

## ISOLATION AND SOLUBILIZATION OF CASEIN KINASE FROM GOLGI APPARATUS OF BOVINE MAMMARY GLAND AND PHOSPHORYLATION OF PEPTIDES

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Phosphate incorporation from [ $\gamma$ - $^{32}$ P]ATP into native and dephosphorylated  $\alpha_{s1}$ -casein is catalyzed by a casein kinase localized in the Golgi apparatus of lactating bovine mammary gland. Casein kinase from the Golgi is activated with either  $Mg^{2+}$  or  $Ca^{2+}$ , and increased specific activity is observed with dephosphorylated casein as the substrate. The casein kinase can be solubilized from Golgi apparatus by the non-ionic detergent, Triton X-100. Gel permeation chromatography on Sepharose CL-4B yields a Stokes radius of 10 nm for the detergent-solubilized casein kinase. Dephosphorylated  $\beta$ -peptide, the amino-terminal peptide from  $\beta$ -casein, is a good substrate for the solubilized casein kinase. With dephosphorylated  $\beta$ -peptide, the maximal velocity is 9.1 and 12.0 nmol/min per mg protein with  $Mg^{2+}$  and  $Ca^{2+}$  activation, respectively. The Michaelis constant for  $\beta$ -peptide is greater with  $Ca^{2+}$  than with  $Mg^{2+}$  (4.8 mg/ml compared to 0.97 mg/ml). However, the Michaelis constant for ATP is not greatly influenced by these metal ions. The Triton X-100-solubilized Golgi enzyme can also catalyze the phosphorylation of peptides, such as fibrinopeptide A and  $\alpha$ -melanocyte stimulating hormone.

### Introduction

The casein micelles of milk are secreted by the Golgi vesicles of lactating, bovine mammary glands [1]. Mercier and Gaye [2] have elucidated the nature of the N-terminal signal peptide sequence which causes insertion of the bovine caseins into the lumen of the endoplasmic reticulum; phosphorylation of casein polypeptides, following protein synthesis, has been reported in mice [3] and rats [4]. In addition, a casein kinase that transfers

phosphate from ATP to dephosphorylated bovine casein has been found to be localized in the Golgi apparatus of lactating rat mammary glands [5,6].

It has been postulated that the phosphorylation of specific serine amino acid residues of bovine casein is determined primarily by the amino acid sequence of the substrates [7]. If this premise is correct, bovine casein kinase should be able to phosphorylate small peptides with the appropriate primary amino acid sequence.

In this investigation, we studied the distribution of casein kinase and its solubilization by Triton X-100 from the Golgi apparatus of lactating bovine mammary gland. We investigated the kinetics of the solubilized casein kinase by utilizing a peptide derived from  $\beta$ -casein. We achieved activation of the enzyme with either  $Ca^{2+}$  or  $Mg^{2+}$ . In addition, we tested other peptides, of a non-milk origin

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having the appropriate amino acid sequence, for phosphate incorporation.

### Materials and Methods

Adenosine-5'-[ $\gamma$ - $^{32}$ P]triphosphate and uridine diphospho-D-[U- $^{14}$ C]galactose were purchased from Amersham.

Fibrinopeptide A and  $\alpha$ -melanocyte stimulating hormone were prepared chemically by Bachem (Marina Del Rey, CA). Sleep peptide was purchased from Calbiochem-Behring (Cat. No. 567295).

Triton X-100 was scintillation grade from J.T. Baker Chemical Co. Imidazole was twice-recrystallized from benzene. All other chemicals were reagent grade.

DEAE-cellulose chromatography was used to purify  $\alpha_{s1}$ -casein [8] and  $\beta$ -casein [9].  $\beta$ -Peptide was released by trypsin digestion of  $\beta$ -casein and recovered by precipitation with barium hydroxide [10]. Acid phosphatase (potato) was used to dephosphorylate the caseins and  $\beta$ -peptide; to assure complete dephosphorylation as previously described [11], the concentration of inorganic phosphate liberated was maintained below 0.5 mM. Dephosphorylated  $\beta$ -peptide was isolated on a Sephadex G-50 column (1.5  $\times$  40 cm) equilibrated with 0.1 M ammonium bicarbonate. Polyacrylamide gel electrophoresis was used to verify that the caseins were dephosphorylated [11], and phosphate determination to verify the dephosphorylation of  $\beta$ -peptide [12].

### Preparation of defined membrane fractions

Mammary tissue was obtained from lactating cows and held on ice until processed. The Golgi apparatus, microsomes, rough endoplasmic reticulum and cytosol were fractionated and characterized as previously described [13]. Keenan et al. [13] found that the purity of their Golgi apparatus and vesiculated rough endoplasmic reticulum (RER) fractions ranged from 80 to 90% as determined by marker enzymes and electron microscopy. These workers recommended lactose synthetase and NADPH:cytochrome C reductase as specific marker enzymes for Golgi apparatus and RER, respectively. The ratios of enzyme activities in these fractions to those of the total homogenate are given in Table I and compared to data of Keenan et al. [13]. Since the ratios obtained in this study were comparable to those previously obtained [13] it was assumed that these samples are of comparable purity. Milk fat globule membranes were prepared by the method of Keenan and Huang [14]. The fractions were stored frozen in 0.32 M sucrose/14 mM mercaptoethanol until assay. Prior to assay, each sample was thawed and sonicated for 30 s at 5°C with a Branson sonifier.

