

Phosphorylation of Casein

ROLE OF THE GOLGI APPARATUS

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SUMMARY

A protein kinase that catalyzes the phosphorylation of dephosphorylated α_{s1} -casein by ATP has been found in the Golgi apparatus of lactating rat mammary gland. This enzyme has more than 10 times the specific activity of the protein kinases in other subcellular fractions. When native α_{s1} -casein is used as the substrate, the rate of phosphorus incorporation decreases by 77%. This suggests that phosphorylation of casein may occur in the Golgi apparatus following biosynthesis of the polypeptide chain.

Since the major protein components of milk (the caseins) are phosphoproteins, lactating mammary gland should be an ideal tissue for studying the incorporation of phosphate into protein. There is evidence that the polypeptide chain of casein is synthesized prior to the incorporation of phosphate and that a pool of unphosphorylated casein exists in the mammary gland (1, 2). If this is correct, then dephosphorylated casein should be an appropriate substrate for the study of phosphorylation. We, therefore, examined protein kinases from subcellular fractions of lactating rat mammary gland in order to locate an enzyme that would preferentially act on dephosphorylated casein.

EXPERIMENTAL PROCEDURE

Materials— $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ and $\text{UDP-D-}[U\text{-}^{14}\text{C}]\text{galactose}$ were purchased from Amersham-Searle.¹ The preparation of α_{s1} -casein and dephosphorylated α_{s1} -casein from cow's milk has been described (3). The phosphate contents of α_{s1} -casein and dephosphorylated α_{s1} -casein were 1.04% and 0.05%, respectively.

Tissue Fractionation—Golgi apparatus was isolated by the procedure of Keenan *et al.* (4) and Morr  (5). The mammary glands from first lactation Sprague-Dawley rats, 10 to 15 days postpartum, were excised, immediately chilled, and minced. The cold minced tissue was suspended in 3 volumes of Medium A (37.5 mM Tris-maleate buffer, pH 6.5; 1 mM EDTA; 20 mM mercaptoethanol; and 5 mM MgCl_2) containing 0.5 M sucrose and 1% dextran. All operations were carried out at 4°. Following homogenization for 1 min (medium speed) with a Polytron 10 ST homogenizer, the homogenate was squeezed in rapid succession through two, four, and six layers of cheesecloth and was then centrifuged for 15 min at $4,000 \times g$ in a swinging bucket rotor. The friable top one-third of the pellet was taken up in a minimum volume of Medium A containing 0.5 M sucrose and

1% dextran and suspended with one stroke of a Teflon-glass homogenizer. This sample was layered over 1.5 volumes of Medium A containing 1.25 M sucrose and 1% dextran and centrifuged at $100,000 \times g$ for 30 min. The Golgi fraction, concentrated at the interface, was removed, diluted with Medium A containing 1% dextran and 0.5 M sucrose, and pelleted at $4,000 \times g$ for 15 min.

For subsequent fractionations, all of the solutions and precipitates remaining after recovery of the Golgi apparatus were combined, diluted with Medium A to a final sucrose concentration of 0.25 M, and homogenized gently with a Teflon-glass homogenizer. The remaining fractionations were carried out in Medium A containing 0.25 M sucrose. Nuclei, mitochondria, microsomes, and cytoplasm were prepared by differential centrifugation as described by Schneider (6). All pellets were suspended in Medium A, thoroughly sonicated, and frozen.

Enzyme Assays—Protein kinase activity was measured at pH 7.0 in a 100- μl reaction mixture, containing the following: 5 μmoles of 2-(*N*-morpholino)ethanesulfonate buffer (pH 7.0), 0.03 μmole of ethylene glycol bis(β -aminoethyl ether)-*N,N'*-tetraacetic acid, 0.2 μmole of theophylline, 1.0 μmole of NaF, 0.5 μmole of MgAc_2 , 0.3 mg of casein, 0.5 μmole of cyclic AMP,² 5.0 μl of enzyme solution, and 0.02 μmole of ATP containing 4×10^6 cpm of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$. The reaction was started by the addition of ATP. After incubation for 20 min at 30°, a 50- μl aliquot of reaction mixture was pipetted onto squares (2×2 cm) of Whatman No. 31ET chromatography paper. The papers were washed according to the procedure described by Reimann *et al.* (7), transferred to scintillation vials containing 15 ml of Aquasol (New England Nuclear), and counted. The endogenous phosphokinase activity was measured in the absence of casein and this value was subtracted from the value obtained in the presence of casein to determine the amount of phosphate incorporated. When the enzyme was omitted, phosphate was not significantly incorporated into casein. Activity was expressed as the picomoles of phosphate incorporated into casein in 20 min in the 100- μl reaction mixture.

