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PURIFICATION AND CHARACTERIZATION OF A PROTEIN KINASE MODULATOR FROM RAT MAMMARY GLAND

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Summary

A factor (Modulator II) partially purified from lactating rat mammary gland causes a marked substrate-dependent inhibition of the activity of mammary cytosol cyclic AMP-dependent protein kinase (ATP : protamine *O*-phosphotransferase, EC 2.7.1.70) for the phosphorylation of casein, calf thymus whole, F-1, F-2b, F-3 and F-2a histones and mammary whole histones and mitochondrial, plasma membrane and ribosomal phosphoproteins whereas it has no effect on the phosphorylation of protamine. Modulator II is stable to heating at 100°C for 30 min, is assumed to be a protein since it is inactivated by treatment with trypsin and it has a molecular weight of approximately 18 000 by gel exclusion. The modulator acts noncompetitively with respect to protein kinase substrates, ATP and histones and to activator molecules, cyclic AMP and Co^{2+}. The modulator does not function by destroying ATP nor by acting as a phosphoprotein phosphatase. However, it causes a marked alteration in the pH and metal ion activation properties of the kinase. The reactivities of the modulator were nearly identical with the holoenzyme and the catalytic subunit of protein kinase. The results suggest that the modulator (M) interacts directly with the catalytic subunit (C) of cyclic AMP-dependent protein kinase to cause the formation of a complex (CM) which has an altered pattern of substrate specificity.

Introduction

Previous studies have characterized a cyclic AMP-dependent cytosol protein kinase (ATP : protamine *O*-phosphotransferase, EC 2.7.1.70) of mammary gland and have demonstrated its central role in the hormonal regulation of mammary cells [1—3]. Cyclic AMP-dependent protein kinase (RC) of mammary gland and a large number of other tissues is an inactive complex of a

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catalytic subunit (C) and a regulatory subunit (R) [3—9]. Cyclic AMP causes the activation of the holoenzyme by specifically binding with the regulatory subunit (R) thereby dissociating the regulatory subunit from the catalytic subunit (C) which is the active form of the enzyme. Phosphorylation of several specific phosphoprotein components of highly purified plasma membranes, ribosomes and chromatin in cultured mammary epithelial cells is under hormonal regulation and these proteins may serve as substrates of the hormonally-induced protein kinase [3,10]. Preliminary studies from this laboratory provided evidence for the presence of two additional proteins (Modulator I and II) in rat mammary gland which may further regulate the activity of protein kinase [11]. The present studies characterize the modulator II which causes substrate-specific inhibition of the hormonally induced cyclic AMP-dependent protein kinase in vitro for the phosphorylation of multiple organelle-associated phosphoproteins. The results are consistent with the view that the modulator II (M) interacts directly with the catalytic subunit (C) to cause the formation of a complex (CM), which has an altered pattern of substrate specificity.

Materials and Methods

Chemicals

Adenosine 3′,5′-monophosphate, pancreatic DNAase, *O*-phosphoserine and *O*-phosphothreonine were obtained from Calbiochem. [γ-^{32}P]ATP (21 to 27 Ci per mmol) was a product of International Chemical and Nuclear Corporation. Calf thymus whole and fractionated histones, pancreatic RNAase (five times crystallized) and trypsin (crystallized two times) were from Worthington. DEAE-cellulose, ATP, protamine (salmon sperm), trypsin inhibitor (ovomucoid), arginine-rich histone (calf thymus) and bovine serum albumin were purchased from Sigma. Ovalbumin was from Schwarz-Mann and Sephadex G-75 and G-100 were products of Pharmacia. Casein was from Nutritional Biochemicals.

Homogeneous preparation of catalytic subunit of rabbit skeletal muscle cyclic AMP-dependent protein kinase was generously provided by Dr. J. Beavo of the University of California, Davis.

Isolation of mammary protein kinases

Partially purified cyclic AMP-dependent protein kinase II isolated from lactating rat mammary gland [1] was further purified by Sephadex gel filtration. Active fractions following DEAE-cellulose chromatography were pooled together and concentrated by ultrafiltration through a PM-30 Amicon membrane. The concentrated fraction was dialysed against 4 mM sodium glycerophosphate buffer (pH 6.5) and subjected to gel filtration on a column (2.8 × 35 cm) of Sephadex G-100. Elution was carried out with the same buffer, flow rate was 30 ml per h and volume in each fraction was 4 ml. The cyclic AMP-dependent protein kinase was eluted as a single enzymatic species peaking at fraction No. 15. Enzyme activities in the fractions 13 to 17 were pooled, concentrated by filtration through Amicon PM-30 membrane and

finally preserved in 40% glycerol containing 2 mM sodium glycerophosphate buffer (pH 6.5) at −25° C.

Catalytic and regulatory subunits of cyclic AMP-dependent mammary protein kinase were isolated by the procedure described earlier [3]. All the steps were carried out at 0 to 4° C.

