Lipophilization of α_{s1}-Casein. 1. Covalent Attachment of Palmitoyl Residue

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Palmitoyl residues were covalently incorporated into α_{s1}-casein by base-catalyzed ester exchange using the *N*-hydroxysuccinimide ester of palmitic acid (16:0-Osu). More than 50% of the reactive nucleophiles reacted within 2.5 min when the reaction was at a 16:0-Osu to lysine residue mole ratio of 1.5, pH 9, at 30 °C. During bulk incorporation only the 16:0-Osu to lysine mole ratio was adjusted; reaction time was 60 min. The amino group of lysine was the most reactive nucleophile and the most susceptible residues appeared to be Lys-34 and/or -36. Tyrosine residues seemed to be in a state of uptake and release since the phenolic ester bonds were spontaneously cleaved in the alkaline medium of the reaction mixture. Threonine was less reactive. Palmitoyl proteins showed an enhanced tendency to associate that was the highest when six ligand molecules were attached but decreased as the incorporation increased. The probable micelle forming capacity of the palmitoyl proteins has been discussed.

The existence of hydrophobic amino acid side chains on protein molecules suggests the possibility of interaction between protein and small molecules containing hydrocarbons, such as hydrocarbons themselves, simple amphiphiles, and biological lipids. Interactions of this type presumably play an important role in the formation of functioning biological membranes. There is a large body of experimental data on the binding of amphiphiles to proteins, which has been reviewed in detail by Steinhardt and Reynolds (1969). In the food system too, lipid–protein interactions have long been recognized to play a critical role in determining the functionality of food proteins.

With a view to enhance the amphipathic nature of food proteins, we "lipophilized" it by chemically attaching naturally occurring fatty acids of various lengths (lauric, myristic, palmitic, and oleic acid) to the hydrophilic soybean glycinin molecule (Haque and Kito, 1982). The term "lipophilization" has been used to denote a general increase in the surface hydrophobicity of the protein molecule brought about by covalent attachment of hydrophobic ligands in an effort to increase the protein's affinity for relatively apolar molecules or amphiphiles. In a related piece of work it was seen that covalent attachment of palmitoyl residues to soybean glycinin caused an increase in its emulsification capacity (Haque et al., 1982).

Chemical modification of proteins to improve functionality has been reported by a number of workers. Succinic anhydride has been used to improve the functionality of wheat flour (Grant, 1973), single-cell protein concentrates (McElwain et al., 1975), and fish protein (Groninger and Miller, 1975). Succinylation improves the emulsification capacity and stability of fish protein concentrate (Chen et al., 1975). A number of reagents including acetic anhydride (Means and Feeney, 1971), maleic anhydride (King and Perham, 1971), 2-methylmaleic (citraconic) anhydride (Brinegar and Kinsella, 1980), and phosphoryl chloride (Woo et al., 1982) have been used.

Not much work has yet been done to study chemical modification of food proteins at a molecular level. Available data are even more scarce regarding the chemical attachment of hydrophobic ligands to the protein molecule. Our present work is an effort to study the covalent attachment of hydrophobic ligands to a food protein; the site and type of bonding that occurs when palmitoyl residues are covalently attached to the α_{s1}-casein molecule by base-catalyzed ester exchange by using the *N*-hydroxysuccinimide ester of palmitic acid as the lipophilic electrophile. α_{s1}-Casein was chosen for our experiments since it is a major component of casein (50–55%), which plays an important role in the food functionality of dairy and confectionary products; it does not have a complex subunit structure like soybean glycinin (Bradley et al., 1975), which we used previously (Haque et al., 1982); it is hydrophobic (Bigelow, 1976) compared to the hydrophilic acidic subunit of glycinin (Moreira et al., 1979); its complete sequence is known (Mercier et al., 1971).

EXPERIMENTAL SECTION

Materials. Hammarsten casein (Art. 2242) and thin-layer chromatographic (TLC) plates of 0.25-mm layer thickness (Art. 5721) were obtained from Merck, Darmstadt, West Germany. Pronase-E (*Streptomyces griseus* protease) (1:1 000 000) was purchased from Kaken Chemical Co., Tokyo. Ultrafiltration membrane tubes (1.2 m × 1.4 mm, i.d. 0.8 mm; molecular exclusion 13 000 daltons) were obtained from Funakoshi Chemical Co., Tokyo. Filter type HA of pore size 0.4 μm was from Millipore Corp., Bedford, MA. The dye reagent concentrate for protein assay was purchased from Bio-Rad Laboratories, Richmond, CA. Coomassie Brilliant Blue G was from Sigma Chemical Co., St. Louis, MO. Coomassie Brilliant Blue R-250, palmitic acid (16:0), deionized urea, ethyl alcohol (99.5%), *N*-hydroxysuccinimide (HOsu), dicyclohexylcarbodiimide, 2′,7′-dichlorofluorescein (DCF), 2,4,6-trinitrobenzenesulfonic acid (TNBS), norleucine, and sodium dodecyl sulfate (SDS) were obtained from Nakarai Chemicals, Ltd., Kyoto. *S*-[2-(4-pyridyl)ethyl]-L-cysteine was prepared by the method of Cavins and Friedman (1970).

