

# Properties of Casein Kinase from Lactating Bovine Mammary Gland\*

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Casein kinase, which catalyzes the transfer of the terminal phosphate of ATP to dephosphorylated caseins, was prepared from homogenates of lactating bovine mammary tissue by differential centrifugation, followed by extraction of the 66,000  $\times g$  particulate fraction with Triton X-100. The enzyme required divalent cations and showed comparable activities with 15 mM  $\text{Ca}^{2+}$ , 15 mM  $\text{Mg}^{2+}$ , and 1 mM  $\text{Mn}^{2+}$ . Proteins with known primary structures were examined as substrates for casein kinase. Caseins ( $\alpha_{s1}$ ,  $\beta$ , and  $\kappa$ ), pepsinogen, and pepsin (denatured) showed significant increases in rates of phosphorylation after phosphate groups were removed. The catalytic efficiency (ratio of  $V_{\max}$  to  $K_m$ ) indicates that dephosphorylated pepsin was the best substrate. Dephosphorylated  $\alpha_{s1}$ - and  $\beta$ -caseins were significantly better substrates than native  $\alpha_{s1}$ -casein and dephosphorylated pepsinogen. The susceptibility of pepsinogen (native and dephosphorylated) and  $\alpha$ -lactalbumin to enzymatic phosphorylation could be enhanced by converting the proteins to the reduced, carboxymethylated derivatives.

Further studies of  $\beta$ -casein indicate that the dephosphorylated phosphopeptide (residues 1 to 25) was phosphorylated at a much higher rate than dephosphorylated  $\gamma_1$ -casein (residues 29 to 209). The results suggest that casein kinase catalyzes the phosphorylation of 1 to 4 serine residues in the phosphopeptide region of  $\beta$ -casein. Human  $\beta$ -caseins, which occur in six forms differing only by zero to five phosphate groups, showed differences in specificity. The rate of phosphate incorporation in unphosphorylated human  $\beta$ -casein was 8 times that of human  $\beta$ -casein with two and four phosphate groups. The role of acidic residues (glutamic and aspartic acids and phosphoserine) on the COOH-terminal side of serines phosphorylated by casein kinase is discussed.

Casein kinase in the lactating mammary gland is believed to be the enzyme that converts unphosphorylated polypeptides to the native phosphorylated caseins found in milk. Investigations of milk protein synthesis in the mammary tissue of mice (1) and rats (2) provided evidence that phosphate groups are added to casein following synthesis of the polypeptide chain, thereby establishing that unphosphorylated casein is the intermediate which serves as an acceptor for phosphate. Casein kinase with a high specificity for dephosphorylated caseins has been found in the Golgi apparatus of rat mammary glands (3, 4). The enzyme is a cyclic AMP-independent protein kinase, which catalyzes the phosphoryl-

ation of dephosphorylated (deP)  $\alpha_{s1}$ - and  $\beta$ -caseins by using ATP as a phosphate donor in the presence of divalent cations ( $\text{Mg}^{2+}$ ,  $\text{Ca}^{2+}$ ,  $\text{Mn}^{2+}$ , or  $\text{Co}^{2+}$ ). While investigation of rat casein kinase was useful initially in characterizing the enzyme and defining the parameters required for activity, several studies (5, 6) have shown marked species differences in casein. Since bovine casein is well characterized, studies of casein kinase from the same animal have been undertaken.

An intriguing question concerns the specificity of casein kinase, since the phosphate groups always occur on specific serine (or threonine) residues in casein. The four major components of bovine casein,  $\alpha_{s1}$ -,  $\beta$ -,  $\kappa$ -, and  $\alpha_{s2}$ -casein, contain 8, 5, 1 to 2, and 10 to 13 phosphate groups/molecule, respectively, with minor differences occurring in certain genetic variants (7-9). Mercier *et al.* (10) observed that glutamic acid or phosphoserine occurs 2 residues to the right of every phosphorylated site in casein. These investigators postulated that casein kinase recognizes a potential phosphorylation site corresponding to the tripeptide sequence Ser/Thr-X-Glu/SerP. Manson *et al.* (11) further suggested that aspartic acid could also serve as a recognition site for casein kinase. They showed that  $\alpha_{s0}$ -casein is identical with  $\alpha_{s1}$ -casein in amino acid composition;  $\alpha_{s0}$ -casein has an extra phosphate group located in the tripeptide SerP-Lys-Asp. Thus, aspartic and glutamic acids can be regarded as primary recognition sites for casein kinase, while phosphoserine would be a secondary site that becomes available as a result of the initial phosphorylation. The apparent requirement for an acidic residue near serines phosphorylated by casein kinase contrasts with findings on other types of protein kinases. Basic residues, particularly arginine, on the  $\text{NH}_2$ -terminal side of the phosphorylated serine are determinants of cyclic AMP-dependent protein kinase specificity (12-14).