Isolation of mammary protein kinase modulator II

(a) Heated modulator II. Modulator II was isolated by the method described previously [11] from the lactating rat mammary homogenate previously heated at 90° C for 10 min.

(b) Native modulator II. An important feature of this procedure is that it does not involve any heat treatment during the isolation procedure. Lactating rat mammary gland was homogenized in 4 mM EDTA, pH 7.0, the homogenate was centrifuged at 25 000 × *g* for 30 min and the pellet was discarded. The pH of the supernate was adjusted to 4.6 by addition of 1.0 M acetic acid with constant stirring. After 30 min the suspension was centrifuged at 25 000 × *g* for 10 min and the precipitate was discarded. The pH of the supernatant fluid was adjusted to 6.5 with sodium bicarbonate and solid ammonium sulfate was added to the above solution (48 g/100 ml) with stirring. After 30 min the precipitate was sedimented by centrifugation at 25 000 × *g* for 10 min and the supernate was discarded (Step. 1). The residue was dissolved in 5 mM potassium phosphate buffer (pH 7.0) and dialysed against the same buffer prior to application to a column of DEAE-cellulose (2.8 × 30 cm) previously equilibrated with 5 mM potassium phosphate buffer pH 7.0. The column was washed with 150 ml of the equilibrating buffer prior to further elution with a linear gradient of potassium phosphate (5 to 500 mM) in a total value of 350 ml of the buffer. The flow rate was 36 ml per h and the volume in each fraction was 12 ml (Step 2). Active fractions (fractions 24 to 33) were pooled together, concentrated by ultrafiltration through Amicon membrane PM-30, equilibrated with 4 mM sodium glycerophosphate · HCl buffer (pH 6.5) and subjected to gel filtration on a column (2.8 × 30 cm) of Sephadex G-100. Elution was carried-out with the same buffer, the flow rate was 30 ml per hour and the volume in each fraction was 4 ml. Fractions 26 to 32 containing modulator II activity were pooled together, concentrated by filtration through Amicon membrane UM-2 and the concentrate was preserved at −25° C in 40% glycerol containing 2 mM sodium glycerophosphate · HCl buffer, pH 6.5 (Step 3). All the steps were carried out at 0—4° C.

Assay of protein kinase modulator II

The effect of modulator II on the activity of protein kinase was determined by using a slight modification of the protein kinase assay method described previously [3]. The standard assay system contained in a final volume of 0.2 ml, 10 μmol of sodium glycerophosphate-HCl (pH 6.5), 1 nmol of [γ-^{32}P]ATP containing 2 to 6 · 10^5 cpm, 2 μmol of sodium fluoride, 0.4 μmol of theophylline, 0.5 mg of calf thymus whole histones, 0.06 μmol of ethylene glycol bis-(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid, 2 μmol of cobalt chloride, 200 pmol of cyclic AMP, 20 to 40 units of cyclic AMP-dependent mammary protein kinase (holenzyme) and varying amounts of protein kinase modulator II as

specified. The incubation was carried out at 30° C for 20 min. The reaction was terminated and the degree of histone phosphorylation was measured as described earlier [2]. A unit of the activity of modulator II was defined as the amount of the modulator which would inhibit the activity of protein kinase by 50% under the standard assay conditions.

A unit of protein kinase activity was defined as the amount of the enzyme which causes the transfer of 1 pmol of ^{32}P from [γ-^{32}P]ATP to histones during 20 min of incubation (without modulator II) under the standard assay conditions. The specific activity of the isolated mammary cyclic AMP-dependent protein kinase was 2 units per μg protein.

Preparation of ^{32}P-labeled histones

Histones were phosphorylated enzymatically by the isolated mammary cyclic AMP-dependent protein kinase. The reaction mixture contained 40 μmol of sodium glycerophosphate · HCl (pH 6.5), 2.0 mg whole histones or a histone subfraction, 10 nmol of [γ-^{32}P]ATP containing 6.0 · 10^6 cpm, 0.24 μmol of ethylene glycol bis-(β-amino-ethyl ether)-*N,N'*-tetraacetic acid, 8 μmol of cobalt chloride, 1 nmol of cyclic AMP, 12 μmol of sodium fluoride, 2 μmol of theophylline, 200 units of protein kinase II with or without 140 μg of protein kinase modulator II (heated) in a total volume of 0.8 ml. The mixture was incubated at 30° C for 30 min and the reaction was arrested by addition of 5 ml of 10% trichloroacetic acid and [^{32}P]histones were isolated by the procedure described earlier [2].

