligands fold one upon the other in aqueous solution so as to reduce the hydrocarbon–water interface. The least incorporated protein ($\bar{\nu}$ = 0.3) was perhaps unable to do so, and hence there was a large change in the conformation as the molecule tried to organize itself to minimize free energy and perhaps thereby caused randomization of the aromatic side-chain conformation. A control was run through the reaction and subsequent dialysis steps in the absence of 16:0–Osu and showed a similar CD spectrum as the native protein at a slightly reduced ellipticity. There was a generaly tendency for the negative extrema to decrease, and the helical content increased gradually as the incorporation increased (Figure 1). At pH 1.5 (Table II), too, the ability to form helical structure increases as incorporation increases. The helical content of the highly incorporated samples showed a similar tendency to increase in 80% ethanol (pH 7 and 9) (data not shown) as did that of the less incorporated samples at pH 1.5. The influence of hydrophobic environment on helical formation has been emphasized (Tanford et al., 1960; Timasheff and Townend, 1965).

While attempting renaturation of alkali-denatured aldolase, Fasman et al. (1970) observed that following dialysis to pH 8.5 the protein only regained 65% of its original ellipticity but no circular dichroism was observed above 250 nm. Thus, as has been proposed by Donovan (1964), this procedure regenerates order in the protein without restoring the original native structure.

The solubility behavior of the incorporated samples may perhaps best be attributed to their highly probable ability to form micelles (Haque and Kito, 1983a). At the acidic pH, these palmitoyl proteins are perhaps insoluble since electrostatic repulsive forces were insufficient to hold off hydrophobic aggregation. Friberg (1976) ascribed emulsion stability to a balance of attractive van der Waals forces and electrostatic repulsive forces derived from oppositely charged ions in a double layer surrounding the globules (DLVO theory). It may be noted that as $\bar{\nu}$ increased the net negative charge also increased (Haque and Kito, 1983a). In other words, the potential for Coulombic interaction between charges and for hydrophobic interaction increased as $\bar{\nu}$ increased. The potential energy (U) for interaction of charged particles q_1 and q_2 separated by distance r in a solvent with a dielectric constant ϵ may be expressed as

$$U = \frac{q_1 q_2}{\epsilon r} \quad (6)$$

Since ϵ for bulk water is about 80 as against 24.3 for ethanol at 25 °C (Williams and Wilson, 1975), U would be more in the presence of ethanol, and this may explain the insolubility of the less incorporated samples in its presence. However, in the case of the highly incorporated samples the van der Waals attractive forces between the long-chain hydrophobic ligands may have overcome U, which is a function of r. That the hydrophobic interaction was strong in the highly incorporated samples, whose potential pos-

itive charge sites were mostly masked, is established by their insolubility at pH 1.5, a pH at which the less incorporated samples were easily soluble. When $\bar{\nu}$ was 6.0, the palmitoyl protein was perhaps in the "border line" and formed a hydrophilic–lipophilic arrangement but was unable to do so when the dielectric of the solvent was decreased.

Far-UV CD bands usually indicate secondary structure of the protein whereas the near-UV CD bands reflect the tertiary structure of the protein (Adler et al., 1973). This holds true in our case since the palmitoyl residue itself does not show any ellipticity. However, when comparing modified proteins, one must consider the possibility that the CD difference reflects a chromophoric modification rather than implies a change in conformation.