### Isolation and solubilization of casein kinase of Golgi apparatus

Mammary glands of cows in full lactation, obtained at the time of slaughter, were sectioned and stored at -20°C. Isolation of the Golgi apparatus from the frozen bovine mammary glands required a slight modification of the procedure of Keenan

TABLE I  
ENZYME ACTIVITIES OF GOLGI APPARATUS AND ENDOPLASMIC RETICULUM FRACTIONS OF BOVINE MAMMARY GLAND

Fraction	Ratio <sup>a</sup>		
	Enzyme	This study	Ref. 13
Golgi apparatus	Lactose synthetase	14.8	16.5
	Casein kinase <sup>b</sup>	13.1	—
"Rough endoplasmic reticulum"	Lactose synthetase	1.0	0.9
	Casein kinase <sup>b</sup>	1.2	—
	NADPH-cytochrome C reductase	5.9	6.6

<sup>a</sup> Ratio of specific activity of sample to that of homogenate.

<sup>b</sup> Casein kinase with dephosphorylated casein and Ca<sup>2+</sup> in assay.

et al. [13]; the homogenizing medium contained 25 mM MgCl<sub>2</sub> rather than 5 mM.

Proteins were solubilized by mixing approximately 10 mg of Golgi apparatus and 5 ml of 20 mM imidazole, pH 7.8, 10% glycerol (v/v) and 0.4% Triton X-100 in a Teflon and glass homogenizer. After one stroke, the mixture was decanted and gently stirred in a beaker for 45 min at room temperature. The solution was centrifuged at 200000 × g for 1 h at 4°C in a Beckman SW65 rotor. The supernatant, which contained the casein kinase, was stored at -20°C. The solubilized casein kinase retained its activity for at least 2 months at this temperature.

Electron microscopy of Golgi apparatus was carried out by negative staining as previously described [6].

#### Enzyme assays

Casein kinase in crude membrane fractions was assayed by the filter paper method as previously described [5].

Solubilized casein kinase (approximately 10 μg) was incubated with 0.01 mmol imidazole, 0.2 μmol MnSO<sub>4</sub>, 0.1 mg dephosphorylated α<sub>s1</sub>-casein-B, and 0.08 μmol ATP (10<sup>6</sup> cpm) in a total volume of 0.1 ml, pH 7.8, at 30°C for 20 min. At the end of the incubation, a 50-μl aliquot was placed on a column (0.5 × 2 cm) of AG1 X-8 (Bio-Rad) anion-exchange resin in the formate form. The column had previously been equilibrated with 0.25 M pyridine formate, pH 3. The sample was washed through the column with 5 ml pyridine formate buffer, and the eluant was measured for phosphorus-32 by Cherenkov radiation [15]. The reaction rate was linear for at least 60 min and was also linear with respect to the addition of protein to at least 40 μg for a 20-min incubation. Control values were subtracted from the samples and were identical when M<sup>2+</sup>, phosphate acceptor or enzyme were omitted. 1 unit of casein kinase activity is defined as the number of nanomoles of phosphate incorporated in the polypeptide per min per mg protein at 30°C.

Lactose synthetase was assayed by the procedure described by Palmiter [16].

#### Gel permeation chromatography

Samples (0.25 ml) were placed on a column

(0.9 × 27 cm) of Sepharose CL-4B (Pharmacia) which was previously equilibrated with 20 mM imidazole, pH 7.8, 10% glycerol (v/v) and 0.4% Triton X-100. Protein was eluted with the imidazole buffer. The flow rate was maintained at 2.8 ml/h by the use of a mariotte flask at a pressure of about 25 cm water; fractions were collected at 15-min intervals. Protein was determined with fluorescamine (Pierce Chemical Co.) according to the manufacturer's procedure. The void volume (V<sub>0</sub>) and the total volume (V<sub>t</sub>) of the column were determined with Blue Dextran 2000 (Pharmacia) and Cibacron Blue F3G-A.

#### Kinetics

The kinetic results for the phosphorylation of dephosphorylated β-peptide were consistent with the following equation [17]:

$$v_i = \frac{VAB}{K_{ia}K_b + K_bA + K_aB + AB}$$

where v<sub>i</sub> is the initial velocity and V is the maximum velocity, A and B are the substrate concentrations of ATP and dephosphorylated polypeptide, respectively. K<sub>a</sub> and K<sub>b</sub> are the Michaelis constants of the substrates and K<sub>ia</sub> is the dissociation constant of ATP.