Casein kinases have been observed in the bovine lactating mammary gland (15, 16). These enzymes can phosphorylate deP<sup>1</sup>  $\alpha_{s1}$ -casein, but have low activities toward deP  $\beta$ -casein. This communication describes a bovine casein kinase, which can rephosphorylate both deP  $\alpha_{s1}$ - and deP  $\beta$ -caseins and has properties similar to the enzyme associated with the Golgi membranes of rat mammary gland (4). We have examined protein substrates with known primary structures to determine whether the enzyme specificity conforms to the theory postulated by Mercier *et al.* (10).

## EXPERIMENTAL PROCEDURES

**Materials**—Milk proteins were isolated from the milk of individual cows homozygous for a particular variant.  $\alpha_{s1}$ -Casein (17),  $\beta$ - and  $\gamma_1$ -caseins (18),  $\kappa$ -casein (19), and  $\beta$ -lactoglobulin (20) were prepared as described previously.  $\alpha$ -Lactalbumin and  $\alpha_{s0}$ -casein were gifts from Drs. Marvin P. Thompson and Harold M. Farrell, Jr. of this laboratory. The phosphopeptide was isolated from a tryptic digest of  $\beta$ -

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<sup>1</sup> The abbreviations used are: deP, dephosphorylated; Mes, 2-(*N*-morpholino)ethanesulfonic acid.

casein (21). Human  $\beta$ -caseins were prepared by the method of Groves and Gordon (22). Pepsinogen and pepsin were from Worthington Biochemical Corp.<sup>2</sup> and ovalbumin from Sigma Chemical Co. The basic trypsin inhibitor of bovine pancreas was kindly provided by Dr. M. Laskowski, Sr., of Roswell Park Memorial Institute. [ $\gamma$ -<sup>32</sup>P]ATP was obtained from Amersham/Searle or New England Nuclear.

**Tissue Sources**—Mammary glands were obtained from two cows in full lactation through the cooperation of Dr. J. Bitman from the Beltsville Agricultural Research Center. Following slaughter, the glands were trimmed of extraneous fat, cut into pieces (approximately 400 g), frozen, and stored at  $-20^{\circ}\text{C}$  until needed.

Golgi membranes from lactating bovine mammary glands were kindly provided by Dr. T. W. Keenan (Purdue University). The procedure for preparing Golgi membranes from bovine lactating mammary glands has been described (23).

**Reduction of Proteins**—Reduced, carboxymethylated proteins were prepared by the procedure of Shechter *et al.* (24). Amino acid analyses (25) of the modified proteins indicated that all the cysteines were carboxymethylated, whereas other amino acids were not modified.

**Removal of Phosphate Groups**—Phosphate groups were removed from the proteins by potato acid phosphatase (26). Electrophoretic procedures (26) and phosphate analyses (27) were used to verify that the proteins were more than 90% dephosphorylated.

**Preparation of Casein Kinase**—Casein kinase was prepared from 100-g batches of frozen mammary tissue. All operations were carried out at  $4^{\circ}\text{C}$ . Mammary tissue (100 g) was cut into small pieces and homogenized in 400 ml of Buffer A (0.25 M sucrose, 0.001 M EDTA, and 0.02 M Tris-HCl, pH 7.6) for 1 min in a Waring Blender. The homogenate was filtered twice through fine cheesecloth and centrifuged for 30 min at  $5,000 \times g$ . The supernatant solution was filtered through cheesecloth to remove the fat and was then centrifuged for 1 h at  $66,000 \times g$ . The pellet thus obtained was suspended in 40 ml of Buffer A and mixed in a glass-Teflon homogenizer. The homogenate was centrifuged at  $66,000 \times g$  for 1 h and the resulting pellet was mixed with 40 ml of Buffer A containing 1% Triton X-100. Following homogenization in a glass-Teflon homogenizer, the mixture was sonicated for 30 s at 3 mA with a Branson Sonifier and then centrifuged for 60 min at  $66,000 \times g$ . Glycerol was added to the supernatant solution to a concentration of 10% and the solution was stored at  $-20^{\circ}\text{C}$ .

