

Phosphorylation of β -Casein and α -Lactalbumin by Casein Kinase from Lactating Bovine Mammary Gland

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ABSTRACT

Two milk proteins, β -casein and α -lactalbumin, were compared as substrates for casein kinase from bovine mammary gland. β -Casein could be rephosphorylated after removal of its phosphate groups, whereas α -lactalbumin was an effective substrate after the protein had been reduced and carboxymethylated. The native proteins could not be phosphorylated. Magnesium²⁺, Ca²⁺, and Mn²⁺ stimulated phosphorylation of the modified proteins. Calcium²⁺ was the most effective cation for α -lactalbumin and Mn²⁺ for β -casein. Michaelis constants were 144 μ M for α -lactalbumin in the presence of Ca²⁺ and 142 μ M for β -casein in the presence of Mn²⁺; however, the maximum velocity for α -lactalbumin was three times that of β -casein. After phosphorylation with [γ -³²P]ATP, partial hydrolysis showed that only serine residues were phosphorylated in both proteins. Chymotryptic peptides of phosphorylated α -lactalbumin and tryptic peptides of phosphorylated β -casein were examined by HPLC and selected peptides were analyzed for amino acid content. Comparison of the analyses with sequence data showed that serine at position 47 in α -lactalbumin is the major site of phosphate incorporation. Dephosphorylated β -casein was only partially rephosphorylated. However, the sites identified correspond to the phosphorylated residues in native β -casein, namely, serine at position 35 and the cluster of four serines between residues 15 and 20.

INTRODUCTION

Recent investigations have increased our knowledge of milk protein synthesis. In vitro experiments on lactating ovine mammary gland have shown that milk proteins are synthesized on membrane-bound polysomes (13, 14). The six major lactoproteins, α_{S1} -, α_{S2} -, β -, and κ -caseins, α -lactalbumin, and β -lactoglobulin are synthesized as preproteins with N-terminal hydrophobic amino acid extensions (21). According to the "signal concept" of Blobel and Dobberstein (7), hydrophobic extensions (signal peptides) guide nascent secretory proteins into the lumen of the endoplasmic reticulum, where the signal peptides are cleaved. At this stage, glycosylation and folding of proteins occur. Mercier and Gaye (21) showed that caseins are phosphorylated after their transfer across the endoplasmic reticulum membranes. A casein kinase located in the Golgi apparatus converts unphosphorylated polypeptides to the native phosphorylated caseins found in milk (3). This casein kinase has been found in the mammary glands of rats (2, 3), cows (5, 19), guinea pigs (22), and mice (6). Casein kinases of nonmammary origin have also been described; however, these enzymes differ from the mammary enzyme in several respects [see review (16)].

Research on protein kinases has provided considerable evidence that the phosphorylation of proteins can be attributed to two factors — the amino acid sequence surrounding the phosphorylatable site and the three-dimensional structure of the protein substrate. The influence of these factors has been documented in studies on cAMP-dependent protein kinases [see review (18)].

In this report, two milk proteins (β -casein and α -lactalbumin) were compared as substrates for casein kinase from bovine mammary gland. Both proteins required modification before phosphorylation could occur. Phosphate groups of β -casein were removed to convert β -casein

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into a substrate that could be rephosphorylated. α -Lactalbumin, which is not a phosphoprotein, was converted into a substrate following reduction and carboxymethylation. The fact that native α -lactalbumin could not serve as a phosphate acceptor suggests that potential phosphorylation sites are masked in the folded, native protein and thus unavailable to casein kinases, whereas proteins with open structures, such as caseins, have accessible sites.

MATERIALS AND METHODS

Materials

α_{S1} -Casein B (27), β -casein A¹ (15), and κ -casein B (29) were purified from the milk of individual cows homozygous for the particular variant. The preparation of β -lactoglobulin has been described (1). α -Lactalbumin and lysozyme were obtained from Sigma Chemical Company (St. Louis, MO). [γ -³²P]ATP was from E.I. duPont de Nemours & Co. (Boston, MA). Trypsin (L-1-Tosylamido-2-phenyl-ethyl chloromethyl ketone-treated) and α -chymotrypsin were from Worthington Biochemical Corp. (Freehold, NJ).