The near-UV CD spectra of the palmitoyl proteins at pH 6.8 (Figure 2A) did not differ much from the native spectra except for a slight red shift of the tyrosyl fine structure at 273 and 280 nm. At pH 9.0 (Figuer 2B) the near absence of the phenolate band at 288 nm and the decrease of the dichroism at the short-wavelength zone with the increase in $\bar{\nu}$ could be due to the formation of phenolic ester bonds. This change, therefore, seems more chromophoric than conformational. However, the shifting of the tryptophanyl fine structure, which was similar in the native and the palmitoyl proteins, indicated a change in the polarity of the tryptophanyl environment. In α_{s1}-casein only two tyrosyl residues and half a tryptophan residue are not accessible to water, and all the chromophores are accessible to glycerol (Ono et al., 1974a). In the presence of glycerol (Figure 2C,D) the overall spectra of the palmitoyl proteins were similar to the spectra in the absence of glycerol except for prominant bands at about 288 and 298 nm. These bands are most likely due to the tryptophanyl and its O–O band. In the presence of ethanol (Figure 2E) also a prominent longer wavelength dichroism was seen, the 288-nm band being strong. Perturbants like alcohol and glycol have been reported to enhance the CD in the region where tryptophanyl bands may occur (Ikeda and Hamaguchi, 1970; Barnes et al., 1972; Glazer and Simmons, 1966). The native protein seemed to be much more perturbed in the presence of glycerol than were the palmitoyl proteins. The tyrosyl and tryptophanyl contributions were marked, but unlike in the palmitoyl proteins, the phenylalanyl fine structure was not defined at all. The hydroxy substituent effectively reduces the symmetry of the phenyl chromophore so that the ^1L_b band of tyrosyl is about 8 times stronger than the corresponding band of phenylalanyl (Horwitz et al., 1969).

From the above analysis of the near-UV CD experiments it appears that, except the least incorporated ($\bar{\nu}$ = 0.3) sample, the palmitoyl proteins showed little change in the tertiary structure. The tyrosyl related changes seem more chromophoric than conformational. The change in tryptophanyl band position and contribution of the palmitoyl proteins in the presence of glycerol could reasonably be because of better orientation of the responsible chromophores (Try-164 and -199). Incorporation at Lys-193 (Haque and Kito, 1983a) could affect the orientation of the C-terminal tryptophanyl. Recently, Shimizu et al. (1982) used chymosin and papain to enzymatically cleave the N-terminal and C-terminal side of α_{s1}-casein and figured that the C-terminal end had little to do with the surface activity of the protein. The comparative stability of the palmitoyl proteins is interesting and could give a clue to the protein's stability at a polar-nonpolar interface.

The EA and ES experiments (Figure 3 and Table III) has shown that less incorporated palmitoyl proteins showed a high EA when the oil:water ratio was low but only the highly incorporated samples were able to perform when the oil:water ratio was 3. Even though the initial drainage increased with the incorporation at a oil:water ratio of 0.5 (Table III), the stability following this drainage and at higher ratios was excellent. Even though the basic idea of Figure 4 is to compare the EA of the different palmitoyl proteins at different oil:water ratios, comparison between different ratios was not intended. In analyzing Figure 3 it is important to note that surfactant protein (12 mg, see Methods) and water have been kept constant and for each experiment the amount of oil was adjusted to attain the desired oil:water ratio. Hence, protein in the system was inversely proportional to the oil:water ratio; higher the ratio, lesser the protein in the system (see the legend of Figure 3). This further tested the ability of the protein to adsorb to and stabilize the oil–water interface as the oil:water ratio was increased in the face of decreasing protein concentration. The experiments show that only 12 mg of the highly incorporated palmitoyl α_{s1}-casein was able to emulsify 3 mL of water and 3 times that amount of oil. In other words, only 0.1% (w/v) was enough to form and stabilize an emulsion when the oil:water ratio was 3. It is known that the reduction of interfacial tension is an important factor in the formation and stabilization of emulsions (Lissant, 1974; Friberg, 1976) and that the first molecules of a surfactant introduced into a two-phase system act to form a monolayer; additional surfactant molecules form micelles and stabilize the system by hydrophilic–lipophilic arrangements (Stutz et al., 1973).