Lactose synthetase activity was determined by measuring the transfer of galactose from $\text{UDP-}[U\text{-}^{14}\text{C}]\text{galactose}$ to glucose using the column method of Palmiter (8) and the revised assay conditions of Fitzgerald *et al.* (9). Each 100- μl assay mixture contained 20 μg of bovine α -lactalbumin, 0.04 μmole of UDP-galactose (22,000 cpm), 0.7 μmole of MnCl_2 , 10 μmoles of glucose, 5 μmoles of glycine buffer, pH 8.0, and 5 μl of enzyme. Blank incubation mixtures contained enzyme but no glucose. After 30 min at 37°, a 50- μl aliquot of the reaction mixture was withdrawn and treated as described by Palmiter (8). The activity is reported as counts per min of galactose incorporated into lactose per 30 min in the 100- μl incubation mixture.

Protein Concentration—Protein was estimated according to Lowry *et al.* (10) with bovine serum albumin as the standard.

RESULTS AND DISCUSSION

When protein kinase activity is measured using α_{s1} -casein and dephosphorylated α_{s1} -casein as the substrates, the Golgi apparatus fraction demonstrates the highest specific activity of any

¹ The mention of commercial items is for your convenience and does not constitute an endorsement by the Department of Agriculture.

² The abbreviation used is: cyclic AMP, cyclic adenosine 3',5'-monophosphate.

TABLE I

Subcellular distribution of protein kinase from lactating mammary gland
These results are representative of individual experiments with three rats.

Fraction	Protein kinase activity		Lactose synthetase
	α_{s1} -Casein	Dephosphorylated α_{s1} -casein	
	<i>pmoles/mg protein/ 20 min</i>		<i>cpm/μg protein/30 min</i>
Total homogenate.....	0.16	0.20	33.0
Golgi apparatus.....	5.44	24.02	1101.0
Nuclei.....	0.54	0.48	38.4
Mitochondria.....	0.54	1.10	91.2
Microsomes.....	1.72	1.80	137.4
Cytoplasm.....	0.50	0.22	2.4

subcellular fraction (Table I). Keenan *et al.* (11) have shown that lactose synthetase is associated with the Golgi apparatus from lactating mammary gland and that this enzyme serves as a marker for mammary Golgi apparatus. It is therefore significant that protein kinase activity parallels that of lactose synthetase (Table I). In addition, when classical methods, which do not maintain the integrity of the Golgi apparatus, were used for subcellular fractionation (6), both protein kinase and lactose synthetase were localized in the microsomal fraction. Under the latter conditions, nuclei, mitochondria, microsomes, and cytoplasm were fractionated, but not Golgi apparatus.

The protein kinase activities reported in Table I were assayed in the presence of cyclic AMP. However, experiments conducted in the absence of added cyclic nucleotide indicate that cyclic AMP has essentially no effect on these mammary gland protein kinases, when casein is employed as the substrate.

The enzyme isolated from the Golgi fraction (Table I) phosphorylates dephosphorylated α_{s1} -casein at 4 times the rate of native α_{s1} -casein. These results suggest that dephosphorylated α_{s1} -casein is the preferred substrate for the Golgi apparatus protein kinase. If this enzyme is responsible for the *in vivo* phosphorylation of casein, the hypothesis of Turkington and Topper (2), that casein is phosphorylated following synthesis of the polypeptide chain, is confirmed.

In contrast to these findings, Rabinowitz and Lipmann (12) showed that 60 to 100% dephosphorylated phosvitin was a poor

substrate for pigeon brain protein kinase. However, native phosvitin and partially (<60%) dephosphorylated phosvitin were readily phosphorylated. It is interesting that the protein kinase from cytoplasm (Table I) has similar properties, in that native α_{s1} -casein is phosphorylated at a faster rate than dephosphorylated α_{s1} -casein. This suggests that the protein kinase in cytoplasm may be different from the Golgi apparatus enzyme.

The biosynthesis of casein has been shown to occur *in vitro* (13) and *in vivo* (14) in the endoplasmic reticulum of the mammary gland. It also has been shown by Turkington and Topper (2) that a pool of unphosphorylated casein occurs in mammary gland. Furthermore, *in vivo*, radioautograms (14) show that the caseins migrate to the Golgi apparatus and are finally secreted by Golgi vacuoles. The results presented here suggest that phosphorylation of the casein occurs in the Golgi apparatus. In other secretory tissues, the Golgi apparatus has been reported to be the site of carbohydrate addition to form glycoproteins (15, 16) and sulfate addition (16) to form sulfoproteins. The incorporation of phosphate into casein by mammary gland Golgi apparatus is another example of polypeptide chain modification by this cell fraction.

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