Activation of casein kinase by Ca<sup>2+</sup> or Mg<sup>2+</sup> required 25 mM of the divalent metal for maximal activity in the phosphorylation of dephosphorylated β-peptide. Under these conditions, virtually all ATP was present as the metal-ATP complex, (M-ATP), which was determined by the following equation [18]:

$$[M-ATP] = \frac{1}{2} \left[ (K_d + [M]_t + [ATP]_t) - \sqrt{(K_d + [M]_t + [ATP]_t)^2 - 4[M]_t[ATP]_t} \right]$$

where K<sub>d</sub> is the dissociation constant of metal-ATP, and [M]<sub>t</sub> and [ATP]<sub>t</sub> are the total concentrations of metal and ATP, respectively; K<sub>d</sub> values were from published pK<sub>a</sub> values [19]. In the enzyme assay in which Mn<sup>2+</sup> was the activator, the metal-ATP complex also was overwhelmingly dominant. The enzyme solubilized from Golgi apparatus, as described above, was first incubated with EDTA at 1 mM. The preincubated enzyme was then ad-

ded to the reaction mixture (10  $\mu$ l per 100  $\mu$ l total volume); in the absence of added  $M^{2+}$  no activity was observed; this was also true if EDTA was omitted.

Kinetic data were the average of duplicate assays for each point. Results were analyzed by the computer method of Cleland [20]. Kinetic constants were calculated from analysis of secondary plots [21].

Protein concentrations of whole membrane fractions were determined by the method of Lowry et al. [22] with bovine serum albumin as the standard.

## Results

### Localization of casein kinase activity

Since phosphorylation of casein can be considered to be a post-translational modification, assays for casein kinase activity were carried out on a number of defined membrane fractions from fresh lactating bovine mammary gland, as well as on the cytosol and the milk fat globule membrane, which is thought to be derived from the apical plasma membrane of mammary secretory cells [13,14]. In the presence of 8 mM  $Mg^{2+}$ , the Golgi fraction exhibited the highest specific activity with  $\alpha_{s1}$ -casein or dephosphorylated  $\alpha_{s1}$ -casein as the substrate (Table II). However, the specific activity of both homogenate and cytosol decreased when de-

phosphorylated  $\alpha_{s1}$ -casein was used as the substrate. With dephosphorylated  $\alpha_{s1}$ -casein as the substrate and  $Ca^{2+}$  replacing  $Mg^{2+}$  in the casein kinase assay, the activity of the Golgi apparatus enzyme increased slightly (Table II), while the activity of other cellular membrane fractions decreased, as did the activities of the homogenate and the cytoplasm. In the absence of exogenous metal ions no reaction occurred. Hence, a  $Ca^{2+}/Mg^{2+}$ -stimulated casein kinase which phosphorylates dephosphorylated bovine casein is localized in the Golgi apparatus of lactating, bovine mammary gland.

### Isolation of Golgi apparatus and solubilization of casein kinase with Triton X-100

Studies on the purification of casein kinase were carried out on Golgi apparatus isolated from frozen mammary glands. The procedure of Keenan et al. [13] was modified to increase the  $MgCl_2$  content to 25 mM; the two methods are compared in Table III. The Golgi apparatus obtained by both methods had similar ratios of enzyme activity for lactose synthetase but both are lower than those obtained from fresh tissue (Table I). Upon examination in the electron microscope, the Golgi apparatus at 5 and 25 mM  $MgCl_2$  appeared morphologically similar, with approximately 80% purity and many plate-like structures; however, both preparations appear to have less tubule-like struc-

TABLE II

#### CASEIN KINASE ACTIVITY OF MEMBRANE FRACTIONS FROM LACTATING BOVINE MAMMARY GLAND

Assay mixture of 100  $\mu$ l containing 10  $\mu$ mol Tris-HCl buffer (pH 7.6), 0.8  $\mu$ mol  $MgCl_2$  or  $CaCl_2$ , 0.3 mg protein and 0.02  $\mu$ mol ATP ( $10^6$  cpm). Each assay was run three times on two different preparations from mammary glands and averaged.

Fraction	Casein kinase activity (nmol $^{32}P$ /mg protein per 20 min)		
	$\alpha_{s1}$ -casein + $Mg^{2+}$	Dephosphorylated $\alpha_{s1}$ -casein	
		+ $Mg^{2+}$	+ $Ca^{2+}$
Homogenate	0.443	0.288	0.260
Golgi apparatus	1.15	3.14	3.40
Microsomes	0.290	1.25	1.08
'Rough endoplasmic reticulum'	0.589	0.845	0.304
Milk fat globule membrane	0.001	0.001	0.023
Cytosol	0.410	0.135	0.050

TABLE III

EFFECT OF  $MgCl_2$  CONCENTRATION IN HOMOGENIZATION MEDIUM UPON CASEIN KINASE AND LACTOSE SYNTHETASE ACTIVITIES IN GOLGI APPARATUS ISOLATED FROM FROZEN MAMMARY GLAND

Casein kinase was assayed for in 110  $\mu$ l containing 0.02 mmol imidazole, pH 7.8, 0.6  $\mu$ mol  $CaCl_2$ , 0.2 mg dephosphorylated  $\alpha_{s1}$ -casein and 0.08  $\mu$ mol ATP. The specific activity is defined as nmol P incorporated per min per mg protein at 30°C.