**Casein Kinase Assay**—Casein kinase activity was determined at pH 7.6 in an 80- $\mu\text{l}$  reaction mixture, containing 0.1 M Tris-HCl buffer (pH 7.6), 10 mM  $\text{MgCl}_2$ , 2 mg/ml of protein, casein kinase, and 1 mM [ $\gamma$ -<sup>32</sup>P]ATP (20 to 40 cpm/pmol). The phosphorylation reaction was initiated by the addition of [ $\gamma$ -<sup>32</sup>P]ATP and  $\text{MgCl}_2$  in 20  $\mu\text{l}$ . After incubation for 20 min at  $30^{\circ}\text{C}$ , the reaction was terminated by applying 50- $\mu\text{l}$  aliquots onto squares ( $2 \times 2$  cm) of Whatman No. 31ET chromatography paper. The paper squares were treated by the method described by Reimann *et al.* (28). Radioactivity was determined by scintillation counting. Duplicate samples were run for each experiment and all values were corrected for endogenous phosphorylation in the casein kinase preparation. One unit of casein kinase activity was defined as the amount of enzyme which transferred 1 pmol of phosphate to protein in 1 min at  $30^{\circ}\text{C}$ . Under the assay conditions used, casein kinase activity increased linearly with the amount of added enzyme and with the time of incubation.

The procedure was modified when the  $\beta$ -casein phosphopeptide (native or dephosphorylated) was the substrate. Assay conditions were identical except that 0.25 mg/ml of peptide replaced the protein. The reaction was terminated by the addition of 100  $\mu\text{l}$  of 60% acetic acid. The peptide was applied to an anion exchange column (2 ml of AG 1-X8 resin, Bio-Rad) equilibrated with 30% acetic acid, as described by Kemp *et al.* (14). The peptides were eluted with 6 ml of 30% acetic acid directly into liquid scintillation vials and Cerenkov radiation was measured. Casein kinase activity measured on  $\beta$ -casein was similar in both procedures (filter paper assay and column method).

**Analyses of Kinetic Data**—Kinetic constants were obtained by fitting the data to rate equations. The computer method described by Cleland (29) was used to obtain the double reciprocal plots.

## RESULTS AND DISCUSSION

**Solubilization of Casein Kinase**—Several procedures were

<sup>2</sup> Reference to brand or firm name does not constitute endorsement by the United States Department of Agriculture over others of a similar nature not mentioned.

tested for their ability to solubilize the  $66,000 \times g$  pellet. Release of casein kinase into the supernatant solution after being centrifuged for 1 h at  $66,000 \times g$  was the criterion for solubilization. Alcohol and acetone at several concentrations were ineffective and only 20% of the casein kinase could be extracted with 1-butanol. Urea (4 M) produced complete loss of activity. Treatment of the pellet with phospholipase C was ineffective and trypsin destroyed casein kinase activity. Extraction of the pellet with Tris (0.4 M, pH 7.5),  $\text{MgCl}_2$  (0.1 M), or EDTA (0.02 M) followed by sonication for 1 min also failed to solubilize the enzyme. In contrast to these procedures, extraction of the pellet with Triton X-100 ( $>0.5\%$ ) solubilized an amount of casein kinase equivalent to that initially present in the pellet and resulted in a 6-fold increase in specific activity. The solubilized casein kinase required glycerol (10%) to maintain the stability of the enzyme at  $-20^{\circ}\text{C}$ .

**Casein Kinase in Golgi Membranes**—In the original studies on casein kinase, rat Golgi membranes were used as the enzyme source (3). Therefore, it seemed pertinent to compare the activity of the solubilized casein kinase prepared from lactating bovine mammary glands with the enzyme activity associated with Golgi membranes from the same tissue. The results (Table I) show that both enzymes were activated by  $\text{Ca}^{2+}$  as well as  $\text{Mg}^{2+}$ . No activity was seen in the absence of divalent cations (data not shown). The solubilized casein kinase incorporated phosphate into deP  $\alpha_{s1}$ -casein at  $2\frac{1}{2}$  times the rate obtained for native  $\alpha_{s1}$ -casein; similar results were obtained with Golgi casein kinase. Although these experiments are limited in scope, the results suggest that the solubilized casein kinase is derived from Golgi membranes.

**Effect of pH on Casein Kinase Activity**—Fig. 1 *a* and *b* illustrates the effect of pH on phosphate incorporation into deP  $\alpha_{s1}$ - and  $\beta$ -caseins. Maximum activity in Tris and Mes buffers occurs as a broad peak from pH 7 to pH 8. Imidazole buffer enhances the activity of casein kinase with both substrates at the higher pH values, the effect being most pronounced at pH 8. The nature of this activation has not been investigated.