Subcellular fractionation

Nuclei were isolated from homogenates of lactating rat mammary gland and were purified by discontinuous sucrose density gradient centrifugation as previously described [1]. Histones were extracted in 0.25 M HCl according to Marzluff et al. [12]. Purified mammary plasma membranes were isolated by a slight modification of the method of Neville [13] as described earlier [2]. For the isolation of mitochondria and ribosomes lactating mammary gland was homogenized in 0.05 M Tris · HCl buffer, pH 7.6 containing 0.25 M sucrose in a Dounce homogenizer, the homogenate was centrifuged at 1000 × *g*, for 10 min and the pellet was discarded. Mitochondria were isolated from the resulting supernate by centrifugation at 17 000 × *g* for 20 min and microsomes were sedimented by centrifugation of post-mitochondrial supernate at 110 000 × *g* for 60 min. Purified ribosomes were isolated from the crude microsomal pellet by a slight modification of the method of Moldave and Skogerson [14] as described earlier [2].

Other analytical methods

The protein contents of all samples were measured by the method of Lowry et al. [15] using bovine serum albumin as standard. Isolated ^{32}P-labeled histones were subjected to amino acid analysis and polyacrylamide gel electrophoresis as previously described [1].

Results

Purification of protein kinase modulation II

One peak of native modulator II which inhibits the activity of the cyclic

AMP-dependent mammary protein kinase was obtained by DEAE-cellulose chromatography (Fig. 1). The DEAE-cellulose eluate of the modulator containing large amount of endogenous cyclic AMP-dependent protein kinase was subjected to molecular sieving on a column of Sephadex G-100 (Fig. 2). One peak of native modulator was obtained which was clearly separated from the cyclic AMP-dependent protein kinase peak. The preparation of native modulator II as obtained by Sephadex gel filtration was essentially free from the activity of protein kinase. Results of a typical purification procedure are shown in Table I. The activity of the modulator was undetectable prior to the ammonium sulfate fractionation procedure. The activities of both the native and heated modulator preparations further increased (approx. 200%) following DEAE-cellulose chromatography. Native modulator II was purified to 40-fold and the heated modulator to 50-fold compared to the corresponding ammonium sulfate fraction. The specific activities of the native and heated modulator preparations were 33 and 40 units per mg protein respectively. Efforts to purify further both the heated and native modulator were unsuccess-

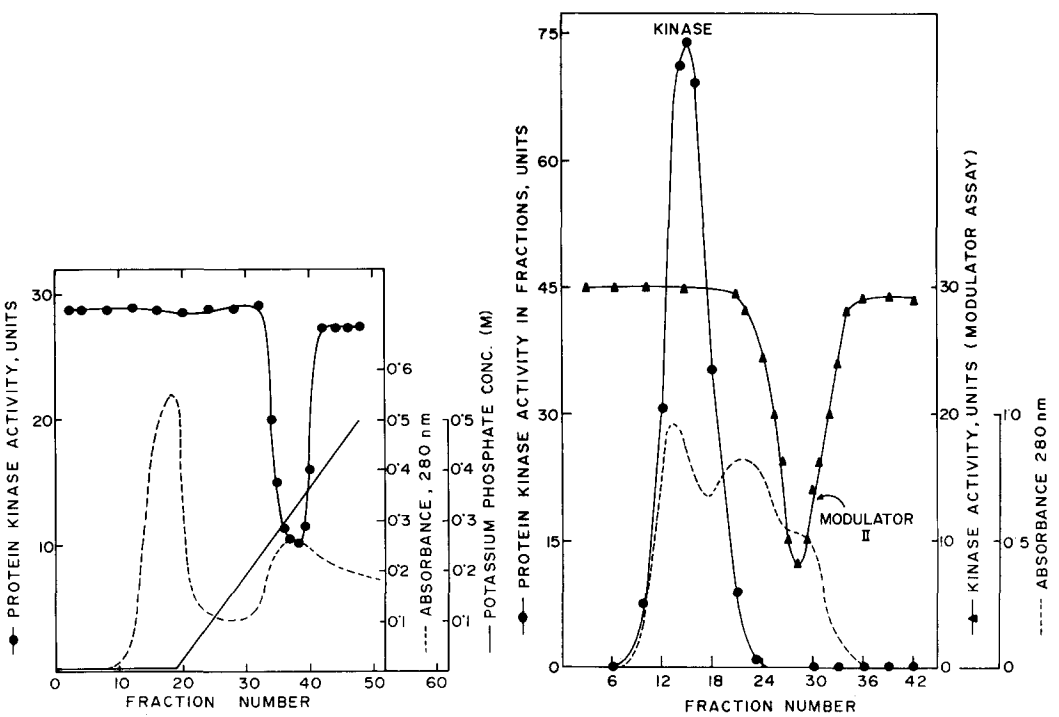

Fig. 1. DEAE-cellulose chromatography pattern of native modulator II (●——●). Ammonium sulfate fraction of the modulator obtained from 70 g lactating rat mammary gland was applied to the column (2.8 X 30 cm). An aliquot of 50 μl of each fraction was heated in a boiling water bath for 2 min to destroy the endogenous protein kinase activity prior to assay of modulator activity using 30 units of cyclic AMP-dependent protein kinase under the standard assay conditions.