Specific activity of lactose synthetase is defined as nmol lactose formed per min per mg protein at 30°C.

	5 mM $MgCl_2$					25 mM $MgCl_2$				
	Casein kinase			Lactose synthetase		Casein kinase			Lactose synthetase	
	nmol P/min per ml	Spec. act.	Yield	Spec. act.	Yield	nmol P/min per ml	Spec. act.	Yield	Spec. act.	Yield
Homogenate (75 ml)	0.840	0.0545	—	1.38	—	1.58	0.125	—	1.42	—
Golgi apparatus (15 ml)	2.49	0.526	59%	3.60	17%	3.01	1.10	38%	4.40	16%

tures than are present in fresh tissue [6,13]. The higher  $MgCl_2$  content apparently increases the stability (and so the specific activity) of casein kinase from frozen tissue without drastically altering other aspects of the preparation. Typically the same tissue gave a 2-fold higher specific activity of casein kinase from Golgi apparatus upon homogenization of tissue in 25 mM  $MgCl_2$ .

Solubilization of the casein kinase activity from Golgi apparatus is best achieved with the non-ionic detergent, Triton X-100. The solubilization procedure results in a 15 to 20% increase in total activity, as well as an increase in specific activity. The Triton X-100 may thus uncover enzyme activity not detected in the sonicated membranes. Solubilized casein kinase preparations can be stored for 2 months without any significant decrease in enzymatic activity if 10% glycerol (v/v) is included. Sodium deoxycholate solubilized similar quantities of protein from Golgi apparatus as Triton X-100, but with lower specific activity. Deoxycholate inhibits the casein kinase activity of Triton X-100 solubilized enzyme with an  $I_{50} = 0.8$  mg/ml; hence, inhibition of casein kinase activity may be the reason for the low activity of the deoxycholate-solubilized Golgi enzyme.

Removal of Triton X-100 from the solubilized preparation by Bio Beads SM-2 (Bio-Rad) [23] precipitates protein and significantly reduces casein kinase activity in the supernatant. Casein kinase activity cannot be solubilized after cold acetone precipitation of Triton X-100-solubilized enzyme. Dialysis fails to lower the concentration of Triton X-100. In all cases removal of detergent resulted in highly significant losses in enzyme activity.

#### *Gel permeation chromatography of Triton X-100-solubilized casein kinase*

A profile of the enzyme solubilized from Golgi apparatus eluted from the Sepharose CL-4B column is depicted in Fig. 1. The presence of a marker enzyme for Golgi apparatus, lactose synthetase, which is also membrane-bound and extracted by Triton X-100, is shown in the elution pattern. The elution position,  $K_D$ , of casein kinase activity is about 0.5. ( $K_D$  is determined by the relation  $(V_p - V_0)/(V_t - V_0)$  where  $V_p$  is the elution volume of protein). Previous calibration of the elution position of a Sepharose 4B column versus known

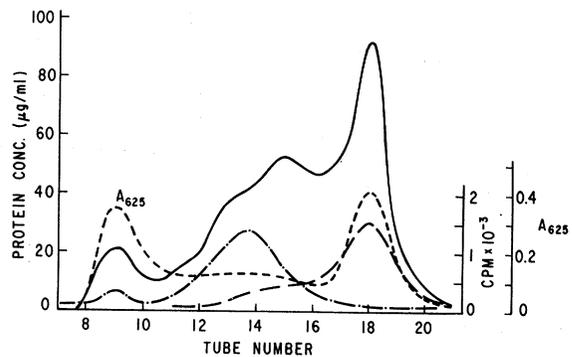


Fig. 1. Elution pattern of proteins solubilized by Triton X-100 from Golgi apparatus on a Sepharose CL-4B column. The sample, 0.25 ml solubilized Golgi apparatus protein, in 0.02 M imidazole, pH 7.8, 10% glycerol (v/v), 0.4% Triton X-100, was placed on the column (0.9×27 cm) and eluted with the imidazole buffer. Fractions were collected at 15-min intervals. Protein concentrations in each fraction were determined with fluorescamine (solid line); protein kinase (---) and lactose synthetase (— — (long)) were determined.  $A_{625}$  (----- (short)) represents Blue dextran and Cibacron blue dye used for calibration of column.