**Apparent  $K_m$  for ATP and Casein**—The effect of ATP concentration on the phosphorylation of deP  $\beta$ -casein is shown in Fig. 2. The apparent  $K_m$  value for ATP, computed from a double reciprocal plot (Fig. 2, *inset*), is 131  $\mu\text{M}$  for deP  $\beta$ -casein. For deP  $\alpha_{s1}$ -casein, an apparent  $K_m$  value of 148  $\mu\text{M}$  was obtained under similar conditions. The two values are similar but are higher than the apparent  $K_m$  (80  $\mu\text{M}$ ) for rat Golgi casein kinase (4).

The rate of phosphorylation of deP  $\alpha_{s1}$ - and deP  $\beta$ -caseins was studied as a function of substrate concentration. The results for deP  $\beta$ -casein are shown in Fig. 3. The apparent  $K_m$  value obtained from a double reciprocal plot of the data (Fig.

TABLE I

Comparison of the rate of phosphate incorporation into casein by the solubilized casein kinase preparation and by Golgi membranes

Casein kinase activity was measured in the presence of 8 mM  $\text{Ca}^{2+}$  or 8 mM  $\text{Mg}^{2+}$  by the method described under "Experimental Procedures."

Substrate	Metal ion	Casein kinase activity	
		Solubilized casein kinase	Golgi membranes
<i>Relative activity</i>			
Dephosphorylated $\alpha_{s1}$ -casein B	$\text{Mg}^{2+}$	100	100
Dephosphorylated $\alpha_{s1}$ -casein B	$\text{Ca}^{2+}$	90	109
$\alpha_{s1}$ -Casein B	$\text{Ca}^{2+}$	38	41

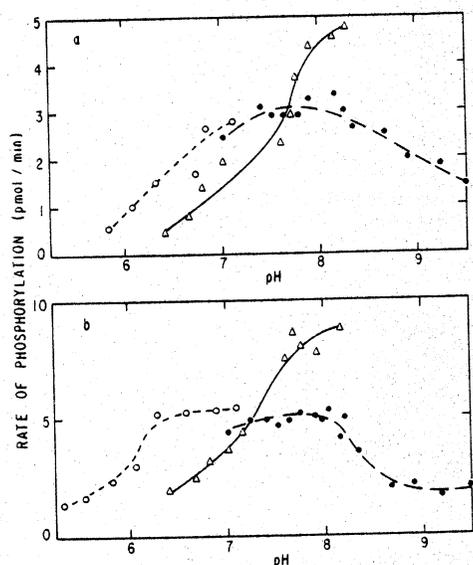


FIG. 1. Effect of pH on casein kinase activity. Substrates for the pH activity curves were dephosphorylated  $\beta$ -casein A<sup>2</sup> (a) and dephosphorylated  $\alpha_{s1}$ -casein B (b). Assay conditions are described under "Experimental Procedures." Buffers were 50 mM Mes (○), 50 mM Tris (●), and 50 mM imidazole (△).

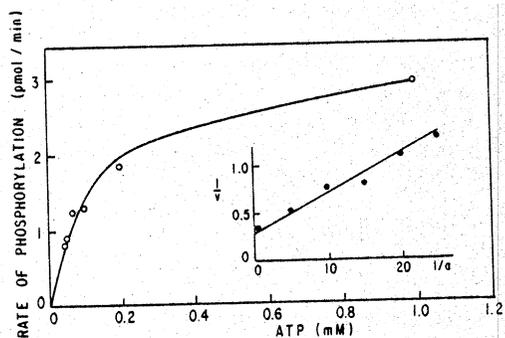


FIG. 2. Effect of ATP concentration on phosphate incorporation into dephosphorylated  $\beta$ -casein A<sup>2</sup>. Assay conditions are similar to those described under "Experimental Procedures," except that the casein concentration was 6 mg/ml and the concentration of ATP was varied. *Inset* shows a double reciprocal plot of the data.

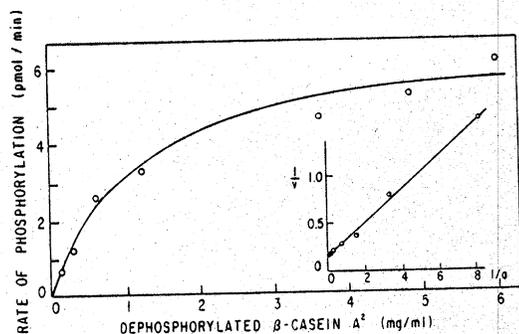


FIG. 3. Effect of the concentration of dephosphorylated  $\beta$ -casein A<sup>2</sup> on phosphate incorporation. Casein kinase activity was measured by the method described under "Experimental Procedures," except that the concentration of dephosphorylated casein was varied. *Inset* shows a double reciprocal plot of the data.