Methods. *Preparation of α_{s1}-Casein.* α_{s1}-Casein was prepared according to the method of Zittle and Custer (1963). Hammarsten casein was used instead of acid casein, and the α_{s1}-casein thus derived (15% yield) was extensively dialyzed against distilled water maintained at neutrality and then stored for short periods in 4.7% concentration at −20 °C. Polyacrylamide gel electrophoresis was carried out in the presence of 7 M urea to confirm purity.

Determination of Protein Concentration. The protein concentration of the native α_{s1}-casein was routinely determined from the relationship $E_{280}^{1\%,1cm} = 10.0$ (Nobel and Waugh, 1965), which was seen to agree well with the dye-binding method of Bradford (1976) using a Bio-Rad protein assay kit. Palmitoyl proteins, however, gave lower readings as the incorporation increased, and hence such

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methods were effective only prior to incorporation following which the solution volume was carefully monitored and the concentration ascertained.

Esterification of Palmitic Acid with N-Hydroxysuccinimide. Esterification was carried out as reported earlier (Haque and Kito, 1982), and the *N*-hydroxysuccinimide ester for palmitic acid (16:0-Osu) was finely ground, thoroughly desiccated, and stored as such at 4 °C for long periods (1 year) without noticeable detrimental effect. Purity and activity were confirmed by TLC using the solvent system of petroleum ether–diethyl ether–acetic acid (80:20:1 v/v/v). 16:0-Osu was detected on the plate as described below.

Lipophilization of α_{s1}-Casein. The molar concentration of α_{s1}-casein in the reaction mixture was fixed at 0.2 mM and the reaction temperature was 30 °C. In the case of "bulk preparation", the reaction time was fixed at 60 min, whereas in the case of the "time course", only the reaction time was varied. The solvent used was 50 mM sodium borate (adjusted to pH 9.0 with HCl) and ethanol (99.5%) in the ratio of 2:8. In the case of the time course experiment, the mole ratio of 16:0-Osu to lysine residues (in α_{s1}-casein) in the reaction mixture was fixed at 1.5 and the degree of incorporation was controlled by adjusting the reaction time. On the other hand, in the case of the bulk preparation, the degree of incorporation was limited by only adjusting the above-mentioned mole ratio. The mole content of lysine in the protein was considered to be 14 (Mercier et al., 1971). Bulk preparation was carried out at a 16:0-Osu to lysine mole ratio of 0.05, 0.10, 0.25, 0.50, 0.75, 1.00, 1.25, and 1.50. Finely powdered 16:0-Osu was quickly dissolved by vigorous stirring in the ethanol component of the solvent system, which had been preheated to 35 °C and then immediately mixed with the protein, which had been separately solubilized in the buffer. The pH was maintained at 9.0 with NaOH and finally adjusted to 7.0 with HCl to stop the reaction. All the palmitoyl protein solution was then exhaustively dialyzed at 4 °C against distilled water maintained at neutrality and concentrated to 0.5% by using a "Mini-Module Ultra-Filtration Unit" (NM-3) (Funakoshi Chemical Co., Tokyo) and stored as such for short periods at 4 °C. A control was run where the reaction pH was adjusted to 7.0 and dialysis started immediately. Portions were lyophilized and desiccated thoroughly to ascertain the protein concentration. Palmitoyl proteins obtained when the mole ratios were 0.5–1.25 were considered for further study since 1.50 gave an incorporation that was essentially similar to that of the 1.25 mole ratio sample.

Determination of the Degree of Incorporation. The degree of incorporation was determined by gas–liquid chromatography (GLC) as reported earlier (Haque et al., 1982). An aliquote (2 mL, i.e., 10 mg) of the palmitoyl protein solution was washed 5 times with the same volume of diethyl ether, centrifuged each time (2500 rpm, 5 °C, 5 min) to break the resulting emulsion, and then dried in vacuo over phosphorus pentoxide. The residue was then acid hydrolyzed with 6 N HCl to 8 h at 110 °C. Stearic acid was used as the internal standard. The diethyl ether extract was then dried in a flow of nitrogen and the residue was methylated for 20 min at room temperature with freshly prepared diazomethane, which only methylates unesterified fatty acids (Christie, 1973). The fatty acid methyl ester thus formed was resolubilized in *n*-hexane prior to GLC analysis. In the case of the TLC separation of palmitoyl peptides (described below), the organic extract of the acid-hydrolyzed TLC scraping was directly methylated and subjected to GLC as above.