Stokes radius of marker proteins [24] indicates that the Stokes radius for the casein kinase is approximately 10 nm. This radius is within the range normally found for Triton X-100-solubilized membrane proteins [24]. Neither sonication nor 2% Triton X-100 (rather than 0.4%) increases the elution position of protein kinase activity on Sepharose 4B. However, omitting Triton X-100 from the eluting buffer results in a casein kinase particle of smaller size, but the enzyme activity recovered in these cases was so low as to preclude further purification.

#### *Phosphorylation of proteins and peptides by casein kinase*

The relative rates of reaction of the Triton X-100-solubilized casein kinase toward various caseins both native and dephosphorylated are given in Table IV. These values are compared with the substrate reactivities obtained in previous reports [25,26]. However, a kinetic study of the phosphorylation of casein polypeptides presents a special problem in that these proteins are known to undergo self-associations which are both metal ion and concentration dependent [27]. In a preliminary investigation using dephosphorylated  $\alpha_{s1}$ -

TABLE IV

REACTIVITY OF BOVINE MAMMARY GLAND CASEIN KINASES TOWARD PROTEINS IN THE PRESENCE OF  $Mg^{2+}$ 

Substrate	Golgi casein kinase <sup>a</sup>	Microsomal casein kinase <sup>b</sup>	Membrane-bound casein kinase <sup>c</sup>
Dephosphorylated $\alpha_{s1}$ -casein	100	100	100
$\alpha_{s1}$ -Casein	35	44	4
Dephosphorylated $\beta$ -casein	96	91	38
$\beta$ -Casein	12	9	13

<sup>a</sup> Calculated from activities obtained in the presence of 8 mM  $Mg^{2+}$ ; expressed as spec. act. units relative to dephosphorylated  $\alpha_{s1}$ -casein.

<sup>b</sup> Taken from the data of Bingham and Groves [25] and calculated as spec. act. units relative to dephosphorylated  $\alpha_{s1}$ -casein (10 mM  $Mg^{2+}$ ).

<sup>c</sup> Taken from the data of MacKinlay et al. [26] and calculated as spec. act. units relative to dephosphorylated  $\alpha_{s1}$ -casein (8 mM  $Mg^{2+}$ ).

casein as the substrate, the 'apparent  $K_m$ ' values for  $Mg^{2+}$  and  $Ca^{2+}$  were estimated at 7 and 4 mM, respectively, with maximum activation occurring at above 20 mM for both metal ions. Dephosphorylated  $\alpha_{s1}$ -casein, however, is precipitated by divalent metal ions [6,28] such as  $Mg^{2+}$ ,  $Ca^{2+}$  and  $Mn^{2+}$  at the concentration necessary for maximum activation of the enzyme, and the state

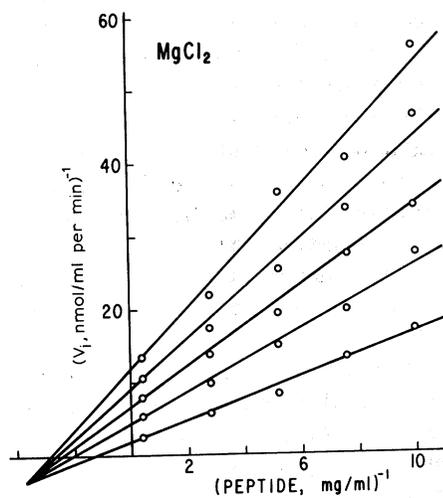


Fig. 2. Kinetics of the phosphorylation of dephosphorylated  $\beta$ -peptide:  $Mg^{2+}$  activation of casein kinase. The reactions were run in a total volume of 0.1 ml containing 0.01 mmole imidazole, pH 7.8, with 16.6  $\mu$ g protein, and 2.5  $\mu$ mole  $MgCl_2$ ; reactions proceeded at 30°C for 20 min. Each line represents an individual concentration of ATP: 0.25 mM, 0.073 mM, 0.038 mM, 0.026 mM, and 0.020 mM, respectively.

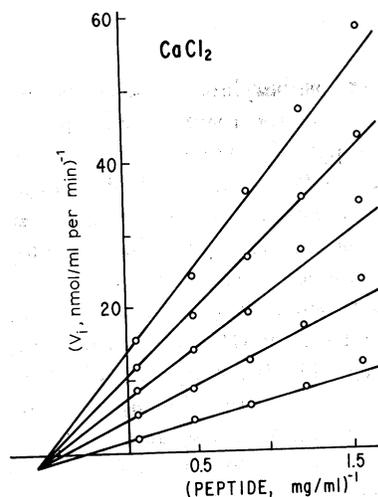


Fig. 3. Kinetics of the phosphorylation of dephosphorylated  $\beta$ -peptide:  $Ca^{2+}$  activation of casein kinase. The reaction (0.1 ml total volume) was run in 0.01 mmol imidazole, pH 7.8, with 8.3  $\mu$ g Golgi protein and 2.5  $\mu$ mol  $CaCl_2$  at 30°C for 20 min. The variable concentrations of ATP are the same as in Fig. 2.