Preparation of Casein Kinase

Mammary glands were obtained from cows in full lactation through the cooperation of R. Miller from the Beltsville Agricultural Research Center. Following slaughter, the glands were trimmed of extraneous fat, cut into pieces (approximately 100 g), and stored at -20°C until needed. Casein kinase was prepared by a modification of a procedure previously described (5). All operations were carried out at 4°C . Mammary tissue (25 g) was cut into small pieces and homogenized for 1 min in a Waring blender with 100 ml of Buffer A (.02 M Tris-HCl and .001 M EDTA, pH 7.6) containing .25 M sucrose. The homogenate was filtered twice through fine cheesecloth and centrifuged for 30 min at $5000 \times g$. The supernatant solution was filtered through cheesecloth to remove fat and then centrifuged for 1 h at $66,000 \times g$. The resulting pellet was suspended in 15 ml of

Buffer A containing 1% n-octyl- β -D-glucopyranoside, mixed in a glass-teflon homogenizer, and centrifuged for 1 h at $66,000 \times g$. Solid ammonium sulfate was added to the supernatant to obtain a concentration of 30%; after standing for 15 min the mixture was centrifuged for 30 min at $10,000 \times g$. The supernatant was then made 60% with ammonium sulfate, and the mixture after standing for 15 min was centrifuged again for 30 min at $10,000 \times g$. The pellet was dissolved in a minimum volume of Buffer A containing 10% glycerol and dialyzed overnight against this buffer. The dialysate (approximately 4 ml) was stored at 4°C and retained full activity for a week.

Phosphorylation of Substrates by Casein Kinase

Incorporation of phosphate into milk proteins was determined at pH 7.5 in a 75 μl reaction mixture, containing .1 M Tris-HCl buffer, 2 mg/ml protein, casein kinase, 1 mM [γ -³²P]ATP (20 to 100 cpm/pmol), and cations (Mg^{2+} , Ca^{2+} or Mn^{2+}) as indicated. The reaction, which was initiated by the addition of [γ -³²P]ATP, was carried out for 20 min at 30°C . The phosphate incorporated into protein was measured by the chromatographic method of DePaoli-Roach et al. (11). Values were corrected for zero time. Endogenous phosphorylation was accounted for by assaying the sample without the protein substrate and subtracting this value from the total phosphate incorporation. Casein kinase activity was expressed as nanomoles of phosphate per milliliter incorporated into protein in 1 min at 30°C .

Protein Determination

Protein concentration was determined from the absorbance at 280 nm. The extinction coefficients, $E_{280}^{1\%}$, are 4.7 and 20.6 for β -casein and α -lactalbumin (12).

Denaturation of Proteins

Lysozyme and α -lactalbumin were reduced and S-carboxymethylated (RCM) by the procedure of Shechter et al. (25). Amino acid analyses of the products indicated that all cysteines were converted to carboxymethyl cysteine, whereas other amino acids were not modified.

¹Reference to brand or firm name does not constitute endorsement by the US Department of Agriculture over others of a similar nature not mentioned.

Removal of Phosphate Groups

Phosphate groups were removed from the caseins by potato acid phosphatase as previously described (4). Electrophoresis and phosphate analysis were used to verify that the proteins were more than 90% dephosphorylated.

Preparation of Phosphoproteins

β -Casein (20 mg) was rephosphorylated in a reaction mixture containing .1 M N-ethylmorpholine (pH 7.5), 50 mM NaF, 1 mM ATP, 1 mg/ml dephosphorylated β -casein, casein kinase, and 4 mM $MnCl_2$. The reaction proceeded for 18 h at 30°C. Simultaneously, 10 mg protein was labeled with [γ - ^{32}P]ATP. The RCM- α -lactalbumin was phosphorylated in a similar manner except that 10 mM $CaCl_2$ replaced the $MnCl_2$.

Reactions were terminated by the addition of 100% TCA to a final concentration of 25%. After they stood for 15 min in an ice bath, the mixtures were centrifuged for 5 min. The precipitates were washed successively with cold 5% TCA, 2:1 ethanol:ether and ether. Final precipitates were dissolved in .1 M NH_4HCO_3 , pH 8.2, and dialyzed against the same buffer for 24 h. The proteins were then freeze-dried, dissolved in distilled water, and freeze-dried a second time.

Digestion of Proteins with Proteolytic Enzymes

Rephosphorylated β -casein was dissolved in .1 M NH_4HCO_3 buffer, pH 8.4, at a concentration of approximately 1 mg/ml. The protein (3 to 5 mg) was digested for 2.5 h at 37°C with 1% trypsin. More trypsin was added and 2 h later a third batch of trypsin was added. The total trypsin concentration was 3%, and the total digestion time was 6.5 h. The final product was freeze-dried three times to eliminate the NH_4HCO_3 .

Phosphorylated RCM- α -lactalbumin was treated with chymotrypsin (1:25) in .1 M NH_4HCO_3 , pH 8.4, for 16 h at 37°C and freeze-dried three times.

Mapping of Peptides

The tryptic and chymotryptic peptides were dissolved in .1% (wt/vol) trifluoroacetic acid and centrifuged to remove insoluble material.