Fig. 2. Elution profile of the chromatography of the native modulator II on Sephadex G-100 (▲——▲). DEAE-cellulose eluate of the modulator obtained from 70 g of lactating rat mammary gland was applied to a column (2.8 X 30 cm). An aliquot of 50 μl of each fraction was assayed for protein kinase activity in presence of 2 μM cyclic AMP without any exogenous modulator under the standard assay conditions. Modulator activity in the fractions was assayed by the procedure described in legend for Fig. 1.

TABLE I

SUMMARY OF THE PURIFICATION OF MODULATOR II FROM LACTATING RAT MAMMARY GLAND

The fractions obtained at various stages of the purification of the native modulator II were subjected to heat treatment at 100°C for 2 min to destroy endogenous protein kinase activity prior to assay of modulator activity. The activities of all the fractions were measured under the standard assay conditions using 30 and 25 units of cyclic AMP-dependent protein kinase for the native and heated modulator fractions respectively.

Fraction	Preparation of modulator II	Yield (%)	Purification * (-fold)
1. Ammonium sulfate precipitate		30	1
2. DEAE-cellulose eluate	Native	100	5
3. Sephadex G-75 eluate		75	40
1. Ammonium sulfate precipitate		35	1
2. DEAE-cellulose eluate	Heated	100	6
3. Sephadex G-100 eluate		70	50

* For the calculation of the purification it has been assumed that 100% of the modulator activity is present in the ammonium sulfate fraction.

ful because of the instability of the modulator. Modulator preparations purified through Sephadex gel filtration step were used in these studies.

Time course

Modulator II inhibited the rate of phosphorylation of calf thymus whole histones by the cyclic AMP-dependent mammary protein kinase and the reaction was linear for at least 30 min in presence of either heated or native modulator II.

Modulator concentration

The effect of varying concentrations of heated and native modulator II on the phosphorylation of histones by the cyclic AMP-dependent protein kinase is shown in Fig. 3. Both the modulator preparations showed similar patterns of progressive increase in the degree of inhibition of protein kinase with increase in the concentration of the modulator.

Protein kinase concentration

The effect of varying concentrations of mammary cyclic AMP-dependent protein kinase (5 to 100 units) on the action of modulator II (heated or native) for the phosphorylation of histones was measured. The degrees of inhibition of protein kinase activity by the modulator II were nearly identical at varying concentrations of the protein kinase indicating that the inhibitory action of modulator is independent of the concentration of protein kinase.

pH optima

Heated modulator II caused a marked alteration in the pH-optimum curve of the cyclic AMP-dependent mammary protein kinase (Fig. 4). The optimum pH

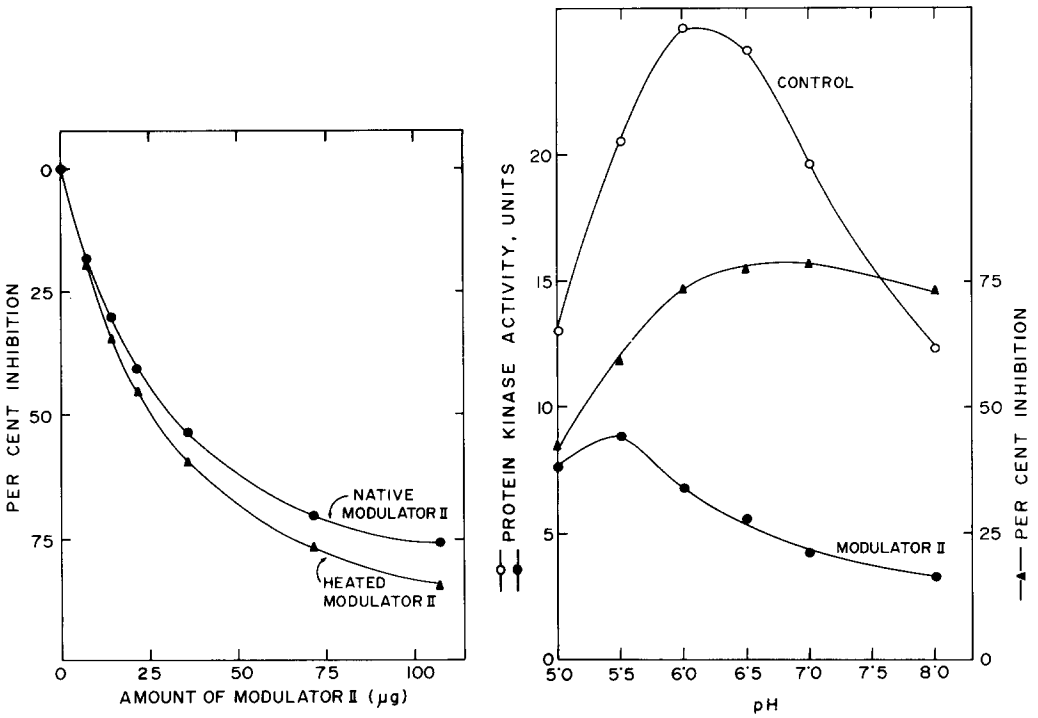

Fig. 3. Standard curve for the assay of mammary modulator II. Extent of phosphorylation of histones catalysed by cyclic AMP-dependent protein kinase (30 units) was measured as a function of modulator concentration (native, ●——●; heated, ▲——▲) in the standard assay procedure.

