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Acylated β -Caseins. Effect of Alkyl Group Size on Calcium Ion Sensitivity and on Aggregation*

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ABSTRACT: A series of acylated β -caseins ending with hexanoylated β -casein has been prepared. The nature of the alkyl group affects both the calcium ion sensitivity and aggregation of modified β -casein. The increase in net negative charge resulting from acylation apparently reduces calcium ion sensitivity and aggregation through electrostatic repulsion. A marked de-

pendence of solubility in 0.025 M CaCl_2 of some acylated β -caseins upon pH near 6 was observed. Ionization of imidazole groups and/or phosphate groups is implicated in calcium ion binding. Increase of alkyl chain length of the acylating agent increased the sedimentation coefficient of the aggregate. Hydrophobic bonding is likely responsible for this enhanced aggregation.

β -Casein is one of the major components of bovine casein. In neutral solution this protein aggregates above 4° (von Hippel and Waugh, 1955; Sullivan *et al.*, 1955; Payens and van Markwijk, 1963) and above 18° exhibits calcium ion sensitivity, *i.e.*, it can be precipitated by calcium ions (Zittle and Walter, 1963). A high proline content, coupled with the absence of cysteine and cystine (Peterson *et al.*, 1966), confer a disordered conformation upon β -casein monomers (Herskovits, 1966; Noelken and Reibstein, 1967). Succinylation of the amino groups of β -casein reduces aggregation markedly and prevents precipitation by calcium ions (Hoagland, 1966). At pH 7, the net negative charge of β -casein A¹ of 11/molecule (mol wt 23,000–24,000; Peterson *et al.*, 1966; Sullivan *et al.*, 1955; McMeekin, 1954) is increased some threefold by near-complete succinylation of the 12 amino groups. Succinylated β -casein is presumably maintained in monomeric form by intermolecular electrostatic repul-

sion. Bound calcium ions cannot reduce the electrostatic repulsion sufficiently to permit precipitation. On the other hand, acetylation of the amino groups of β -casein roughly only doubles the net negative charge at pH 7. Aggregation of this derivative occurs, but is less than for β -casein; moreover, acetylated β -casein exhibits calcium ion sensitivity, although to a lesser extent than β -casein (Hoagland, 1966). Since alkyl groups probably participate in aggregation of β -casein, which is rich in hydrophobic amino acid residues (von Hippel and Waugh, 1955), the methyl groups of acetylated β -casein were replaced by alkyl groups of increasing chain length. The effect of larger alkyl groups upon calcium ion sensitivity and aggregation of acylated β -casein is the subject of this report.

Experimental Section

Materials. β -Casein A¹ (Peterson *et al.*, 1966) was prepared from milk of a typed cow by the urea fractionation method of Aschaffenburg (1963). A 4.5 × 25 cm DEAE-cellulose column was equilibrated at 20° with 0.01 M imidazole–3.3 M urea–0.1% mercaptoethanol (Thompson, 1966) buffer adjusted to pH 7.0 with 1 N HCl. A 100-ml solution of 5 g of β -casein in this buffer was added to the column. Contaminating κ -

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casein and γ -casein were stripped from the column with 1.5 l. of the buffer 0.075 M in NaCl, final pH 7.0 at 20°. The β -casein was then eluted by a salt gradient in the same buffer from 0.075 to 0.35 M NaCl in 4 l. Flow rate was 100 ml/hr. Elution was monitored at 280 m μ by a Vanguard Model 1056OD ultraviolet analyzer.¹ Only electrophoretically homogeneous β -casein (Peterson, 1963; Thompson *et al.*, 1964) recovered from column chromatography was used for preparation of derivatives.

Acid anhydrides and imidazole were Eastman products. Inorganic salts and urea were reagent grade. Dimethyl sulfoxide, obtained from Fisher, was redistilled. On several occasions, spectrophotometric grade Fisher dimethyl sulfoxide was used directly.