All the ^{32}P remained with the supernatant. The peptides were subjected to HPLC on a Vydac C₄ reversed phase column (4.6 x 250 nm).

The column was developed with a water:acetonitrile gradient (5 to 65%) containing .1% trifluoroacetic acid for 1 h at 1 ml/min. The peptide elution pattern was measured at 220 nm. Fractions of .5 ml were collected to analyze for ^{32}P by Cherenkov counting. From 82 to 98% of the ^{32}P applied was recovered in the peaks.

Amino Acid Analyses

Selected peptides from HPLC were pooled from repeated injections, evaporated to dryness, and the residue was hydrolyzed in evacuated tubes with 5.7 N HCl containing phenol (.05%) at 110°C for 24 h. Amino acid analyses was performed on a Beckman 119CL analyzer (Beckman Instruments, Fullerton, CA).

RESULTS

Phosphorylation of Milk Proteins

Milk proteins were tested as substrates for casein kinase (Table 1). The results confirm previous findings; removal of phosphate groups from α_{s1} -, β -, and κ -caseins increased their susceptibility to phosphorylation by casein kinase (5). Because the phosphate groups on the native proteins occur as phosphomonoesters of serine, it is assumed that casein kinase is transferring phosphate groups from ATP to specific serine residues. The whey proteins, β -lactoglobulin and α -lactalbumin, were not phosphorylated; these proteins do not contain phosphate groups in their native form (12). However, when α -lactalbumin was modified by reduction and carboxymethylation, it became an excellent substrate for casein kinase, but modified β -lactoglobulin was not appreciably phosphorylated by casein kinase.

Comparison of α -Lactalbumin and Lysozyme as Substrates for Casein Kinase

Browne et al. (9) have shown that the gross structure of chicken egg white lysozyme is similar to that of bovine α -lactalbumin in conformation and primary structure. Both proteins are relatively small molecules (approximately 14,000 daltons) and have compact

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TABLE 1. Phosphorylation of milk proteins by casein kinase.¹

Substrate	Incorporation of phosphate in milk proteins		
	Native protein	Dephosphorylated protein	Reduced, carboxymethylated protein
(nmol·min ⁻¹ ·ml ⁻¹)			
α_{S1} -Casein B	1.83	13.12	
β -Casein A ²	.33	8.2	
κ -Casein B	.03	2.46	
α -Lactalbumin	0		13.01
β -Lactoglobulin	.11		.11

¹ All assays were measured in the presence of .01 M Mg²⁺.

structures with four disulfide bonds. Lysozyme was tested as a substrate for casein kinase and compared with α -lactalbumin. Both proteins were poor phosphate acceptors in their native form. When the native proteins were reduced in the presence of excess dithiothreitol and alkylated with iodoacetic acid, RCM- α -lactalbumin, but not RCM-lysozyme, showed a significant improvement in acceptor activity. Although α -lactalbumin and lysozyme have many similarities, the results indicate that α -lactalbumin is a much better phosphate acceptor.

Effect of Divalent Cations on Rate of Phosphorylation

The phosphorylation of RCM- α -lactalbumin and dephosphorylated β -casein requires divalent cations (Table 2). No phosphorylation occurs in

the absence of divalent cations (data not shown). The relative rates indicate that the effect of Mn²⁺ and Mg²⁺ is similar for the two substrates; both proteins are phosphorylated at a greater rate with Mn²⁺ than with Mg²⁺. Significant differences are evident in the presence of Ca²⁺; rate of phosphorylation for RCM- α -lactalbumin is six times that of dephosphorylated β -casein.

Kinetic Constants

The apparent Michaelis constants (K_m) and maximum velocities (V_{max}) were determined for both substrates. The linearity of the double reciprocal plots (Figure 1) is indicative of classical Michaelis-Menten kinetics. The K_m value for RCM- α -lactalbumin in the presence of Ca²⁺ is 144 μ M, while the K_m value for de-

TABLE 2. Effect of divalent cations on phosphorylation.

Cation	Concentration (mM)	Phosphate incorporation			
		Dephosphorylated β -casein		RCM ¹ - α -lactalbumin	
		(nmol·min ⁻¹ ·ml ⁻¹)	(Relative rate)	(nmol·min ⁻¹ ·ml ⁻¹)	(Relative rate)
Mg ²⁺	4	3.6	.5	6.7	.6
Mg ²⁺	10	7.4	1.0	11.7	1.0
Ca ²⁺	4	1.2	.2	13.7	1.2
Ca ²⁺	10	3.3	.4	34.7	3.0
Mn ²⁺	4	18.0	2.4	26.0	2.2
Mn ²⁺	10	14.3	1.9	16.2	1.4

¹ Reduced and carboxymethylated.