Fig. 4. Effect of pH on the activity of modulator II (heated, 65 μg) for its action on cyclic AMP-dependent mammary protein kinase (25 units) was determined under the standard assay conditions except for the variation in the pH of the sodium glycerophosphate/HCl buffer.

for the activity of the kinase without and with modulator II were 6.0 to 6.5 and 5.5, respectively. The degree of inhibition of protein kinase activity by the modulator II was maximal at pH 6.5 to 7.0. The optimum pH for the action of native modulator II was nearly identical to that of the heated modulator.

Concentrations of histones, ATP and cyclic AMP

The effects of varying concentrations of whole histones and [γ^{32}-P]ATP for the action of heated modulator II on cyclic AMP-dependent protein kinase are shown in Fig. 5. The degrees of inhibition of kinase by the modulator were observed to be independent of the concentration of the kinase substrates, histones and ATP. Studies with varying concentrations (0.01 to 10 μM) of cyclic AMP showed that the inhibitory action of modulator was nearly identical at these concentrations of the cyclic nucleotide. These results demonstrate that modulator II inhibits mammary protein kinase noncompetitively with respect to histones, ATP and cyclic AMP. Nearly identical results were also observed with the native modulator II.

Metal ions

The activity of mammary cyclic AMP-dependent protein kinase is dependent

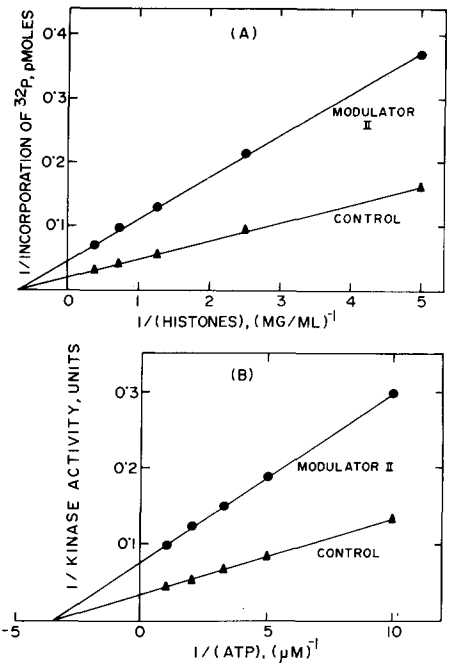

Fig. 5. Double-reciprocal plots of the initial velocity of the phosphorylation of histones by cyclic AMP-dependent protein kinase versus (A) calf thymus whole histones concentration and (B) ATP concentration at constant level of the heated modulator II (30 μg). Standard assay conditions were used except the variations in the concentration of the constituents as specified.

on a divalent metal ion Co^{2+}, Mn^{2+} or Mg^{2+} [1] and the effects of these metal ions on the action of heated modulator II with the mammary protein kinase are shown in Table II. The extent of inhibition of protein kinase by the modulator is dependent on the nature of the activator metal ion. The inhibitory activity of the modulator was observed in presence of all these metal ions and the inhibition was maximal in presence of Co^{2+}. The metal ion activation property of the enzyme was markedly altered by the modulator. In its absence Co^{2+} caused maximal activation of the kinase whereas in its presence Mg^{2+} was most effec-

TABLE II

EFFECT OF METAL IONS ON THE ACTIVITY OF MODULATOR II FOR ITS ACTION ON CYCLIC AMP-DEPENDENT MAMMARY PROTEIN KINASE

The standard assay system was used except the indicated changes in the nature and concentration of the divalent metal ions. 35 units of cyclic AMP-dependent mammary protein kinase and 40 μg of heated modulator II were used in this experiment. In absence of the divalent metal ion the activity of protein kinase was insignificant.

Metal ion (14 mM)	Incorporation of ^{32}P into protein (pmol/20 min)		
	Control	Modulator II	% inhibition
Cobalt chloride	34.5	12.1	65
Magnesium acetate	24.9	13.4	46
Manganese chloride	7.2	3.4	53

tive. Further studies with varying concentrations of Co^{2+} demonstrated the noncompetitive nature of inhibition of protein kinase by the modulator with respect to the metal ion.