TABLE V

KINETIC PARAMETERS FOR PHOSPHORYLATION OF  $\beta$ -PEPTIDE BY CASEIN KINASE

$V$  is expressed in nanomoles per min per mg protein.

	$Mg^{2+}$	$Ca^{2+}$
$V$ —	9.1	12.0
$K_a$ [ATP]	150 $\mu$ M	232 $\mu$ M
$K_{ia}$ [ATP]	47 $\mu$ M	85 $\mu$ M
$K_b$ [ $\beta$ -peptide]	0.97 mg/ml	4.8 mg/ml

TABLE VI

## PHOSPHORYLATION OF PEPTIDES BY CASEIN KINASE

Assay run in 0.01 mmol imidazole, pH 7.8, 2.5  $\mu$ mol  $MgCl_2$ , 0.1 mg polypeptide and 0.08  $\mu$ mol ATP in 100  $\mu$ mol at 30°C. Proposed sites of serine phosphorylation and the (N+2) glutamate are underlined; key for amino acids: A=Ala, D=Asp, E=Glu, F=Phe, G=Gly, H=His, I=Ile, K=Lys, L=Leu, M=Met, N=Asn, P=Pro, R=Arg, S=Ser, T=Thr, V=Val, W=Trp, Y=Tyr.  $v_i$  is expressed in nanomoles per min per mg protein.

Peptide substrates	$v_i$
Dephosphorylated $\beta$ -peptide $_2$ HN-R-E-L-E-E-L-N-V-P-G-E-I-V-E-S-L-S-S-S-E-S-I-T-R-COOH	2.3
Fibrinopeptide A (human) $_2$ HN-A-D-S-G-E-G-D-F-L-A-E-G-G-G-V-R-COOH	3.6
$\alpha$ -Melanocyte-stimulating hormone Ac-HN-S-Y-S-M-E-H-F-R-W-G-K-P-V-CONH $_2$	2.0
Sleep peptide $_2$ HN-W-A-G-G-D-A-S-G-E-COOH	0.1

of association of dephosphorylated  $\beta$ -casein is unknown. A suitable choice for a substrate for phosphorylation is the  $\beta$ -peptide, which is the amino-terminal peptide from  $\beta$ -casein. The high charge density and low hydrophobicity of this peptide make it unlikely to associate. Thus, phosphorylation of dephosphorylated  $\beta$ -peptide by Triton X-100-solubilized casein kinase can be achieved with optimal concentrations (25 mM) of either  $Mg^{2+}$  or  $Ca^{2+}$  as an activator. Double reciprocal plots of both  $Mg^{2+}$  and  $Ca^{2+}$  activation of protein kinase are illustrated in Figs. 2 and 3. The data are summarized in the kinetic parameters of Table V.

Mercier et al. [7] postulated that the specificity of phosphorylation of specific serine residues of the caseins is determined by the amino acid sequence. Acidic amino acids such as glutamic acid, phosphoserine or aspartic acid [29], (N+2) residues from a serine, are thought to lead to the phosphorylation of that serine. Thus an amino acid sequence of serine-X-glutamate theoretically will lead to enzymatic phosphorylation of serine by the protein kinase of Golgi. Some 'non-casein' peptides which contain the hypothetical sequence were tested to determine whether the specificity of phosphorylation is truly determined by the primary sequence of the peptide (Table VI). Only sleep peptide does not incorporate significant amounts of phosphate under the conditions of the enzymatic assay.

### Discussion

The high specific activity of the Golgi apparatus toward dephosphorylated  $\alpha_{s1}$ -casein indicates that the casein kinase of lactating bovine mammary gland is similar to the enzyme of lactating rat mammary gland [5,6], and both possess a casein kinase stimulated by  $Ca^{2+}$  and  $Mg^{2+}$ . It is significant that the casein kinase of both species is localized in the Golgi apparatus. Turkington and Topper [3] have shown that lactating cells contain a pool of unphosphorylated casein. Mercier and coworkers [2] have shown very elegantly that each casein contains a signal peptide which causes its insertion into the lumen of the endomembrane system. Hence, after synthesis of the caseins on the ribosomes, these secretory proteins enter the endomembrane system and may then be phosphorylated in the Golgi apparatus prior to secretion by Golgi vesicles.

The crude Golgi apparatus enzyme phosphorylates both native and dephosphorylated  $\alpha_{s1}$ -casein, but at different rates (Table I). This is of interest since Manson et al. [29] have shown that a minor casein ( $\alpha_{s0}$ -) has an amino acid sequence identical to that of  $\alpha_{s1}$ -casein except that it contains one more phosphoserine residue with aspartic acid two residues to the right. The differential rates observed could be related to the occurrence of  $\alpha_{s0}$ -casein in milk.