3, *inset* is 1.1 mg/ml (47  $\mu$ M). A similar experiment with deP  $\alpha_{s1}$ -casein as the substrate gave an apparent  $K_m$  value of 1.0 mg/ml (42  $\mu$ M), which is higher than the value (12  $\mu$ M) obtained for the rat Golgi casein kinase (4).

**Effect of Divalent Cations on Rate of Phosphorylation**—Casein kinase activity is stimulated by  $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$ ,  $\text{Mn}^{2+}$ , and

$\text{Co}^{2+}$  (Fig. 4).  $\text{Mn}^{2+}$  at 1 mM is as effective as 15 mM  $\text{Ca}^{2+}$  or  $\text{Mg}^{2+}$ . Little activity is seen in the absence of divalent cations.

The data presented in Fig. 4 were obtained with deP  $\beta$ -casein, which is soluble in the presence of  $\text{Ca}^{2+}$ ,  $\text{Mn}^{2+}$ , and  $\text{Mg}^{2+}$  but precipitates at high concentrations of  $\text{Co}^{2+}$  (>4 mM). Thus, the low activity of casein kinase with  $\text{Co}^{2+}$  may be attributed to the insolubility of deP  $\beta$ -casein under the assay conditions used. Native caseins and deP  $\alpha_{s1}$ -casein precipitate in the presence of  $\text{Mn}^{2+}$ ,  $\text{Ca}^{2+}$ , and  $\text{Co}^{2+}$  at the concentrations used in this experiment. However, in the presence of 10 mM  $\text{MgCl}_2$ , the protein substrates were completely soluble.

**Effect of Removing Phosphate Groups from Proteins on Casein Kinase Activity**—Casein kinase was tested on a variety of phosphoprotein substrates as well as their dephosphorylated derivatives. The proteins examined included bovine caseins, pepsinogen, and pepsin. Since pepsin is denatured at pH values above 6.0, alkali-denatured pepsin was prepared (30) and used for the experiments. The results (Table II) show that all the proteins became more susceptible to enzymatic phosphorylation after conversion to the dephosphorylated derivatives. Best substrates were deP  $\alpha_{s1}$ - and deP  $\beta$ -caseins and deP pepsin. The high rate of phosphate incorporation in the deP caseins ( $\alpha_{s1}$  and  $\beta$ ) gives support to the theory of Mercier *et al.* (10) that glutamic acid N + 2 residues from serine is essential to casein kinase specificity. The tripeptide Ser-X-Glu occurs five times in deP  $\alpha_{s1}$ -casein, three times in deP  $\beta$ -casein (A<sup>2</sup> and B), and twice in deP  $\beta$ -casein C, but is absent from the phosphorylated native caseins (7). DeP  $\kappa$ -casein can be phosphorylated by casein kinase but at a rela-

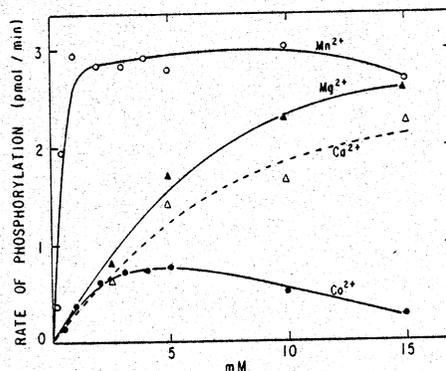


FIG. 4. Effect of cation concentration on casein kinase activity. Phosphate incorporation was measured in the presence of  $\text{Mg}^{2+}$ ,  $\text{Ca}^{2+}$ ,  $\text{Mn}^{2+}$ , and  $\text{Co}^{2+}$ . Enzyme activity was determined by the method described under "Experimental Procedures," except that the cation concentration was varied.

TABLE II

*Effect of dephosphorylation of protein substrates on casein kinase activity*

Conditions for measuring casein kinase activity are described under "Experimental Procedures." Denatured pepsin was prepared by the method of Tang and Hartley (30).

Substrate	Casein kinase activity	
	Phosphoprotein	Dephosphorylated protein
	units	
$\alpha_{s1}$ -Casein B	2.4	5.5
$\alpha_{s0}$ -Casein B	0.6	
$\beta$ -Casein A <sup>2</sup>	0.5	5.0
$\beta$ -Casein B	0.2	4.5
$\beta$ -Casein C	0.8	4.9
$\kappa$ -Casein	0.02	1.5
Pepsinogen	0.1	1.8
Pepsin (denatured)	2.5	8.3

tively slow rate. Serine 49 (SerP-Pro-Glu), the phosphorylated residue in  $\kappa$ -casein, is probably the site modified.