Amino Acid Analysis. A Hitachi KLA-5 amino acid analyzer was used for the quantitative estimation of amino acids using the sodium citrate buffer system of Spackman et al. (1958). *S*-[2-(4-Pyridyl)ethyl]-L-cysteine (Cavins and Friedman, 1970) and norleucine were used as internal standards.

Acid Hydrolysis. Protein and peptides were hydrolyzed with redistilled 6 N HCl at 110 °C for 24 h in deaerated tubes in the presence of 20 μL of 5% phenol for amino acid and combined amino acid and fatty acid analysis. The HCl was saturated with nitrogen prior to use. The sample containing tube was deaerated in vacuo overnight, flushed with nitrogen, and sealed after sonication under vacuum failed to produce bubbles. For 16:0 analysis alone, the deaeration step was not necessary and the hydrolysis was performed in Teflon-stoppered tubes for 8 h.

TLC Analysis of Palmitoyl-α_{s1}-casein. A 0.25% solution of the palmitoyl protein (50 mg) in 20 mL of 0.1 M sodium phosphate buffer was hydrolyzed at pH 7.0, 40 °C, for 4 h with Pronase-E (10 mg). The solution pH was then adjusted to 6.0 with 1 N HCl and extracted 3 times (10, 5, and 5 mL) with a solvent system of chloroform–methanol–1–butanol (2:1:1 v/v/v) and developed alone with parallel references on a TLC plate (0.25 mm), as described in a previous paper (Haque et al., 1982), by using a solvent system of chloroform–methanol–acetic acid (80:20:1 v/v/v) and developed again by using a slightly more polar solvent system of chloroform–methanol–acetic acid (65:25:8 v/v/v) to a position 2 mm lower than the previous solvent front. Peptide-rich sections corresponding to the ninhydrin-visualized parallel references were sprayed faintly with ethanolic dichlorofluorescein to locate sections rich in palmitoyl peptides (as described below) that were then scraped off, acid hydrolyzed, and extracted with diethyl ether prior to amino acid analysis. The 16:0 content in the organic extract was determined by GLC as described before. A control experiment was carried out with α_{s1}-casein to check whether the TLC mobility of the resulting peptide fractions interfered with the separation of palmitoyl peptides. A parallel experiment, beginning at the acid hydrolysis step and involving known amounts of standard amino acids and 16:0 mixed with an appropriate amount of TLC scraping (from below the origin of the control plate), was carried out in the presence of internal standards [norleucine, *S*-[2-(4-pyridyl)ethyl]-L-cystein and steric acid] to determine the mechanical and chemical losses. Correction factors were then calculated.

Visualization of Different Fractions on the TLC Plate. The developed TLC plates were dried in vacuo for 10 min, and then reference strips were cut off and treated as follows for detecting the mentioned materials. (1) Protein and peptide fractions: These were sprayed with a 0.5% (w/v) solution of ninhydrin in 1-butanol and then heated mildly to give purple and violet patches. (2) Palmitic acid: This was sprayed with a 20% (w/v) aqueous solution of ammonium bisulfate and then heated at 200 °C until black patches appeared (Borowski and Ziminski, 1966). 16:0-Osu also gave a positive test. (3) 16-0-Osu: This was sprayed with a molar solution of hydroxylamine in 1 M NaOH followed after 3 min with a spray of 0.2 M ferric chloride in 1.5 N HCl to give characteristic bright orange patches. (4) Palmitoyl peptides: Sections parallel to the ninhydrin-visualized reference strips were sprayed with a 0.1% (w/v) solution of DCF in 99.5% ethanol and viewed under a UV lamp as bright yellow patches against a light orange background.

Sucrose Gradient Centrifugation. The protein samples were centrifuged in 12 mL of a 5–20% (w/v) linear sucrose

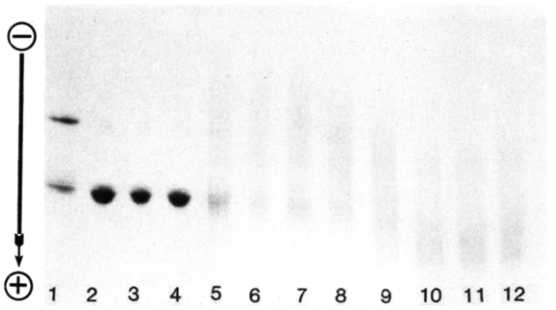

Figure 1. Alkaline PAGE profile of palmitoyl-α_{s1}-casein following the time course experiment. (1) Casein; (2) purified α_{s1}-casein; (3) control; (4) zero time; (5) 2.5 min; (6) 5 min; (7) 10 min; (8) 15 min; (9) 30 min; (10) 60 min; (11) 90 min; (12) 120 min.

gradient in 20 mM sodium phosphate buffer, pH 7.0, at 152000*g* for 20 h at 20 °C in a Hitachi RPS 40T rotor. The gradients were divided into 0.6-mL fractions and measured at 280 nm with an ISCO density gradient fractionator.