Acylation. β -Casein A¹ (0.5 g) recovered by lyophilization of a turbid water solution (pH 5.5) was dissolved in 20 ml of dimethyl sulfoxide. To the solution was added 0.10 ml of acid anhydride. After reaction with vigorous stirring at room temperature, the solution was added to 150 ml of water. Reaction times were determined by test runs in which portions of solution were withdrawn at selected times. Extent of acylation was followed by examination of withdrawn samples with polyacrylamide gel electrophoresis at pH 9.2. The reaction times selected gave maximum migration without any deterioration of the gel pattern. Acetylation required 5 min, whereas propionylation, butyrylation, and isobutyrylation required 10 min. Higher homologous anhydrides required 15 min. Succinylation of β -casein was carried out by reaction for 15 min with 0.1 g of succinic anhydride. The aqueous solution obtained after reaction was adjusted to pH 7 with 1 N NaOH. The derivative was then recovered by dialysis and lyophilization. Each preparation was purified by DEAE-cellulose chromatography at 20° as described earlier (Hoagland, 1966).

Zone Electrophoresis. Polyacrylamide gel electrophoresis in Tris-EDTA-borate buffer (pH 9.1–9.3) (Peterson, 1963; Thompson *et al.*, 1964) was used with 6.5% acrylamide gel 4.5 M in urea.

Free Amino Groups. Extent of acylation of β -casein was measured by loss of ninhydrin-positive color compared with β -casein (Fraenkel-Conrat, 1957; Hoagland, 1966).

Ultraviolet spectra were obtained for pH 6.98 phosphate buffer ($\mu = 0.2$) solutions of acylated β -casein (0.1%) at room temperature with a Cary Model 14 recording spectrophotometer.

Ca²⁺ Sensitivity. An adaptation of the method of Zittle and Walter (1963) was used. Aqueous protein solutions (40 ml), about 2%, were adjusted to within pH 7.5–8.0 and dialyzed against cold 0.025 M calcium chloride, adjusted to pH 8 with CaO. The absorbance at 275 m μ of a portion of the dialyzed solution diluted 1:1 with water to avoid turbidity was determined. The stock protein solution was then adjusted to 1.5% concentration by addition of an appropriate amount of 0.025 M calcium chloride. The temperature throughout was kept low to prevent precipitation. A 5-ml portion of the solution was then brought to 30° for 15 min. The insoluble protein was separated with a clinical

centrifuge. The supernatant pH and absorbance at 275 m μ were recorded. To cold 5-ml portions of remaining protein-calcium chloride solution were added microliter quantities of either 1 N KOH or 1 N HCl to give a spread of pH values. The solutions were brought to 30° for 15 min, and after centrifugation both the pH and absorbance at 275 m μ of the supernatants were determined. Slightly turbid supernatants were clarified by either slight cooling or by addition of 1 drop of saturated sodium citrate solution. Absorbance ratios, using the value for the stock solution adjusted to 1.5% protein concentration, were converted into per cent solubility.

Stabilization of acylated β -casein to precipitation by calcium ions by κ -casein was determined by the same procedure. The protein-calcium chloride solutions in this instance contained 0.15% κ -casein (Zittle and Walter, 1963).

Results

Each derivative eluted as a single peak during DEAE-cellulose column chromatography at pH 7. Elution profiles were similar to that for acetylated β -casein (Hoagland, 1966). In each of these cases a concentration of sodium chloride higher than for β -casein was required to initiate elution.

Acylated β -casein migrated toward the anode as a single band during polyacrylamide gel electrophoresis at pH 9.1–9.2 in Tris–4.5 M urea buffer (Hoagland, 1966). The altered β -caseins migrated faster than β -casein. Relative mobilities are listed in Table I.

Acylation was practically complete according to measured loss of ninhydrin-positive color. The per cent of color loss with respect to β -casein is presented in Table I. The ultraviolet spectrum of β -casein was unaltered by derivatization in dimethyl sulfoxide. Acylation of tyrosyl residues was judged to be negligible by the method of Fraenkel-Conrat and Colloms (1967).

A pH profile of the solubility of β -casein in 0.25 M calcium chloride is shown in Figure 1. Minimum solu-

TABLE I: Electrophoretic Mobilities and Extent of Acylation.