Substrate specificity

As demonstrated previously, the mammary cytosol cyclic AMP-dependent protein kinase has high degree of substrate specificity for the basic proteins histones and protamine [1]. Of all the histone species F-2b histone was most effective as protein substrate of the mammary holoenzyme (Table III). The rates of phosphorylation of calf thymus whole histones and histone subfractions and casein by protein kinase were markedly inhibited in presence of modulator II and the degree of this inhibition is substrate specific. Of all these proteins tested the rate of phosphorylation of F-1 histone was most strongly inhibited (75%) by heated modulator II as compared to 65% inhibition for F-2b, 57% for F-3 and 50% for F-2a histone. Whereas it had no significant effect on the phosphorylation of protamine. The patterns of inhibition of protein kinase for the phosphorylation of the various proteins by native and heated modulator II were nearly identical.

The effects of varying concentrations (25 to 220 μg) of calf thymus histone species F-1, F-3, F-2a and F-2b on the activity of mammary cyclic AMP-dependent protein kinase (30 units) at constant level of the heated modulator II (40 μg) were measured under the standard assay conditions. The extents of inhibition of protein kinase by the modulator II were independent of the concentration of each of the protein substrates. Modulator II inhibited the rate of phosphorylation of F-1, F-2b, F-3 and F-2a histones by 75%, 66%, 60% and 50%, respectively. These studies further extend the view that the inhibitory action of modulator II on protein kinase is substrate specific.

Characterization of phosphorylated histones

^{32}P-labeled F-1, F-2a, F-2b and F-3 histones obtained by phosphorylation

TABLE III

EFFECTS OF MODULATOR II ON THE ACTIVITY OF THE CYCLIC AMP-DEPENDENT MAMMARY PROTEIN KINASE FOR THE PHOSPHORYLATION OF VARIOUS MODEL PROTEIN SUBSTRATES

The effects of heated modulator II (40 μg protein) on the activity of the cyclic AMP-dependent mammary protein kinase (35 units) were measured using 250 μg of each of various calf thymus histones and protamine and 2 mg of casein as substrates under the standard assay conditions.

Substrate	Incorporation of ^{32}P into protein (pmol/20 min)		
	Control	Modulator II	Inhibition (%)
Whole histones	30	11	63
F-1 histone	60	15	75
F-2b histone	120	42	65
F-3 histone	36	15	57
F-2a histone	20	10	50
Lysing rich histone	14	6	57
Casein	14	8	43
Protamine	16	17	0

with mammary cyclic AMP-dependent protein kinase in presence and absence of heated modulator II were fractionated by polyacrylamide gel electrophoresis. With each of these preparations one major ^{32}P-labeled band was identified and of all these species of histones the rate of phosphorylation of F-1 histone was maximally inhibited by modulator II and the order of inhibition of the phosphorylation of other histone species by modulator II was F-2b, F-3 and F-2a as also observed previously using standard assays (Table III).

^{32}P-labeled whole histones obtained by phosphorylation with kinase in presence and absence of the modulator were also analysed for the measurement of radioactivity in *P*-serine and *P*-threonine residues (Table IV). Approximately 95% of radioactivity was recovered in *P*-serine as opposed to 5% in *P*-threonine and modulator II showed little specificity for the phosphorylation of serine and threonine residues of whole histones.

Incubation of isolated [^{32}P]histones with mammary protein kinase in presence and absence of modulator II (heated) in the standard modulator assay system at 30° C for periods upto 60 min did not cause any significant loss of ^{32}P from the histones indicating that the inhibitory action of the modulator is not due to dephosphorylation or proteolysis of [^{32}P]histones. There was no appreciable loss of ^{32}P when [γ^{32}-P]ATP was incubated at 30° C periods upto 30 min with and without protein kinase in the presence of the modulator under the standard assay conditions (minus histones), as demonstrated by the analysis of the products of this reaction with high voltage electrophoresis [1]. Thus the inhibitory action of modulator II is not due to the presence of ATPase activity either in the modulator or in the protein kinase preparation.

Molecular weight

Both the native and heated modulator II preparations were nondialysable and 100% stable when heated at 100° C for 30 min. As shown in Table V, the activity of modulator II was markedly lost when subjected to the proteolytic action of trypsin indicating the polypeptide nature of the modulator. Whereas treatments with DNAase and RNAase had little effect on the activity of the modulator. Molecular weight of the modulator II (native or heated) was observed to be approximately 18 000 as determined by the method of molecular sieving on Sephadex G-100 (Fig. 6).

TABLE IV

DISTRIBUTION OF [^{32}P]PHOSPHOSERINE AND [^{32}P]PHOSPHOTHREONINE IN CALF THYMUS [^{32}P]HISTONES

^{32}P-labeled whole histones were hydrolysed with 2 M HCl and subjected to high voltage electrophoresis by the procedure described in the text. Approximately 70% of the [^{32}P]histone radioactivity was recovered in the *O*-phosphoserine and *O*-phosphothreonine spots.