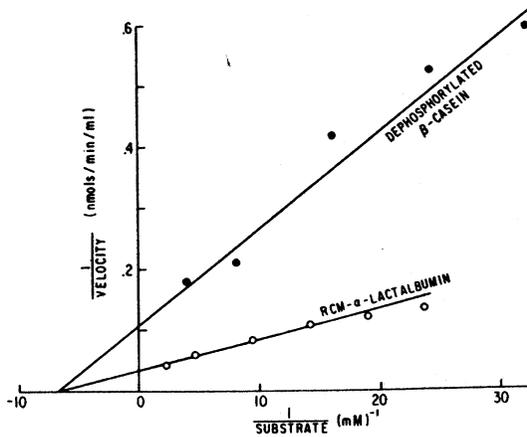


Figure 1. Double reciprocal plot of the rate of phosphorylation as a function of substrate concentration. Dephosphorylated β -casein (\bullet - \bullet) and reduced and carboxymethylated (RCM)- α -lactalbumin (\circ - \circ) were assayed in the presence of 4 mM Mn^{2+} and 10 mM Ca^{2+} , respectively. The slope and intercept of the lines were computed by the method of Cleland (10).

phosphorylated β -casein in the presence of Mn^{2+} is 142 μ M. The V_{max} values differ by threefold; values for RCM- α -lactalbumin and dephosphorylated β -casein are 28 and 9 nmol/min per ml, respectively.

Time Course

Figure 2 shows the extent of phosphorylation as a function of time for dephosphorylated β -casein and RCM- α -lactalbumin. For the two proteins, the incorporation of ^{32}P /mol approached a plateau at approximately .75 mol of ^{32}P /mol of protein.

Phosphoserine and Phosphothreonine Determinations

The RCM- α -lactalbumin and dephosphorylated β -casein were phosphorylated for 15 h with [γ - ^{32}P]ATP using casein kinase. Following partial hydrolysis, the proteins were analyzed for phosphoserine and phosphothreonine by high voltage electrophoresis at pH 1.9 (Figure 3). Upon autoradiography, two radioactive spots were observed, one migrating with phosphate and the other comigrating with phosphoserine. No radioactive spots corresponding to phosphothreonine were observed.

In both proteins, serine was the amino acid phosphorylated by casein kinase.

Identification of Phosphorylation Site in β -Lactalbumin

The RCM- α -lactalbumin was phosphorylated by casein kinase using [γ - ^{32}P]ATP. Following chymotryptic digestion, the resulting peptides were analyzed by reverse-phase chromatography (Figure 4). Samples (.5 ml) were collected and analyzed for radioactivity. Two labeled peptides were eluted at 21 min and 24 min. The major peak (21 min) represented 80% of the radioactivity recovered from the column.

The major phosphopeptide peak (21 min) was pooled from five HPLC runs and analyzed for amino acid composition (Table 3). Analyses indicate that peptides, Arg-10 to Leu-15 and Asp-37 to Tyr-50, are found in this peak (see Figure 5). Because α -lactalbumin contains only one arginine, the chymotryptic peptide (Arg-Glu-Leu-Lys-Asp-Leu) can be considered to be one component of the peak. When 2.5 mol of this peptide are subtracted from the

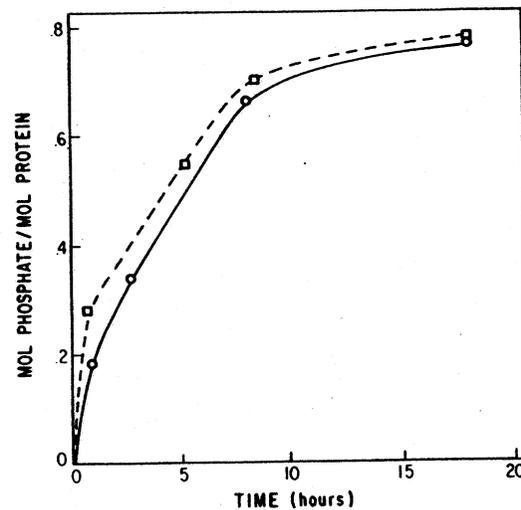


Figure 2. Time course of phosphate incorporated into reduced and carboxymethylated (RCM)- α -lactalbumin (\square - \square) and dephosphorylated β -casein (\circ - \circ) in the presence of 10 mM Ca^{2+} and 4 mM Mn^{2+} , respectively. Phosphate incorporation was measured by the method described under Materials and Methods except that the substrate concentration was .93 mg/ml.