Polyacrylamide Gel Electrophoresis (PAGE). Polyacrylamide slab gel electrophoresis was carried out by using the buffer system of Davis (1964), with and without 7 M urea and with 7 M urea plus 0.1% SDS (w/v). The separating gel concentration was 10% and the stacking gel was 2.5%. Pigmentation was carried out for 30 min in 0.1% Coomassie Brilliant Blue G in 50% TCA or for 12–16 h in 0.1% Coomassie Brilliant Blue R-250 in 25% methanol–10% acetic acid (w/v) following fixation for 30 min in 50% trichloroacetic acid (TCA) (w/v) and then destained in methanol–acetic acid–water (20:7:73 v/v/v). In the presence of SDS, the voltage was controlled at 40 V for the first hour followed by 120 V for about 3.5 h, whereas in its absence, in all cases, the current was adjusted to 15 mA for 1 h followed by 60 mA for about 1.25 h. The gel were preelectrophoresed for 20 min at 40 V in the presence of SDS or at 15 mA in its absence, and then electrophoresis was carried out at 4 °C with precooled electrode solution to prevent heating of the gel. The protein samples were pretreated prior to PAGE by incubation at 90 °C for 3 min in 2% SDS, 20% glycerol, and 0.002% bromophenol blue (w/v).

Available Lysine. Available lysine was determined with TNBS by using the method of Fields (1971).

RESULTS

Lipophilization of α_{s1}-Casein. The covalent incorporation of palmitoyl residue (16:0) into α_{s1}-casein seemed to progress very fast for the initial 2 min or so and then slowed down. This was apparent from the pH drop that had to be neutralized with 1 N NaOH (data not shown) and from the time course experiment. The reaction could be stopped by lowering the pH to 7.0 and did not progress further after 60 min even when the mole ratio of 16:0-Osu to lysine in the reaction mixture was increased to 2 (data not shown). The imidazole group of histidine, which is ionized at pH 6.5 (Feeney, 1977), did not seem to react with 16:0-Osu as is apparent from the lack reactivity at pH 7.0 and from the Pronase-E experiment that follows, even though there are five histidine residues in the α_{s1}-casein molecule (Mercier et al., 1971). Figure 1 shows the urea–alkaline PAGE profile of the palmitoyl proteins derived from the time course experiment. The profile of native α_{s1}-casein, control, and zero-time samples look exactly alike whereas that of the 2.5-min sample is quite different. The incorporation, i.e., the average moles of 16:0 covalently bound to 1 mole of α_{s1}-casein ($\bar{\nu}$), at 2.5, 5, 10, 15, 30, and 60 min as determined by GLC was 5, 7, 9, 9.5, 11, and 11.5, respectively. The incorporation did not increase any

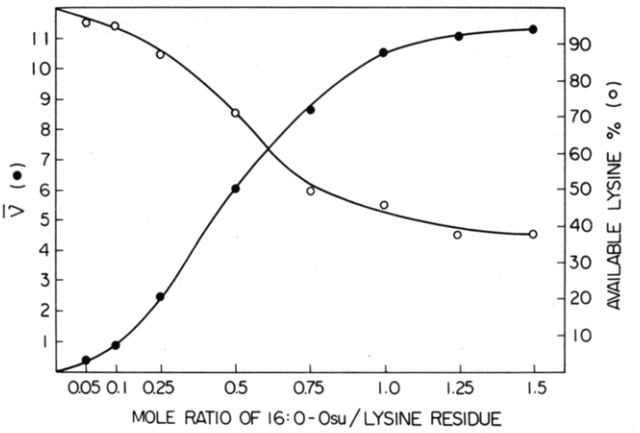

Figure 2. Increase in incorporation as a function of increase of the mole ratio of 16:0-Osu to lysine as compared to the decrease of available lysine.

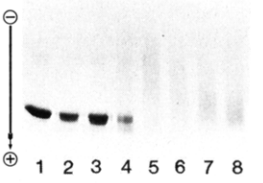

Figure 3. Urea–alkaline PAGE profile of palmitoyl-α_{s1}-casein when the degree of incorporation was controlled by adjusting the mole ratio of 16:0-Osu to lysine in the reaction mixture. (1) α_{s1}-casein; (2) $\bar{\nu}$ = 0.3; (3) $\bar{\nu}$ = 0.8; (4) $\bar{\nu}$ = 2.5; (5) $\bar{\nu}$ = 6.0; (6) $\bar{\nu}$ = 8.8; (7) $\bar{\nu}$ = 10.5; (8) $\bar{\nu}$ = 11.1.