Native  $\alpha_{s1}$ -casein showed a significant rate of phosphate incorporation despite the fact that the protein contains no Ser-X-Glu sequence. However, serine 41 occurs in the sequence Ser-Lys-Asp. Since  $\alpha_{s0}$ -casein is identical with  $\alpha_{s1}$ -casein except for an extra phosphate group located on serine 41, this serine is a possible site for phosphorylation. Manson *et al.* (11) noted that no other Ser-X-Asp occurs in  $\alpha_{s1}$ - and  $\beta$ -caseins. The low rate of phosphate incorporation into  $\alpha_{s0}$ -casein (Table II) indicates a lack of potential sites and supports the concept that casein kinase converts  $\alpha_{s1}$ -casein to  $\alpha_{s0}$ -casein.

If glutamic and aspartic acids are recognition sites for casein kinase, pepsin and pepsinogen should be excellent substrates. The one phosphate group in pepsin is located at position 68 in the configuration SerP-Gln-Glu (31). In addition, two Ser-X-Glu and two Ser-X-Asp segments occur and may be responsible for the phosphorylation of pepsin as shown in Table II. The rate of phosphorylation increased more than 3-fold when pepsin was dephosphorylated. It is possible that serine 68 in pepsin exists in a unique environment that makes it particularly susceptible to enzymatic phosphorylation. The lower rates observed for native and dephosphorylated pepsinogen compared to denatured pepsin may reflect an influence of tertiary structure on the availability of potential phosphorylation sites.

**Kinetic Constants**—The apparent  $K_m$  and  $V_{max}$  values were determined for protein substrates (Table III). The caseins ( $\alpha_{s1}$ -casein and dephosphorylated  $\alpha_{s1}$ - and  $\beta$ -caseins) had similar  $K_m$  values, higher than those observed for pepsin and pepsinogen. The catalytic efficiency (ratio of  $V_{max}$  to  $K_m$ ) indicates that denatured deP pepsin is the best substrate of the proteins tested, while deP  $\alpha_{s1}$ - and deP  $\beta$ -caseins are better substrates than  $\alpha_{s1}$ -casein and deP pepsinogen.

**Effect of Protein Denaturation on Rate of Protein Phosphorylation by Casein Kinase**—Bylund and Krebs (32) indicated that the tertiary structure of proteins can mask phosphorylation sites, which become accessible when the protein is denatured. Therefore, the rate of phosphate incorporation in several proteins and their denatured derivatives (reduced and carboxymethylated) was examined (Table IV). The results show that chemical modification of pepsinogen doubled its rate of phosphorylation by casein kinase. Even greater enhancement of casein kinase activity (4.7 units) was observed when deP pepsinogen was tested after base denaturation (pH 10 for 3 days at 4°C). Thus, denaturation by two methods converts deP pepsinogen into a better substrate and provides an explanation for the differences between native pepsinogen and denatured pepsin in phosphorylation rates observed in Table II.

TABLE III  
Kinetic constants for protein substrates

Protein phosphorylation was measured as described under "Experimental Procedures" except that the concentration of protein substrates was varied.  $K_m$  and  $V_{max}$  values were estimated by fitting the data to the Michaelis-Menten equation according to Cleland (29).  $K_m$  values were computed by assuming that the molecular weights of the proteins were 23,613 ( $\alpha_{s1}$ -casein B), 23,980 ( $\beta$ -casein A<sup>2</sup>), 41,000 (pepsinogen), and 35,000 (pepsin).

Substrate	$K_m$ app $\mu M$	$V_{max}$ units	$V_{max}/K_m$ app
$\alpha_{s1}$ -Casein	48	2.2	0.05
Dephosphorylated $\alpha_{s1}$ -casein	48	6.9	0.15
Dephosphorylated $\beta$ -casein A <sup>2</sup>	48	6.6	0.14
Dephosphorylated pepsinogen	28	1.3	0.05
Dephosphorylated pepsin (denatured)	18	6.4	0.36

TABLE IV

Phosphorylation of proteins by casein kinase

The assay conditions for measuring casein kinase activity are described under "Experimental Procedures."