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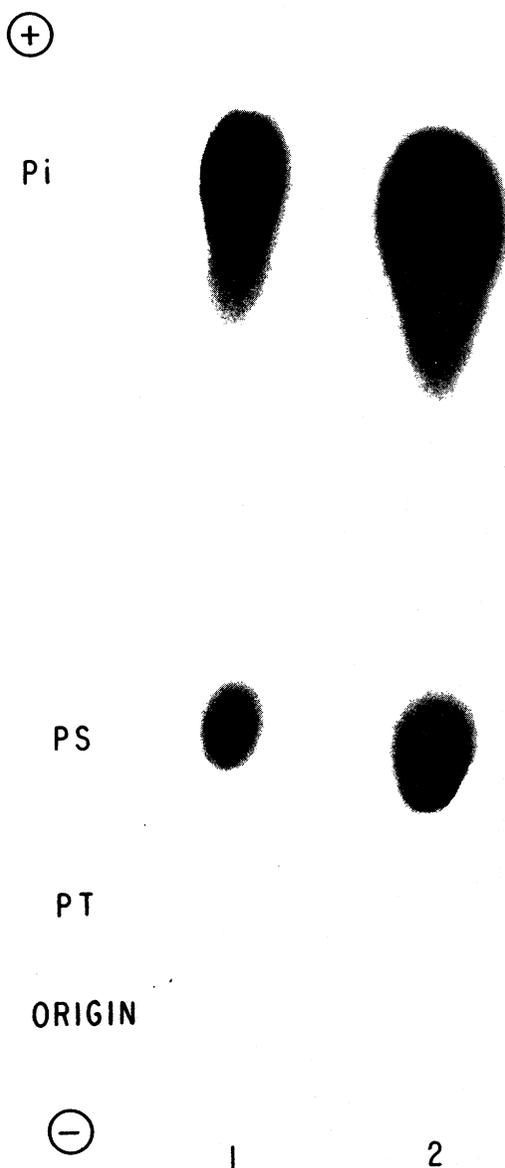


Figure 3. Phosphoamino acid analysis of reduced and carboxymethylated- α -lactalbumin (lane 1) and dephosphorylated β -casein (lane 2). The procedure of Tuazon *et al.* was used to locate the phosphorylated amino acids (28). The [32 P] proteins were hydrolyzed for 4 h at 110°C in 4 N HCl in evacuated sealed tubes. The hydrolysates were freeze-dried, then resuspended in a small volume of water and analyzed by thin-layer electrophoresis in 1.8% formic acid and 7.3% acetic acid, pH 1.9, at 500 V for 2 h. The labeled spots were analyzed by autoradiography with Kodak XAR-2 X-ray film. Carrier phosphoserine (PS) and phosphotreonine (PT) were added to the samples and stained with ninhydrin to identify the spots.

observed results (Table 3), the remaining peptide is almost identical to Asp-37 to Tyr-50 (see Figure 5). Only one serine residue occurs in this peptide, and serine at position 47 is undoubtedly the specific site that is phosphorylated.

The chymotryptic peptides can be compared with those obtained by Brew and Hill (8) in their studies of the sequence of α -lactalbumin. They found that Arg-10 to Leu-15 consisted of two peptides (Arg-Glu-Leu and Lys-Asp-Leu). Because the two peptides are similar in charge, size, and hydrophobicity, they could elute from the column at the same position. Brew and Hill (8) also found the chymotryptic peptide, Asp-37 to Tyr-50. Our low yield of this peptide relative to the arginine-containing peptide could be attributed to the fact that α -lactalbumin was not completely phosphorylated.

Identification of Phosphorylation Sites in β -Casein

β -Casein A² contains 16 serine residues, five of which (residues 15, 17, 18, 19, and 35) are specifically phosphorylated in the protein as isolated from milk (20). The sequence of β -casein is shown in Figure 6 with sections relevant to the discussion underlined. Native and dephosphorylated β -caseins were digested with trypsin and their HPLC profiles obtained. Comparison of the chromatograms (Figure 7a) showed that upon complete dephosphorylation, peaks labelled A, B, and C drastically decreased in intensity, whereas two new peaks (D and E) appeared. Rephosphorylation of the dephosphorylated β -casein by the mammary kinase, followed by trypsin digestion, showed the reappearance of peaks A, B, and C as well as the diminution of peaks D and E (Figure 7b). Radioactivity from γ - 32 P ATP was incorporated into A, B, and C with the majority of the activity (Figure 7c) centering about peak C. In order to pinpoint the nature of the peptides involved in rephosphorylation, all of these peaks were collected following repetitive injections and their amino acid compositions determined (Table 4).