further at 90 and 120 min. It was therefore evident that under our reaction conditions, at 2.5 min almost 50% of the reactive nucleophile sites had reacted, bringing about a distinct change in the PAGE profile whereupon the bands became diffused and there was extensive trailing. In the presence of 0.1% SDS (w/v), however, bands were still slightly diffused but the trailing was not seen (figure not shown). It is therefore evident that the palmitoyl proteins were not fragmented and aggregation had most probably occurred due to hydrophobic or electrostatic bonds or a combination of both and that these were destroyed by the action of SDS. When the mole ratio of 16:0-Osu to lysine was adjusted and the reaction time kept constant at 60 min (see Methods), bulk preparations were possible having very low degrees of incorporation as shown in Figure 2, which shows at different mole ratio of 16:0-Osu to lysine as compared to the loss of available lysine. It is apparent that under our reaction conditions about nine lysine residues were reactive. Since the mole content of lysine in α_{s1}-casein has been reported to be 14 (Mercier et al., 1971), a 16:0-Osu to lysine mole ratio of 0.05, 0.10, 0.25, 0.50, 0.75, 1.00, 1.25, and 1.50 means 0.7, 1.4, 3.5, 7.0, 11.5, 14.0, 17.5, and 21 mol of 16:0-Osu/mol of α_{s1}-casein, respectively. Figure 2 shows that the percentage of 16:0-Osu in the reaction mixture that covalently attached to the protein molecule was high when the mole ratio of 16:0-Osu to lysine was 0.25, 0.50, 0.75, and 1.00 and was the highest (85%) when the mole ratio was 0.50, which gave a palmitoyl protein ($\bar{\nu}$ = 6) that had the greatest trailing behavior. Figure 3 shows the urea–alkaline PAGE profiles of the various palmitoyl proteins thus derived. These above palmitoyl proteins, as observed before with the samples derived from the time course experiment, did not show any distinct sign of trailing during urea–alkaline PAGE in the presence of 0.1% (w/v) SDS (data not shown). This further substantiates our time course experimental observation of decreased reactivity after $\bar{\nu}$

reached 5. The trailing of the proteins in native PAGE (Davis, 1964) was similar to the urea–alkaline PAGE (Figure 3) and has not been shown.

The sucrose gradient centrifugation experiments showed that the control samples and palmitoyl proteins when $\bar{\nu}$ was 0.3 and 0.8 moved to a position similar to the native α_{s1}-casein, the peak being fractionated at 3 mL of a total of 12 mL (BSA was at 6 mL). When $\bar{\nu}$ reached 2.5, the protein sedimented further and the peak was broader at 4.5 mL, whereas when $\bar{\nu}$ was more, the protein remained more or less in the same position (about 5 mL).

Type and Site of Incorporation. Palmitoyl α_{s1}-caseins of different degrees of incorporation were obtained by the bulk preparation method and subjected to Pronase-E digestion. Even though Pronase-E is a very powerful proteolytic enzyme (Nomoto et al., 1960), it has been shown that endopeptidases and exopeptidases are unable to split off peptide bonds that are close to phosphate groups (Mercier et al., 1971). Even though alkaline phosphatase would have solved the problem, complete hydrolysis was not our goal. TLC separation and visualization (see Methods) of the enzymatic hydrolysate gave TLC fractions A, B, C, and D. Fraction A had a R_f value (considering the front of the second solvent system) of 0.10 when $\bar{\nu}$ was 2.5 and 0.12 when $\bar{\nu}$ was 6.0, 8.8, and 10.5. Fraction B, on the other hand, had a R_f value that was 0.16 when $\bar{\nu}$ was 0.3, 0.8, and 2.5 and was 0.25 and $\bar{\nu}$ was 6.0, 8.8, and 10.5. The lower R_f values of the less incorporated palmitoyl peptide fractions indicate that there were unreacted nucleophiles in these fractions that reacted when $\bar{\nu}$ increased. Positive dichlorofluorescein identification of ninhydrin-reactive bands was possible for fraction B when $\bar{\nu}$ = 0.3 and 0.8, fractions A and B when $\bar{\nu}$ was 2.5, fractions A–C when $\bar{\nu}$ = 6, and fractions A–D when $\bar{\nu}$ were 8.8 and 10.5. The R_f value of 16:0 and 16:0-Osu were 0.9 and 0.99, respectively. 16:0 was detected in all samples including the lowest incorporated samples ($\bar{\nu}$ = 0.3 and 0.8). 16:0-Osu appeared only when the mole ratio of 16:0-Osu to lysine in the reaction mixture was 0.25 and then, as in the case of 16:0, increased as the mole ratio increased. Since the 16:0-Osu did not contain TLC-detectable amounts of 16:0, it is evident that the detected 16:0 evolved during the reaction. On the other hand, when 16:0-Osu (15 mg) was solubilized in 99.5% ethanol (0.15 solution, w/v) for 4 h at 30 °C, 16:0 detected from the mixture was negligible (55 μg). The 16:0 observed after the reaction could have mostly originated from the spontaneous cleavage of phenolic ester bonds that 16:0-Osu may have formed with tyrosine. On repetition of the time course experiment after having blocked the amino groups with TNBS (5 mg of TNBS/mg of α_{s1}-casein) (Goldfarb, 1966) for 2 h at 20 °C, it was observed that at 15 min $\bar{\nu}$ was 4.5 and almost all of this could be released within 2 h in alkaline hydroxylamine (final 0.5 M) at pH 8.5. However, at 30 min, $\bar{\nu}$ was only about 1.5 and almost half of it could be released in the same way.