Substrate	Casein kinase activity	
	Native protein	Reduced, carboxymethylated protein
	units	
Pepsinogen	0.60	1.69
Dephosphorylated pepsinogen	1.37	3.16
$\alpha$ -Lactalbumin	0.55	4.12
$\kappa$ -Casein	0.15	<0.05
Ovalbumin	<0.05	0.60
Dephosphorylated ovalbumin	0.25	0.65
$\beta$ -Lactoglobulin	<0.05	<0.05
Basic trypsin inhibitor	<0.05	<0.05

Native  $\alpha$ -lactalbumin also became more susceptible to enzymatic phosphorylation after reduction and carboxymethylation. The unfolding obtained by the cleavage of four disulfide bonds must expose potential sites. The sequence of  $\alpha$ -lactalbumin has two Ser-X-Asp but no Thr/Ser-X-Glu segments (7). Thus, if acidic residues are determinants of specificity, aspartic acid residues must be involved.

Denatured ovalbumin is a better substrate than native ovalbumin; however, its rate of phosphorylation is low compared with other substrates.  $\kappa$ -Casein (7),  $\beta$ -lactoglobulin (7), and basic trypsin inhibitor (33) each contain at least one tripeptide Ser-X-Glu segment but no Ser-X-Asp segments. When these proteins were reduced and carboxymethylated, there was little change in the rates of phosphate incorporation. While glutamic acid might be necessary for casein kinase catalysis, the data suggest that additional factors must be involved or that the sites are still unavailable in the derived proteins.

**Rate of Phosphate Incorporation into  $\beta$ -Casein and Fragments Derived from  $\beta$ -Casein**—To determine which sites are phosphorylated by casein kinase, we examined peptides from  $\beta$ -casein. Fig. 5 shows the sequence of  $\beta$ -casein A<sup>2</sup>, indicating the location of the phosphopeptide (residues 1 to 25) and of  $\gamma_1$ -casein (residues 29 to 209). There are four phosphate groups in the phosphopeptide, which are well separated from the one phosphate group in  $\gamma_1$ -casein. When these peptides were tested as substrates for casein kinase (Table V), deP phosphopeptide was phosphorylated at a much faster rate than the  $\gamma_1$ -caseins (native and dephosphorylated). The results suggest that casein kinase rapidly phosphorylates from one to four serines in the phosphopeptide region of the molecule.  $\beta$ -Casein C (see Fig. 5) has only four phosphate groups, all in the phosphopeptide. Therefore, the high rate of phosphorylation of deP  $\beta$ -casein C (shown in Table II) must be entirely attributed to the serines in the phosphopeptide region.

**Rate of Phosphorylation of Human  $\beta$ -Caseins**—Human  $\beta$ -casein is an excellent model for specificity studies since it occurs in six forms, containing from zero to five phosphate groups on an identical polypeptide chain (22). Sequence studies of four forms indicate that all the phosphate groups are located at the NH<sub>2</sub>-terminal end of the molecule (34). When the human  $\beta$ -caseins were examined as substrates for casein kinase, the unphosphorylated human  $\beta$ -casein was phosphorylated at a much greater rate than the human  $\beta$ -caseins containing two and four phosphate groups per molecule (Table VI). Thus, serines 9 and 10 must be available for a high rate of phosphorylation to occur and are likely sites phosphorylated by casein kinase. The low activity of casein kinase toward the di- and tetraphosphorylated human  $\beta$ -caseins in-



obtained with the tetraphospho human  $\beta$ -casein, which has one Thr-X-Glu segment, and with native  $\kappa$ -casein, which has two Thr-X-Glu segments, indicate that these proteins were poor substrates for casein kinase.

Results obtained with the diphospho human  $\beta$ -casein indicate that Ser-X-SerP is not a sequence readily recognized by casein kinase. According to the hypothesis of Mercier *et al.* (10), phosphorylation occurs in a sequential manner; serine residues must be phosphorylated to generate phosphoserines, which can act as specifiers for further phosphorylation. Examination of human  $\beta$ -caseins supports such a mechanism. Two forms of human  $\beta$ -casein predominate, the diphospho form with phosphate groups on primary sites and the tetraphospho form with phosphate groups on secondary as well as primary sites (22, 34). Whether bovine caseins are phosphorylated in a similar manner is not known. However, it is possible that several enzymes might be required, since our results cannot account for complete phosphorylation of casein by one enzyme. The protein substrates used in this study should provide tools for locating casein kinases with different specificities with the ultimate objective of learning how fully phosphorylated caseins are synthesized in the lactating mammary gland.