System	^{32}P-labeled serine radioactivity (cpm)	^{32}P-labeled threonine radioactivity (cpm)
Control	9800	500
Modulator II	3300 (66) *	180 (64) *

* Figures within parentheses represent percent inhibition.

TABLE V

EFFECT OF VARIOUS ENZYME TREATMENTS ON THE ACTIVITY OF MODULATOR II

Four units of heated modulator II (100 μg per 0.2 ml) were incubated with or without the indicated enzymes at 30°C for 3 h in 4 mM sodium glycerophosphate buffer, pH 6.5. Magnesium acetate (0.05 M) was present during incubation with DNAase. The incubation with trypsin was stopped by adding 50-fold excess of egg white trypsin inhibitor. All the incubated samples were dialysed against 4 mM sodium glycerophosphate buffer, pH 6.5 at 4°C prior to the determination of the modulator activity under the standard assay conditions using 30 units of protein kinase and 100 μl of the dialysed preparations.

Treatment	Modulator activity remaining
None	100
Trypsin, 10 μg	30
Trypsin, 25 μg	17
RNAase, 25 μg	100
DNAase, 25 μg	95

Action of modulator II on isolated catalytic subunit of protein kinase

The action of modulator II (heated) on the partially purified catalytic subunit (C) of cyclic AMP-dependent mammary protein kinase (RC) was determined. The extents of inhibition of the phosphorylation of whole histones with C and RC by modulator II were nearly identical. Addition of regulatory subunit of protein kinase (R) had little effect on the substrate-specific inhibition of kinase by modulator II when the assays were carried out at 2 μm concentration of cyclic AMP. These results demonstrate that the modulator is clearly different from the regulatory subunit and the observed protein substrate-dependent inhibitory action of the modulator represents the interaction of catalytic subunit (C) with the modulator.

The above view was further strengthened by the studies of the action of

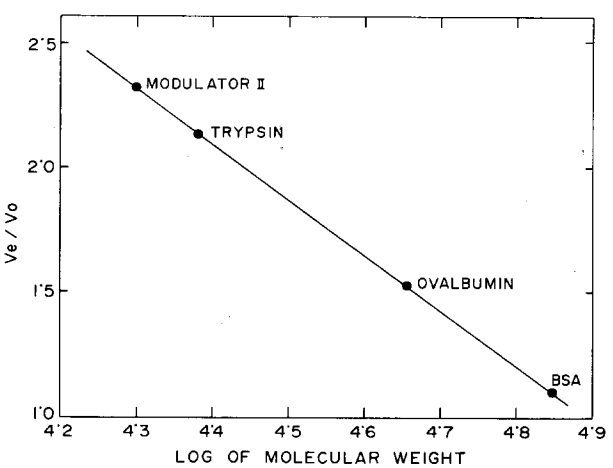

Fig. 6. Determination of the molecular weight of the mammary modulator II by gel filtration. The molecular weight of the modulator was determined using a column of Sephadex G-100 (2.8 × 39 cm) by the method of Whitaker [16]. Gel was equilibrated with 5 mM sodium glycerophosphate · HCl buffer, pH 6.5 and 12 mg of the modulator was applied to the column. Standard proteins and blue dextran were detected by absorption at 280 nm and the activity of the modulator was assayed under the standard assay conditions. The molecular weights of the proteins used as standards were BSA: bovine serum albumin (70 000), ovalbumin (45 000) and trypsin (24 000).

TABLE VI

EFFECT OF MAMMARY MODULATOR II ON THE SUBSTRATE SPECIFICITY OF PURIFIED CATALYTIC SUBUNIT OF RABBIT SKELETAL MUSCLE CYCLIC AMP-DEPENDENT PROTEIN KINASE

The effect of heated modulator II (40 μg protein) on the activity of the purified catalytic subunit of the rabbit skeletal muscle cyclic AMP-dependent protein kinase (30 units) was determined using 250 μg of each of the various calf thymus histones and protamine and 3 mg of casein as substrates under the standard assay conditions (minus cyclic AMP).

Substrate	Incorporation of ^{32}P into protein (pmol/20 min)		
	Control	Modulator II	% inhibition
Whole histones	20	8	60
F-1 histone	45	14	70
F-2b histone	160	53	66
F-3 histone	34	14	58
F-2a histone	24	11	55
Lysine rich histone	13	6	53
Casein	9	4	55
Protamine	20	20	0

mammary modulator II on the purified catalytic subunit (C) of the rabbit skeletal muscle cyclic AMP-depndent protein kinase (Table VI). Modulator II as well produced substrate-specific inhibition of the muscle protein kinase for the phosphorylation of a variety of protein species and in this respect rat mammary and rabbit muscle kinases behaved similarly. Cyclic AMP had no effect on the action of modulator with the catalytic subunit.