Peak A was collected directly from the rephosphorylated sample. The amino acid composition of this peak (Table 4, column 1) is in excellent agreement with a tryptic peptide encompassing residues 33 to 48 and containing phosphoserine-35. The location of its

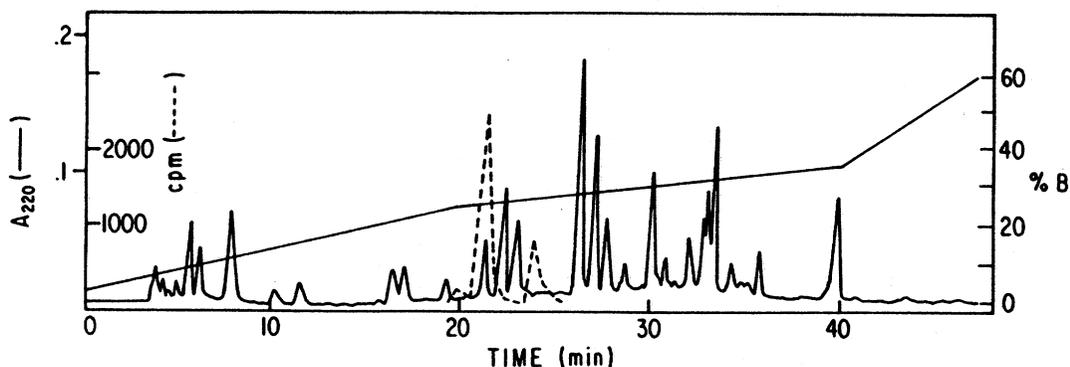


Figure 4. High performance liquid chromatogram of the chymotryptic phosphopeptides from RCM- α -lactalbumin, which was phosphorylated by mammary casein kinase. The peptides (—) were monitored at an absorbance of 220 nm by continuous recording. Phosphopeptides (---) were identified by radioactivity measured by Cerenkov counting from fractions (.5 ml) collected at 30-s intervals. The acetonitrile gradient (—) is indicated.

dephosphorylated counterpart was not determined.

The amount of Peak B collected from the rephosphorylated sample was not sufficient for amino acid analysis. Peak B was collected from native β -casein and found to represent the N-terminal phosphopeptide 1 to 25, which contains four of five phosphoserines, residues 15, 17, 18, and 19. In the case of peak C good recovery above background was obtained from

the rephosphorylated sample and also from native β -casein. Analysis of the data suggest that this region contains residues 2 to 25 as well as another peptide. The generation of 2 to 25 is not unexpected, because trypsin has a tendency to remove partially the N-terminal arginine. The identity of the other species is not unequivocal, but they could represent residues 106 to 117. Normally lysine 113 would be cleaved by trypsin but this cleavage was not observed to a large ex-

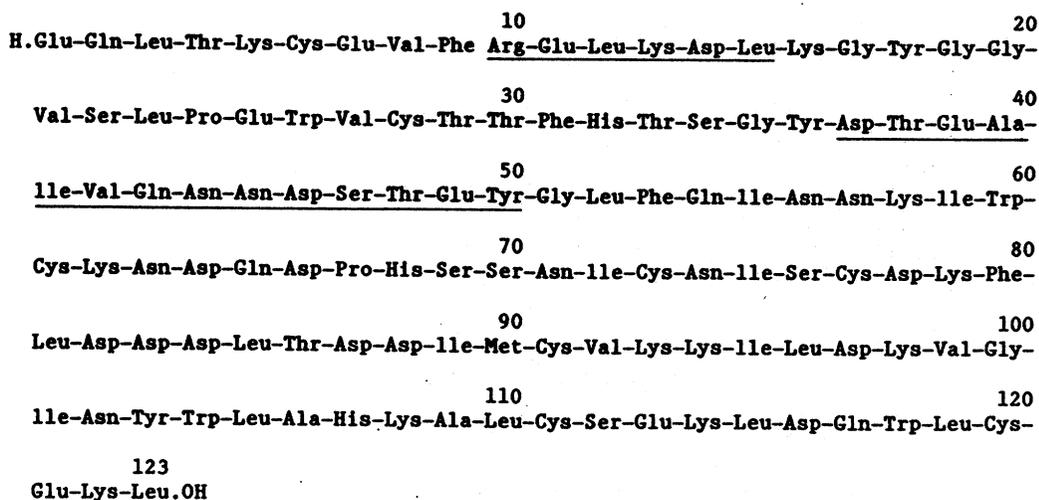


Figure 5. Primary structure of α -lactalbumin (26). Asn = Asparagine, Asp = aspartic acid, Arg = arginine, Cys = cysteine, Glu = glutamic acid, Gln = glutamine, Gly = glycine, His = histidine, Ile = isoleucine, Leu = leucine, Lys = lysine, Met = methionine, Phe = phenylalanine, Pro = proline, Ser = serine, Thr = threonine, Trp = tryptophan, Tyr = tyrosine, Val = valine.