Material	Rel Mobility ^a	% Acylation ^b
β -Casein	1.00	0
Acetylated β -casein	1.32, 1.34	97
Propionylated β -casein	1.28, 1.30	93, 90
Butyrylated β -casein	1.32, 1.33	92
Isobutyrylated β -casein	1.33	88
Valerylated β -casein	1.30	93
Hexanoylated β -casein	1.30	92
Succinylated β -casein	1.52	86

^a Polyacrylamide gel electrophoresis at pH 9.2.

^b Per cent loss of ninhydrin positive color compared to β -casein.

TABLE II: S Values and Relative Distribution of Monomers and Aggregates of Acylated β -Caseins.^a

Material	NHCOR, R =	Monomer		Rel Peak Area %	
		(S)	Aggregate (S)	Monomer	Aggregate
β -Casein		1.34	9.43	29	71
Acetylated β -casein	CH ₃	1.43	4.14	51	49
Propionylated β -casein	CH ₂ CH ₃	1.35	5.28	31	69
Butyrylated β -casein	(CH ₂) ₂ CH ₃	1.39	5.83, 5.85	19, 16	81, 84 ^b
Isobutyrylated β -casein	CH(CH ₃) ₂	1.46	6.19	16	84
Valerylated β -casein	(CH ₂) ₃ CH ₃	^c	7.58	14	86
Hexanoylated β -casein	(CH ₂) ₄ CH ₃	^c	8.45	12	88
Succinylated β -casein	(CH ₂) ₂ COO ⁻	1.13	None	100	0

^a Sedimentation at 52,640 rpm at 20.0° in pH 6.98 phosphate buffer, $\mu = 0.2$, 1% protein concentration; relative peak areas determined by weighing projections from plates. ^b Separate preparations. ^c Peak too small and diffuse for calculations of S value, but qualitatively similar to other monomer peaks.

bility occurs near pH 7. Below pH 7 the solubility increases only slightly before approaching the isoelectric point of 4.9. Butyrylated β -casein above pH 6 is more soluble than β -casein. Below pH 6 the derivative becomes less soluble. Acetyl, propionyl, and isobutyryl derivatives of β -casein were all more soluble than β -casein in the presence of calcium ions. A sharp drop in calcium sensitivity was observed for these three acylated β -caseins as the pH fell to just below 6. The derivatives were stabilized against precipitation in 0.025 M CaCl₂ over the pH range 6–8 by a 1:10 weight ratio of κ -casein to β -casein.

Sedimentation conditions were chosen to favor determination of S values of aggregates of modified β -casein. Because aggregation of β -casein is concentration dependent (Payens and van Markwijk, 1963), sedimentation coefficients cannot be calculated with accuracy by extrapolation to infinite dilution. Therefore, for comparative purposes S values at 1% concentration of

the acylated β -caseins are presented in Table II. S values for the monomer peak were calculated whenever possible and are listed in Table II. The changes of the S value of monomer β -casein produced by acylation were small. However, acylation greatly affected the S value for the aggregate, which sedimented always as a well-defined peak. A general increase in the S value of acylated β -casein accompanied increasing length of the alkyl group of the acylating agent. Also, for β -casein acylated with larger alkyl groups, a larger proportion of material was found in aggregated form, judging by the relative areas of monomer and aggregate peaks (Table II). No aggregation was observed for succinylated β -casein.

Discussion

At pH 7 β -casein has a net negative charge of 11 per molecule (McMeekin, 1954). When the 11 lysyl amino groups plus the N-terminal amino group of the β -casein A¹ variant are acylated, the net negative charge is slightly more than doubled through the loss of 12 cationic groups/molecule. Therefore, changes in some physical properties of β -casein resulting from acylation should reflect an electrostatic effect due to the increased net negative charge. Acylation of β -casein increases the concentration of sodium chloride required for elution of the derivative from a DEAE-cellulose column at pH 7 (Hoagland, 1966). The nature of the alkyl group of the acylating agent had no observable effect on this requirement for a higher sodium chloride concentration. This indicates that tighter binding to DEAE-cellulose by acylated β -casein is a consequence of the large net negative charge at pH 7. The nature of the alkyl group of the acylating agent also had little effect on the relative mobility during alkaline polyacrylamide gel electrophoresis (Table I). Of course, the increase in electrophoretic mobility of β -casein resulting from acylation is direct evidence for a larger net negative charge. The uniformity of the increase in relative mobility indicates that the net negative charge governs