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#### REFERENCES

1. Turkington, R. W., and Topper, Y. J. (1966) *Biochim. Biophys. Acta* **127**, 366-372
2. Singh, V. N., Dave, S. S., and Venkatasubramanian, T. A. (1967) *Biochem. J.* **104**, 48C-50C
3. Bingham, E. W., Farrell, H. M., Jr., and Basch, J. J. (1972) *J. Biol. Chem.* **247**, 8193-8194
4. Bingham, E. W., and Farrell, H. M., Jr. (1974) *J. Biol. Chem.* **249**, 3647-3651
5. Jenness, R. (1975) in *Lactation: A Comprehensive Treatise* (Larson, B. L., and Smith, V. R., eds) Vol. 3, pp. 3-107, Academic Press, New York
6. McKenzie, R. M., and Larson, B. L. (1978) *J. Dairy Sci.* **61**, 885-889
7. Whitney, R. McL., Brunner, J. R., Ebner, K. E., Farrell, H. M., Jr., Josephson, R. V., Morr, C. V., and Swaisgood, H. E. (1976) *J. Dairy Sci.* **59**, 795-815
8. Vreeman, H. J., Both, P., Brinkhuis, J. A., and Van der Spek, C. (1977) *Biochim. Biophys. Acta* **491**, 93-103
9. Brignon, G., Ribadeau Dumas, B., Mercier, J. C., Pelissier, J. P., and Das, B. C. (1977) *FEBS Lett.* **76**, 274-279
10. Mercier, J. C., Grosclaude, F., and Ribadeau Dumas, B. (1972) *Milchwissenschaft* **27**, 402-408
11. Manson, W., Carolan, T., and Annan, W. D. (1977) *Eur. J. Biochem.* **78**, 411-417
12. Pomerantz, A. H., Alfrey, V. G., Merrifield, R. B., and Johnson, E. M. (1977) *Proc. Natl. Acad. Sci. U. S. A.* **74**, 4261-4265
13. Kemp, B. E., Graves, D. J., Benjamini, E., and Krebs, E. G. (1977) *J. Biol. Chem.* **252**, 4888-4894
14. Kemp, B. E., Bylund, D. B., Huang, T. S., and Krebs, E. G. (1975) *Proc. Natl. Acad. Sci. U. S. A.* **72**, 3448-3452
15. Mackinlay, A. G., West, D. W., and Manson, W. (1977) *Eur. J. Biochem.* **76**, 233-243
16. Chew, L. F., and Mackinlay, A. G. (1974) *Biochim. Biophys. Acta* **359**, 73-82
17. Thompson, M. P. (1966) *J. Dairy Sci.* **49**, 792-795
18. Groves, M. L., Gordon, W. G., Kalan, E. B., and Jones, S. B. (1973) *J. Dairy Sci.* **56**, 558-568
19. Zittle, C. A., and Custer, J. H. (1963) *J. Dairy Sci.* **46**, 1183-1188
20. Aschaffenburg, R., and Drewry, J. (1957) *Biochem. J.* **65**, 273-277
21. Manson, W., and Annan, W. D. (1971) *Arch. Biochem. Biophys.* **145**, 16-26
22. Groves, M. L., and Gordon, W. G. (1970) *Arch. Biochem. Biophys.* **140**, 47-51
23. Keenan, T. W., Huang, C. M., and Morr , D. J. (1972) *J. Dairy Sci.* **55**, 1577-1585
24. Shechter, Y., Patchornik, A., and Burstein, Y. (1973) *Biochemistry* **12**, 3407-3413
25. Moore, S., and Stein, W. H. (1963) *Methods Enzymol.* **6**, 819-831
26. Bingham, E. W., Farrell, H. M., Jr., and Dahl, K. J. (1976) *Biochim. Biophys. Acta* **429**, 448-460
27. Meun, D. H. C., and Smith, K. C. (1968) *Anal. Biochem.* **26**, 364-368
28. Reimann, E. M., Walsh, D. A., and Krebs, E. G. (1971) *J. Biol. Chem.* **246**, 1986-1995
29. Cleland, W. W. (1967) *Adv. Enzymol.* **29**, 1-32
30. Tang, J., and Hartley, B. S. (1970) *Biochem. J.* **118**, 611-623
31. Sepulveda, P., Marciniszyn, J., Jr., Liu, D., and Tang, J. (1975) *J. Biol. Chem.* **250**, 5082-5088
32. Bylund, D. B., and Krebs, E. G. (1975) *J. Biol. Chem.* **250**, 6355-6361
33. Kassell, B., and Laskowski, M., Sr. (1965) *Biochem. Biophys. Res. Commun.* **20**, 463-468
34. Greenberg, R., Groves, M. L., and Peterson, R. F. (1976) *J. Dairy Sci.* **59**, 1016-1018
35. Milstein, C. P. (1968) *Biochem. J.* **110**, 127-134