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TABLE 3. Amino acid analysis of the phosphorylated chymotryptic peptide from RCM¹- α -lactalbumin.²

Amino acid ³	Observed	Subtracted 2.5(Arg ¹⁰ -Leu ¹⁵)	Expected Asp ³⁷ -Tyr ⁵⁰
Asp	5.4	2.9	4
Thr	1.7	1.7	2
Ser	1.3	1.3	1
Glu	5.3	2.8	3
Pro	.4	.4	
Gly	.8	.8	
Ala	1.2	1.2	1
Val	.9	.9	1
Met	
Ile	.9	.9	1
Leu	5.0	...	
Tyr	1.0	1.0	1
Phe	
Lys	2.8	.3	
His	.4	.4	
Arg	2.5	...	

¹ Reduced and carboxymethylated.

² The data in column 1 represent moles of amino acid per mole of peptide. In column 2, 2.5 mol of the peptide (Arg-Glu-Leu-Lys-Asp-Leu) were subtracted from the data presented in column 1. A dotted line indicates a value of less than .25. Tryptophan was not determined.

³ Asp = Aspartic acid, Thr = threonine, Ser = serine, Glu = glutamic acid, Pro = proline, Gly = glycine, Ala = alanine, Val = valine, Met = methionine, Ile = isoleucine, Leu = leucine, Tyr = tyrosine, Phe = phenylalanine, Lys = lysine, His = histidine, Arg = arginine.

ment in the original sequencing of β -casein (24); peptides 106 to 107 and 108 to 113 were obtained in only 22% yield with >40% of the digest in an insoluble form. The quantities of histidine, methionine, phenylalanine, and tyrosine found both in the native and rephosphorylated peaks argue for a chymotryptic split yielding the smaller fragment (106 to 117). It is known that glutamine (Gln) is often cleaved by chymotrypsin (17), which can contaminate tryptic preparations. Regardless of the true identity of the contaminant, subtraction of these residues (106 to 117) yields peptides in agreement with 2 to 25 both in the native and rephosphorylated forms.

Peaks D and E were collected from the dephosphorylated β -casein and found to be the dephosphorylated forms of 1 to 25 and 2 to 25, respectively; these peptides contain Ser 15, 17, 18 and 19. Rephosphorylation of the casein led to a reappearance of peaks B and C at the expense of D and E. Thus, the C region, which reappears on rephosphorylation, accounts for Ser 15, 17, 18 and 19, and the A peak accounts for Ser 35. Both of these taken together (C with A) contain 70% of added label. All of

these results suggest that enzymatically dephosphorylated β -casein is being rephosphorylated by casein kinase in vitro at the identical serine residues found phosphorylated in the native molecule.

DISCUSSION

The amino acid sequences surrounding the phosphorylated residues in milk proteins have been examined in over 50 caseins from a variety of species [see review (20)]. Without exception, an acidic residue (glutamic acid, aspartic acid, or phosphoserine) occurs two residues to the right of every phosphorylated hydroxyamino acid in the caseins. Mercier and his coworkers (21) have postulated that casein kinase recognizes a potential phosphorylation site corresponding to the tripeptide sequence -Ser/Thr-X-A where X represents any amino acid and A is an acidic residue, which can be glutamic acid, aspartic acid, or phosphoserine. Thus, aspartic and glutamic acids can be regarded as primary recognition sites for casein kinase, but phosphoserine would be a secondary site that becomes available as a result of the initial phosphorylation.