Figure 4 shows the combined yields of amino acids of palmitoyl peptide fractions A–D. It is noteworthy that the most predominant amino acids are lysine and glutamic acid/glutamine (Glx). Such a presence of lysine is understood since it appears to be the most reactive species (Figure 2). We see that the palmitoyl proteins with the least incorporation ($\bar{\nu}$ = 0.3 and 0.8) gave only fraction B that contained lysine, Glx, valine, leucine, serine, and aspartic acid/asparagine (Asx) where the ratio of lysine and Glx was about twice that of the other amino acids. From the primary sequence it is apparent that this peptide fraction most likely represents the segment 34–40, indi-

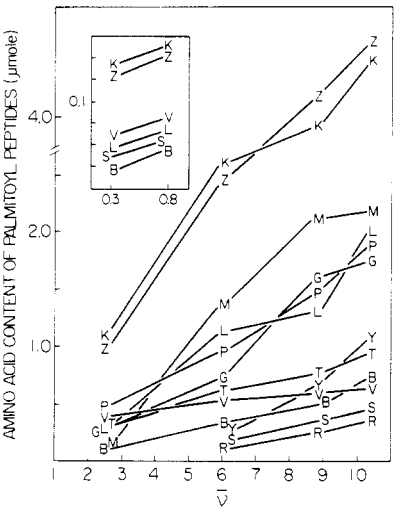

Figure 4. Combined amino acid yield of different TLC separated palmitoyl peptides. IUPAC–IUB-recommended one-letter notations for amino acids have been used as follows: B = Asx (Asp plus Asn); G = Gly; K = Lys; L = Leu; M = Met; P = Pro; R = Arg; S = Ser; T = Thr; V = Val; Y = Tyr; Z = Glx (Glu plus Gln).

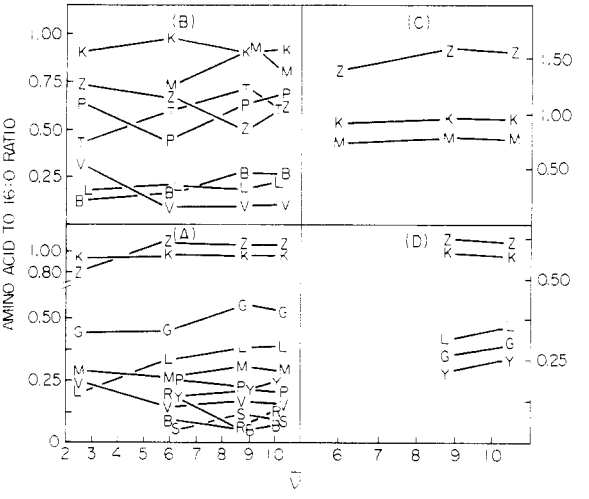

Figure 5. Amino acid to 16:0 ratio of palmitoyl peptides in different TLC fractions. One-letter notations as in Figure 4.

cating that Lys-34 and/or -36 are the most susceptible nucleophiles. When $\bar{\nu}$ was 2.5, fraction A was visible for the first time and the combination of fractions A and B showed the additional appearance of proline, threonine, methionine, and glycine, the occurrence of lysine and Glx still being the highest. On thoroughly studying the primary structure and considering adjacent residues of the lysine residues in the protein, it appears that the likely nucleophiles that are next in the line of reactivity are Lys-132, which has both proline and methionine its proximity, and Lys-193, which has proline, threonine, and methionine. Lys-34 has glycine residue next to it. When $\bar{\nu}$ reaches 6.0, we see a further increase in the occurrence of lysine and Glx with a concomitant increase of methionine, leucine, proline, glycine, and threonine in that order of occurrence. It was at this stage that a tripeptide comprising of lysine, methionine, and Glx appears (Figure 5C). Tyrosine appears here for the first time at a detectable level (Figure 5C) and may have originated from segment 90–100, which also contains arginine (trace detected but not shown), glycine, and a number of leucine residues. It may be noted that fraction A was the richest in amino acids and contributed the most to the total yield as shown in Figure 4. Even though the amino acid to fatty acid ratio at $\bar{\nu}$ = 6 of