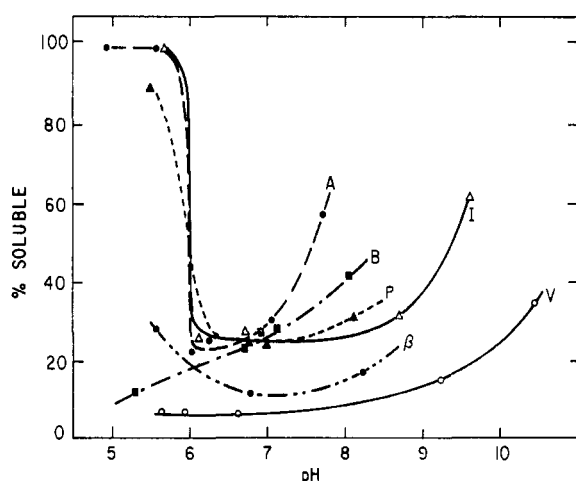


FIGURE 1: pH profile for solubility of acylated β -caseins (1.5%) in 0.025 M CaCl₂ at 30°: (β) β -casein, (A) acetylated β -casein, (P) propionylated β -casein, (B) butyrylated β -casein, (I) isobutyrylated β -casein, and (V) valerylated β -casein.

electrophoretic mobility much more than does the nature of the substituent alkyl group. This interpretation is reinforced by the behavior of succinylated β -casein whose calculated net negative charge, at pH 7, some three times that of β -casein, is likely responsible for tighter binding to DEAE-cellulose and for greater electrophoretic mobility than β -casein modified with uncharged acyl groups (Table I). Moreover, comparable increases in electrophoretic mobilities from acetylation or from succinylation of bovine serum albumin, of bovine γ -globulin, and of bovine β -lactoglobulin have been reported by Habeeb *et al.* (1958). In these instances, though, modification produced extensive conformational changes. Since β -casein probably has a loose coil conformation (Herskovits, 1966; Noelken and Reibstein, 1967) and is unfolded, large conformational changes are not expected to result from acylation. The similarity of monomer S values for β -casein and for acylated β -caseins (Table II) suggests that large conformational changes did not result from acylation.

Calcium ions bound to β -casein in solution at pH 6–8 effectively lower the net negative charge with consequent precipitation of the highly aggregated protein. Removal of positive charges through acylation of lysyl amino groups increases the net negative charge of β -casein and, as expected, increases the solubility of the protein in dilute calcium chloride solution above pH 6. The great solubility of acetylated, propionylated, and isobutyrylated β -caseins between pH 5 and 6 (Figure 1) was an unexpected result. This sharp transition in solubility around pH 6 suggests that ionization of the five phosphate groups and six imidazole groups (Peterson *et al.*, 1966) per protein molecule is governing the interaction of acylated β -casein with calcium ions. Removal of positive charges from the imidazole groups probably reduces electrostatic repulsion of calcium ions, which can bind to the protein by displacing protons from the phosphate groups. Aggregation of the resulting Ca^{2+} -protein may promote complete conversion of the protein into its calcium ion complex over the observed narrow range of pH centered near 6.

Ester-linked phosphate groups of β -casein have been implicated as primary binding sites for calcium ions by Ho and Waugh (1965) through infrared spectroscopy studies. Earlier, Zittle *et al.* (1958) suggested that ionization of phosphate groups promoted binding of calcium ions to casein. Their calcium ion binding studies also implicated phosphate groups as primary binding sites. In this present report, the pH-solubility profiles of some acylated β -caseins give substance to the idea that phosphate groups are involved in the binding of calcium ions to β -casein.