Triton X-100 has been used for solubilizing membrane proteins, especially enzymes [30]; it was the detergent of choice for this study. The size distribution observed for casein kinase on Sepharose CL-4B is large but in the typical range found for membrane proteins solubilized by a Triton X-100 [24]. In general, the particles formed by Triton X-100 solubilization are characterized by a high Stokes radius and a low sedimentation coefficient, which is related to the fact that the partial specific volume of Triton X-100 is greater than the average partial specific volume of proteins [31]. Triton X-100 solubilized membrane proteins are also characterized by abnormally high frictional coefficient ratios [24]. Hence, the large size observed (10-nm radius) may be due to the fact that the casein kinase is complexed with Triton X-100, forming large particles which are either highly asymmetric or highly hydrated [32]. Lactose synthetase has been purified following Triton X-100 solubilization from Golgi apparatus of lactating sheep mammary gland by omitting the detergent in subsequent purification steps [33]. Such a procedure leads to smaller particle size with casein kinase on the Sepharose CL-4B column, but the accompanying losses of enzymatic activity prevented further purification.

MacKinlay et al. [26] reported a protein kinase from bovine mammary gland that will phosphorylate casein. However, this enzyme is extracted by, and is active in, deoxycholate, while our enzyme is inhibited by this bile acid; the enzyme reported by MacKinlay is recovered from a supernatant fraction obtained at low speed, while our enzyme is from the corresponding precipitate. MacKinlay's enzyme (Table IV) shows a lower degree of incorporation of phosphate into dephosphorylated  $\beta$ -casein, while our enzyme which is obtained from purified Golgi apparatus fractions is reactive. More recently, Bingham and Groves [25] characterized a casein kinase which was solubilized by Triton X-100 from a crude microsomal fraction of bovine mammary gland that phosphorylated both dephosphorylated  $\beta$ -casein and  $\beta$ -peptide and has properties similar to the enzyme from bovine Golgi apparatus studied in this work (Table IV). It is important to note that in bovine mammary gland the proportion of other cell types to secretory

epithelial cells is much higher than in rat mammary gland. Thus, a microsomal preparation from bovine mammary gland will include contributions from a variety of cell types. The Golgi apparatus, however, is unique to the secretory epithelial cells [1,13,14] and so the results presented here link this enzyme more closely to the secretory process.

The casein kinase activity associated with the Golgi apparatus is activated by both  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  as shown in Tables II and V. This makes the enzyme rather unusual, since most kinases are activated by one of these divalent metal ions and often inhibited by the other. The role of metal ion in this reaction is somewhat speculative. Certainly, the metal-ATP complex predominates under even the lowest metal ion concentrations used (8 mM) by all workers. However, in this work  $V_{\max}$  conditions with respect to metal ions are not achieved until 25 mM. Activation of the enzyme at higher metal concentrations could be due to metal binding by other proteins in the preparations or to real activation phenomena involving the enzyme. It may be possible that different forms of casein kinase are present in the enzyme preparation solubilized from Golgi apparatus, but confirmation of this theory awaits further purification of the enzyme.

Nevertheless, an intriguing finding exists (Table V) in the fact that phosphorylation of the dephosphorylated  $\beta$ -peptide proceeds at a higher maximum velocity with  $\text{Ca}^{2+}$  than with  $\text{Mg}^{2+}$ . There is also a significant difference in  $K_b$ , the Michaelis constant for the  $\beta$ -peptide. These differential effects with  $\text{Ca}^{2+}$  may be important, since  $\text{Ca}^{2+}$  is an integral part of the casein micelle, which is assembled in the Golgi and secreted into the alveolar lumen during lactation [1]. Furthermore, dephosphorylated casein exhibits reduced  $\text{Ca}^{2+}$  binding as well as incomplete micelle formation [28]. Thus,  $\text{Ca}^{2+}$  in the Golgi may serve a dual purpose, acting initially in the activation of casein kinase (which occurs prior to phosphorylation). Subsequent calcium binding by casein phosphopeptides would lead to the formation of casein micelles which are ready for secretion.

The ability of casein kinase to phosphorylate  $\beta$ -peptide, Fibrinopeptide A and  $\alpha$ -melanocyte-stimulating hormone supports the hypothesis of

casein phosphorylation made by Mercier et al. [7]. Thus, serine-X-glutamate is the necessary sequence in the polypeptide substrate that enables casein kinase to catalyze phosphorylation. Phosphoserine and aspartate [29] apparently can substitute for glutamic acid. The inconsequential nature of the middle amino acid, X, between the serine and acidic residue is striking. This is shown, for example, by the  $\alpha$ -melanocyte-stimulating hormone, which has a methionine residue between serine and glutamate. There is a phosphoserine-lysine-glutamate sequence in  $\beta$ -casein E [34] and a phosphoserine-lysine-aspartate sequence in  $\alpha_{s0}$ -casein [29]. Finally, phosphoserine itself becomes X in native caseins [7]. Thus, a bulky amino acid such as methionine, a basic amino acid such as lysine, or an acidic amino acid is not deleterious to phosphorylation of the serine. However, the critical sequence in the sleep peptide, which is not phosphorylated, lies at the carboxyl end of the molecule, where an additional negative charge is created on the glutamate by the terminal carboxylate group. None of the glutamic acids that apparently direct phosphorylation in the caseins is at the carboxyl end of the protein or peptide.