glycine in fraction A (Figure 5A) seems essentially same as before, the occurrence of the peptide fraction was higher, resulting in a higher total yield. The tripeptide in Figure 5C was from TLC fraction C and is most likely to represent Lys-58 along with its adjacent residues. The threonine/16:0 ratio (Figure 5B) increases gradually, perhaps indicating the slow reactivity of Thr-194 and -195, and when $\bar{\nu}$ was 8.8, we see a decrease in the lysine/16:0 ratio, indicating that some other residue was reactive at this stage. Figure 2 shows that is was at about this stage that available lysine did not decrease equally with the increased in $\bar{\nu}$. Fraction C (Figure 5C), which was composed of Glx, lysine and methionine, the approximate ratio being 2:1:1, could represent fraction 58–61. Fraction D (Figure 5D) appeared when $\bar{\nu}$ = 8.8 and above and further increased the total yield of lysine and Glx compared to the other residues. This fraction most likely contained a dipeptide and a tripeptide: Glx-Lys, which appeared in a lysine to 16:0 ratio of about 0.6, and Leu-Gly-Tyr, which appeared in the tyrosine to 16:0 ratio of 1:0.25. Of all the tyrosine residues in the protein, only Tyr-91 and Tyr-94 have leucine and glycine adjacent to them. The observed fraction therefore is likely to have originated from segment 91–95. Figure 5 confirms our earlier observation that lysine was predominantly the most reactive residue (Figure 2); tyrosine also reacts (Figure 5D); threonine also seems to react (Figure 5B) but its reactivity was less.

DISCUSSION

Payens and Schmidt (1965) reported the thermodynamic parameters of the association of α_{s1}-casein C. At each consecutive association step the free enthalpy is decreased by a constant amount of 3.2 kcal/mol, corresponding to the formation of three to four hydrophobic bonds. Under extreme conditions such as high concentration of urea or high pH, the α_{s1}-casein molecule exists as the monomer (Schmidt et al., 1967; Noelken, 1967). Figures 1 and 3 show that native α_{s1}-casein did not show any sign of trailing under any electrophoretic conditions. However, the same intact protein acquires a special property after incorporation of hydrophobic ligands whereby the trailing, which is inhibited by SDS, appears on the PAGE gel. As will be published later (Haque and Kito, 1983) we have seen that the conformation of the palmitoyl proteins did not show much change. Perhaps the trailing indicates that the palmitoyl proteins show "soap-like behavior", indicating the ability to form micelles. Figures 2 and 4 show that the ϵ-amino groups of lysine are the most reactive residues. Hence, incorporation resulted in a net gain in the negative charge of the molecules, which explains the increased mobility of the highly incorporated palmitoyl proteins (samples 9–12, Figure 1). Association as indicated by the trailing (decreased mobility), especially when $\bar{\nu}$ = 6 (Figures 1 and 3), does not reflect an increase in net positive charge but was most likely due to hydrophobic interactions and argues for a continuum of modification rather than discrete ones. As the net negative charge increased with incorporation, the hydrophobic interaction may have been overcome by charge repulsion. This observation was substantiated by SDS–PAGE experiments (figure not shown), which showed that there was no trailing at all when the protein molecules were all negatively charged in the presence of SDS. Aggregation was seen to be reduced by the increase in net negative charge that accompanied acetylation of β-casein, while the larger increase in net negative charge resulting from succinylation effectively prevents aggregation (Hoagland, 1966). The driving force for the spontaneous aggregation of surfactant molecules to form micelles is hydrophobic (Helenius and Simons, 1975). Perhaps when $\bar{\nu}$ was 6 the palmitoyl protein was best able to associated with each other by hydrophilic–lipophilic arrangement but the required amphipathicity deteriorates as $\bar{\nu}$ increases further.

While working with β-casein, Hoagland (1968) carried out a series of acylations ending with hexanoylation and observed uniformity in the increase in relative electrophoretic mobility, indicating that the net negative charge governs mobility much more than does the nature of the substituent alkyl group. Succinylation showed highest mobility. He also observed by ultracentrifugation that the S value and specially the relative peak area (percent) of the aggregated acylated protein seemed to be directly related to the length of the alkyl group; longer the chain length, higher the aggregation.

Robbins et al. (1965) introduced nonpolar groups into α-lactalbumin using ethyl acetimidate hydrochloride and ethyl butyrimidate hydrochloride and reported that the modified proteins were much more susceptible to association and aggregation than the starting material itself.

The sucrose gradient centrifugation experiments shows the tendency for the palmitoyl proteins to associate when $\bar{\nu} \geq 2.5$. This could be motivated purely due to the free energy advantage of a resulting decrese in the hydrocarbon–water interface. It has been reported that native α_{si}-casein itself tends to associate when the ionic strength is high (Schmidt and Van Markwijk, 1968). The associating tendency of the palmitoyl proteins seem to be decreased in the presence of a high concentration of sucrose but is nevertheless there. While studying Semliki Forest virus, Simons et al. (1973) observed two proteins which gave protein–Triton X-100 complexes that had sedimentation coefficients (s) values of 4.5 and 23.5 S. They observed that removal of sucrose leads to association of the 4.5S form to the rosette-like 23.5S aggregate; addition of sucrose leads to dissociation.