β -Casein does not have the relatively great solubility of its acetylated, propionylated, or isobutyrylated derivative in dilute aqueous calcium chloride below pH 6 (Zittle and Walter, 1963, and Figure 1) since β -casein is very insoluble at its isoelectric point of 4.9 (Hipp *et al.*, 1950). In these cases, acylation of β -casein lowers the isoelectric point sufficiently to permit complete solution at pH 5 at a protein concentration of 0.15%. However, this is not the case for butyrylated β -casein or for valerylated β -casein. The longer alkyl chains

presumably cause a general decrease in solubility below pH 6 as shown by the respective pH-solubility profiles in Figure 1. Evidence for this is the observation that a cold, dilute calcium chloride solution of butyrylated or valerylated β -casein became turbid when the pH was lowered from near 7 to below 6. Calcium ion induced precipitation of β -casein and its acylated derivatives can only be effected at temperatures above 18° (Waugh, 1958, 1961; Zittle and Walter, 1963; Hoagland, 1966). The pH-solubility profile of isobutyrylated β -casein (Figure 1) is more like that of acetylated β -casein than that of butyrylated β -casein. This reflects the normally less hydrophobic nature and more compact conformation of a branched alkyl group compared with its straight-chain isomer. The quantitatively and qualitatively different solubility behavior of butyrylated β -casein and of valerylated β -casein may reflect a different mechanism for the conglomeration of aggregates that results in precipitation. For a speculative example, intermolecular hydrophobic bonding between bulky alkyl side chains may displace intermolecular salt bridges involving calcium ions and phosphate groups as the means for causing aggregates to precipitate.

κ -Casein at a weight ratio of 1:10 with respect to β -casein prevents precipitation of the Ca^{2+} -protein complex (Zittle and Walter, 1963; Hoagland, 1966). Since stabilization of the acylated β -caseins to precipitation by calcium ions resulted from addition of κ -casein, the amino groups of β -casein are not critically involved in formation of a κ -casein- β -casein complex.

Early work by von Hippel and Waugh (1955) and by Sullivan *et al.* (1955) and later work by Payens and van Markwijk (1963) established that β -casein aggregates in solution above 4° near pH 7. Evidence for a disordered conformation for β -casein has been gathered from optical rotatory dispersion by Herskovits (1966) and from viscosity studies by Noelken and Reibstein (1967). Such a disordered conformation is compatible with the amino acid composition of β -casein (Peterson *et al.*, 1966). A high content of glutamic acid confers a net negative charge upon the protein under physiological conditions, while a high content of proline precludes extensive helical regions (Guzzo, 1965). Aggregation is favored by a high content of hydrophobic amino acid residues. Aggregation is reduced by the increase in net negative charge that accompanies acetylation of β -casein, while the larger increase in net negative charge resulting from succinylation effectively prevents aggregation (Hoagland, 1966). If, however, the length of alkyl group of the acylating agent, $(\text{RCO})_2\text{O}$, is increased, the aggregate sedimentation coefficient is increased as shown in Table II. Assuming that changes in hydrodynamic characteristics of aggregated acylated β -caseins brought about by variation of the alkyl substituent are not large, then the increase in sedimentation coefficient can be largely attributed to an increase in aggregate size. One interpretation is that greater hydrophobic bonding by the longer substituent alkyl groups enhances aggregation of the acylated β -casein. This interpretation is supported by the monomer-aggregate distribution data in Table II that show that butyrylated, valerylated, or hexanoylated β -casein is more extensively

aggregated than either acetylated or propionylated β -casein.

The physical state of β -casein in solution would therefore appear to be under the sensitive control of net negative charge, intermolecular hydrophobic bonding, and probably intermolecular hydrogen bonding. To date, however, no satisfactory explanation for the temperature dependence of aggregation of β -casein has been advanced.

Compact proteins such as bovine serum albumin, bovine γ -globulin, and bovine β -lactoglobulin have been found by Habeeb *et al.* (1958) to unfold upon acetylation or, particularly, upon succinylation. Supporting evidence was a sharp decrease in sedimentation coefficient coupled with an increase in intrinsic viscosity that resulted from acylation. The fact that no large change in monomer sedimentation coefficient (Table II) resulted from acylation of β -casein can be taken as further evidence for an unfolded native conformation for β -casein in solution. The conformation of β -casein in the milk micelle is yet unknown and remains a subject for further investigation.

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