In summary, the Golgi apparatus of lactating bovine mammary gland contains a  $\text{Ca}^{2+}$ - and/or  $\text{Mg}^{2+}$ -stimulated casein kinase capable of phosphorylating dephosphorylated caseins, dephosphorylated casein peptides and peptides of 'non-milk' origin which contain the sequence serine-X-glutamic acid.

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#### References

- Patton, S. and Jensen, R.G. (1976) *Biomedical Aspects of Lactation*, pp. 32-35, Pergamon Press, Elmsford, NY
- Mercier, J.C. and Gaye, P. (1980) *Ann. N.Y. Acad. Sci.* 343, 232-251
- Turkington, R.W. and Topper, Y.L. (1966) *Biochim. Biophys. Acta* 127, 366-372
- Singh, V.N., Dave, S.S. and Venkitasubramanian, T.A. (1967) *Biochem. J.* 104, 48-50
- Bingham, E.W., Farrell, H.M., Jr. and Basch, J.J. (1972) *J. Biol. Chem.* 247, 8193-8194
- Bingham, E.W. and Farrell, H.M., Jr. (1974) *J. Biol. Chem.* 249, 3647-3651
- Mercier, J.C., Grosclaude, F. and Ribadeau-Dumas, B. (1972) *Milchwissenschaft* 27, 402-425
- Thompson, M.P. and Kiddy, C.A. (1964) *J. Dairy Sci.* 47, 626-632
- Thompson, M.P. and Pepper, L. (1964) *J. Dairy Sci.* 47, 633-637
- Peterson, R.F., Nauman, L.W. and McMeekin, T.L. (1958) *J. Am. Chem. Soc.* 80, 95-99
- Bingham, E.W., Farrell, H.M., Jr. and Dahl, K.J. (1976) *Biochim. Biophys. Acta* 429, 448-460
- Bartlett, G.R. (1959) *J. Biol. Chem.* 234, 466-468
- Keenan, T.W., Huang, C.M. and Morrè, J.D. (1972) *J. Dairy Sci.* 55, 1577-1585
- Keenan, T.W. and Huang, C.M. (1972) *J. Dairy Sci.* 55, 1013-1015
- Clausen, T. (1968) *Anal. Biochem.* 22, 70-73
- Palmiter, R.D. (1969) *Biochim. Biophys. Acta* 178, 35-46
- Cleland, W.W. (1970) in *The Enzymes* (Boyer, P.D., ed.), Vol. 2, 3rd edn., pp. 8-32, Academic Press, New York
- Infante, J.P. and Kinsella, J.E. (1976) *Int. J. Biochem.* 7, 483-493
- Sillen, L.G. and Martell, A.E. (eds.) (1964) *Stability Constants of Metal Ion Complexes*, Chemical Society of London, Special Publication No. 17, pp. 651-652, Burlington House, London
- Cleland, W.W. (1967) in *Advances in Enzymology* (Meister, A., ed.), Vol. 29, pp. 1-32, John Wiley and Sons, New York
- Segal, I.H. (1975) *Enzyme Kinetics*, pp. 279-285, John Wiley and Sons, New York
- Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) *J. Biol. Chem.* 193, 265-275
- Holloway, P.W. (1973) *Anal. Biochem.* 53, 304-308
- Clarke, S. (1975) *J. Biol. Chem.* 250, 5459-5469
- Bingham, E.W. and Groves, M.L. (1979) *J. Biol. Chem.* 254, 4510-4515
- MacKinlay, A.G., West, D.W. and Manson, W. (1977) *Eur. J. Biochem.* 76, 233-243
- Swaisgood, H.E. (1973) *CRC Crit. Rev. Food Technol.* 3, 375-414
- Bingham, E.W., Farrell, H.M., Jr. and Carroll, R.J. (1972) *Biochemistry* 11, 2450-2454
- Manson, W., Carolan, T. and Annan, W.D. (1977) *Eur. J. Biochem.* 78, 411-417
- Helenius, A. and Simons, K. (1975) *Biochim. Biophys. Acta* 415, 29-35
- Tanford, C. and Reynolds, J.A. (1976) *Biochim. Biophys. Acta* 457, 133-170
- Edsall, J.T. (1965) in *Proteins, Amino Acids and Peptides as Ions and Dipolar Ions* (Cohn, E.J. and Edsall, J.T., eds.), ch. 19, Hafner Publishing Co., New York
- Smith, C.A. and Brew, K. (1977) *J. Biol. Chem.* 252, 7294-7299
- Whitney, R.M., 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, 785-815