The time course experiments following TNBS blocking of lysine residues indicates the ready reactivity of about four tyrosyl residues, the phenolic ester bond being unstable. From this we may visualize that tyrosyl ester bonds were being formed and then cleaved in the alkaline (pH 9.0) reaction mixture, resulting in most of the free fatty acid that was detected by TLC. It was also evident that some bond other than that with lysine or tyrosine takes place and that it is stable under the conditions that cleave phenolic ester bonds. Puigserver et al. (1979) reported the cleavage of phenolic ester bonds using a alkaline solution of hydroxylamine. Riordan and Vallee (1964) have also reported the spontaneous hydrolysis of tyrosyl ester bonds while succinylating carboxypeptidase A and suggested the catalytic participation of the carboxylate anion. The slight difference in the intensity of the trailing between the 2.5-min sample of the time course experiment and the sample derived from the bulk preparation experiment, when the mole ratio of 16:0-Osu to lysine was 0.50, may be because the type and site of bonding are somewhat different in the two even though $\bar{\nu}$ is about the same.

The TLC separated palmitoyl peptides showed a high incidence of lysine and Glx (Figures 4 and 5). Such a presence of lysine is understood since it was predominantly the reactive species (Figure 2). However, the high incidence of Glx is highly noteworthy. On the other hand, when we study the primary sequence of α_{s1}-casein (Mercier et al., 1971) we can see that the adjacent amino acids to the lysine residues that do not have glutamic acid in their proximity have not been detected by our method. It may therefore be prudent to assume that the carboxylate anion

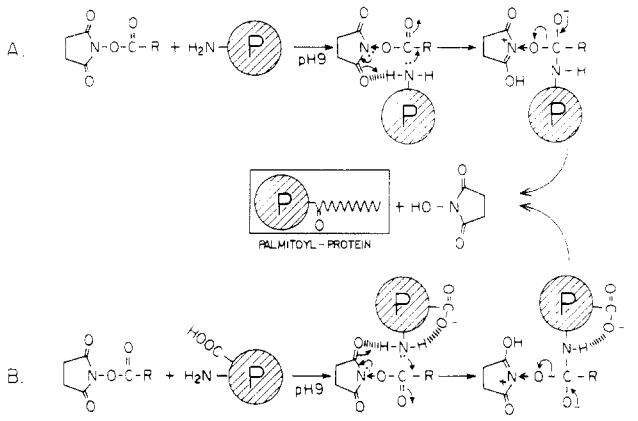

Figure 6. Scheme visualizing the incorporation of the palmitoyl residue at the ϵ-amino group of lysine by (A) general base catalysis and (B) double activation by general base catalysis and neighboring group participation.

of glutamic acid increases the nucleophilicity of adjacent ϵ-amino groups as proposed in Figure 6, where (A) visualizes intermolecular base catalysis and (B) shows increased nucleophilicity due to a combination of intermolecular base catalysis and neighboring group participation, a phenomenon of double activation. In (A) the lone pair on the ϵ-amino group "pushes" whereas the succinimide moety "pulls". On the other hand, in the case of (B) the "push" component of the "push-pull" phenomenon is further strengthened. Since the reaction is very fast, takes place in the alkaline medium, and may be stopped at neutral pH, it has been assumed that (Figure 6) an external base (OH^-) acts primarily by partially deprotonating the ϵ-amino group of lysine and ionizes the carboxyl group of glutamic acid. It has been suggested (Horiki, 1977) that during such intermolecular base catalysis, a transition complex for the formation of the adduct, and the adduct itself, may be stabilized by hydrogen bonding (Goodman and Glaser, 1970); the subsequent proton transfer would be greatly accelerated in the final stage and thereby such reactions can be expected to be fast.

Even though arginine has appeared in our data, the reactivity of this residue is unlikely since the guanidino group is protonated below pH 10 (Feeney, 1977). The proximal residues (histidine and isoleucine) of the N-terminal arginine have not appeared indicating its nonreactivity. Figures 4 and 5 show that this residue appears when tyrosine appears ($\bar{\nu} \geq 6$), and from the primary sequence we know that arginine is the adjacent residue of Tyr-91, which appeared to be highly reactive among the tyrosine residues. It may be noted that the lysine residues at the N-terminal end, Lys-3 and -7, which do not have glutamic acid residues in their proximity, did not seem to react. It is unlikely that this peptide fraction remained at the origin during TLC separation since this part of the polypeptide fraction is itself hydrophobic (Mercier et al., 1971) and if incorporated would migrate in the apolar developing solvent used.

In an effort to further study the effect of lipophilization, the following paper will handle conformational and functional effects of the incorporation of 16:0 to α_{s1}-casein.

Registry No. 16:0-Osu, 14464-31-4; Lys, 56-87-1.

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