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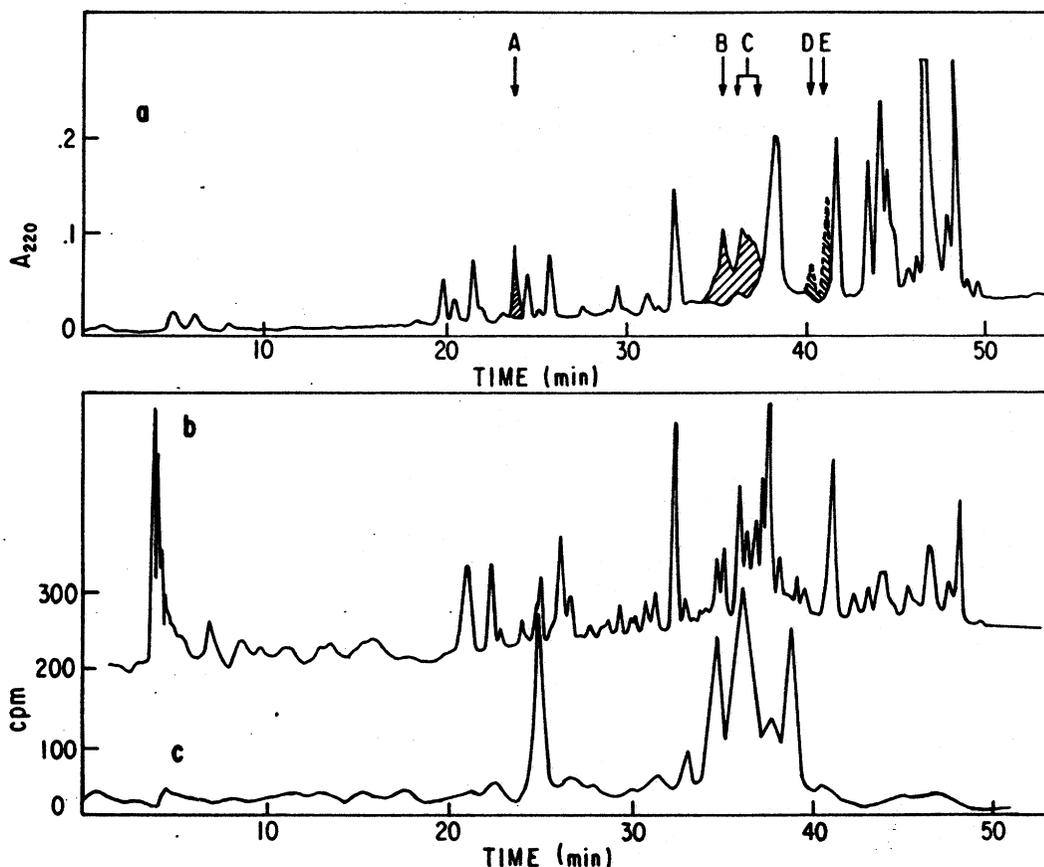


Figure 7. High performance liquid chromatography—profiles of the tryptic peptides of β -casein: a. Chromatograms of the peptides from native and dephosphorylated proteins, superimposed; peaks A, B, and C predominant in the native casein (shaded areas), whereas peaks D and E occur only in the dephosphorylated form (---). A_{220} = Absorbance at 220 nm. b. Profile of the tryptic digest of β -casein rephosphorylated in vitro by casein kinase. Note the reappearance of peaks A, B, and C. c. Radioactivity in fractions collected from profile b. All conditions of the experiment are the same as described in Figure 4.

Val-Gln-Asn-Asn-Asp-Ser-Thr-Glu-Tyr-Gly-Leu-Phe-. The factors that make α -lactalbumin a better substrate for casein kinase than lysozyme are not clear.

α -Lactalbumin could be converted into a substrate for casein kinase following denaturation by reduction and carboxymethylation. Thus, disruption of the native protein configuration was necessary for the recognition of the acceptor site, which is masked in the folded, native protein. A similar finding occurs in experiments on the enzymatic conversion of α -lactalbumin to the glycosylated derivative. Pless and Lennarz (23) showed that the RCM

form of α -lactalbumin was a much better acceptor of oligosaccharides than the native protein. They concluded that disruption of the native protein conformation appears necessary to expose the appropriate site.

β -Casein A² (the variant used in this study) has three primary sites where phosphorylation can occur, -Ser-Ser-Glu-Glu- (residues 18 to 21) with two primary sites and -Ser-Glu-Glu- (residues 35 to 37) with one primary site. Theoretically, trypsin cleavage should yield three phosphorylated peptides (1 to 25, 2 to 25 and 33 to 48). Dephosphorylation showed dramatic decreases in three major peaks (A, B

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and C) in the HPLC-tryptic peptide profiles of β -casein (Figure 7a). These have been shown by recovery and amino acid analysis to represent peptides 33 to 48, 1 to 25 and 2 to 25 respectively. The positions of the dephosphorylated forms of 1 to 25 and 2 to 25 were also identified (peaks D and E, Figure 7a). Rephosphorylation of the β -casein caused the peptides 33 to 48 (peak A) and 2 to 25 (peak C) to reappear in the same positions of the HPLC chromatograms in which they occurred in the native β -casein. Greater than 70% of the incorporated radioactivity was found in these two peaks. No other major peptides were either significantly labelled or showed changed retention times as a result of dephosphorylation and rephosphorylation. These results indicate that the purified casein kinase *in vitro* phosphorylates the β -casein at the same sites which are phosphorylated *in vivo*.

If the present concept of protein synthesis is correct, α -lactalbumin is folded as it goes through the endoplasmic reticulum. When it reaches the Golgi apparatus, phosphorylation sites are no longer exposed. However, unphosphorylated caseins, which contain little secondary structure, are readily phosphorylated by Golgi enzymes. Thus, the three-dimensional structural of proteins in the Golgi apparatus may determine which proteins in milk become phosphorylated.

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