In the foaming experiments (Figure 4) the least incorporated samples, when $\bar{\nu}$ was 0.3 and 0.8, do not show any FS, which increased as the incorporation increased and was best for the highly incorporated samples, which on the other hand gave lower FA as $\bar{\nu}$ increased more than 6.0. It is interesting to note that FA was the highest when the tendency for association of the surfactant protein was the highest (Haque and Kito, 1983a). Reduction of surface (and interfacial) tension is an important function that the surfactant protein must perform concurrently with the formation of structured, continuous, cohesive films around the air vacuoles (Kinsella, 1976). Birdi (1973) found no correlation between surface unfolding and molecular weight or helical content of proteins but observed a fair relationship with the polar:apolar ratio. Protein with low ratio (<1.3) unfolded completely at an interface, with the more hydrocarbon-like residues oriented away from the water. Horiuchi et al. (1978) showed that foam stability occurred when the hydrophobic region of the protein became situated at the interface, causing the molecule to resist migration to the aqueous phase.

It is well established that an ambivalent demand is placed on the food protein during its interfacial and surface function in the food system. Molecular size, flexibility, charge, et. are important factors that determine the functionality of amphiphile molecule but distinctly separate hydrophilic and hydrophobic zones, and favorable geometry may be critical. From the experimental results presented in this paper and the preceding paper, it is seen that the amphipathic nature of α_{s1}-casein improves dramatically with the covalent incorporation of palmitoyl residues and the improvement is primarily due to the incorporation per se and does not reflect a drastic conformational change.

Registry No. Glycerol, 56-81-5.

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Received for review October 14, 1982. Revised manuscript received March 21, 1983. Accepted August 24, 1983.

Sesquiterpene Hydrocarbons in Pineapple Fruit

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The volatile constituents of pineapple fruit have been isolated under enzyme inhibition, enriched by liquid–liquid extraction, and fractionated on silica gel. Analysis of the nonpolar fraction by gas chromatography and coupled GC–MS showed at least 20 sesquiterpene hydrocarbons to be present. α-Copaene, β-ylangene, α-patchoulene, γ-gurjunene, germacrene D, α-muurolene, and δ-cadinene were identified via authentic samples and/or published data. One of the minor compounds of the fraction seems to be responsible for the fragrant odor (reminescent of fresh-cut pineapple).

The first comprehensive studies of pineapple volatiles resulted from collaboration between the Pineapple Research Institute of Hawaii and the Stanford Research Institute and showed the fruit to contain aliphatic, hydroxy, and acetoxy esters, γ-lactones, sulfur compounds, linalool oxide, and 2,5-dimethyl-4-hydroxy-3(2*H*)-furanone (Creveling et al., 1968; Rodin et al., 1965, 1966; Silverstein et al., 1965). Flath and Forrey (1970) reported the presence of a number of additional compounds, mainly carboxylic esters, and also of three monoterpene alcohols. The occurrence of sesquiterpenoid structures ($M^+ = 204$) in pineapple extracts has been mentioned by Näf-Müller and Willhalm (1971), but only one compound, an alcohol with a selinane skeleton, was identified by GLC and mass spectrometry. The interesting sensory properties of the hydrocarbon fraction were the reason to study the chemical composition in more detail.

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EXPERIMENTAL SECTION

Materials. Pineapple fruits (*Ananas comosus* Merr.) from the Ivory Coast were obtained from a local supplier.

Aroma Isolation. Whole ripe pineapples were peeled, cut into methanol, and mixed in a Waring Blendor (final methanol concentration 66%; Drawert et al., 1973). The methanol–juice mixture was separated immediately in a Sorvall-type centrifuge (0 °C, 1500*g*, 10 min) and extracted with a pentane–methylene chloride (2:1) mixture. The solvent was removed by distillation through a Vigreux column (20 cm, 40 °C) (Drawert and Rapp, 1968; Drawert et al., 1969), and the total concentrate was subjected to ascending column chromatography using pentane–ether mixtures as the migrating solvents (Schreier et al., 1974). The hydrocarbon fraction was obtained by elution with

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