TABLE VII

EFFECT OF MODULATOR II ON THE SUBSTRATE SPECIFICITY OF MAMMARY CYCLIC AMP-DEPENDENT PROTEIN KINASE FOR THE PHOSPHORYLATION OF MAMMARY PROTEIN SUBSTRATES.

Highly purified preparations of rat mammary mitochondria, plasma membrane and ribosomes were heated at 100°C for 2 min to destroy intrinsic protein kinase activities. These heat-treated organelles and isolated mammary histones served as protein substrates of the mammary cyclic AMP-dependent protein kinase (50 units) under the standard assay conditions with and without heated modulator II (40 μg).

Mammary protein substrate	Amount of protein (μg)	Incorporation of ^{32}P into protein (pmol/20 min)		
		Control	Modulator II	% Inhibition
Histones	200	30	13	57
	600	50	22	56
Plasma membranes	200	10	5.5	45
	600	16	8.6	46
Mitochondria	200	15	5.3	65
	600	22	8.3	62
Ribosomes	200	20	9.2	54
	600	30	14.4	52

Mammary protein substrates

As demonstrated previously protein kinase activities are tightly bound with the organelles of the mammary epithelial cells which can cause the phosphorylation of the respective endogenous phosphoproteins [3]. Modulator II had little effect on these endogenous phosphorylation reactions. As shown in Table VII modulator II, however, causes varying degrees of inhibition of the cyclic AMP-dependent mammary cytosol protein kinase for the phosphorylation of mammary histones and proteins associated with the heat-treated mitochondria, ribosomes and plasma membranes. The magnitude of inhibition by the modulator was highest for the phosphorylation of mitochondrial proteins (65%) and least for the plasma membrane proteins (45%).

Discussion

The present studies characterize a heat-stable protein (Modulator II) from lactating rat mammary gland which can modulate the activity of mammary cytosol cyclic AMP-dependent protein kinase. The modulator has a molecular weight of approximately 18 000 and it produces a marked substrate-specific inhibition of the activity of the protein kinase for the phosphorylation of casein, calf thymus whole, F-1, F-3, F-2a and F-2b histones and mammary whole histones and mitochondrial, plasma membrane and ribosomal phosphoproteins. Whereas the modulator has no significant effect on the phosphorylation of protamine. The patterns of this substrate-specific inhibition are nearly identical when the holoenzyme or the catalytic subunit is used as the enzyme.

The modulator acts noncompetitively with respect to the substrates of the cyclic AMP-dependent protein kinase, namely calf thymus whole and fractionated histones and ATP and to the activator molecules, cyclic AMP and Co^{2+}. The modulator does not catalyse the breakdown of [γ^{32}-P]ATP and the dephosphorylation or proteolysis of the phosphoprotein product of the kinase. It seems unlikely that the inhibitory action of modulator II on protein kinase is due to any structural modification of the protein substrates (histones) since the gel electrophoretic patterns of histones remain unchanged following incubation with the modulator. The interaction between the catalytic subunit and the modulator thus appears to be direct as represented by the reaction of the simplified Eqn. 1.

$$C + M \rightarrow CM \qquad (1)$$

In this reaction M represents the modulator and CM is a complex of the modulator and the catalytic subunit. The activity of the complex CM is not influenced by either cyclic AMP or the regulatory subunit and it has an altered pattern of substrate specificity for the phosphorylation of proteins. This mechanism of the action of the modulator accounts for its substrate-dependent inhibitory action on protein kinase. The observations that the modulator causes a marked alteration in the pH (Fig. 4) and metal ion (Table II) activation properties of the holoenzyme are consistent with the above view.

The products of the histone phosphorylation reactions catalysed by the holoenzyme were characterized by polyacrylamide gel electrophoresis and amino acid analysis. The modulator has no specificity for the phosphorylation

of seryl and threonyl residues of calf thymus histones. The observations that the native and heated modulator preparations have nearly identical molecular size and reactivities towards the protein kinase strengthens the view that the observed properties of the modulator are not an artifact due to its isolation procedure.

Protein kinase inhibitor [17,18] and modulator [19,20] activities have been demonstrated in several other sources. Rabbit skeletal muscle protein kinase inhibitor interacts directly with the catalytic subunit of the cyclic AMP-dependent protein kinase of the same tissue to cause marked inhibition of the phosphorylation of the protein substrates [18]. The properties of the mammary modulator are clearly different from those of the muscle protein kinase inhibitor particularly in relation to its molecular size and specificity of action on protein kinase [17]. The finding that the mammary modulator II also causes substrate-specific inhibition of the catalytic subunit from the rabbit skeletal muscle for the phosphorylation of several species of proteins (Table VI) further supports this view. A modulator from lobster tail muscle has been shown to act by altering the substrate specificity of both the cyclic GMP-dependent and cyclic AMP-dependent protein kinases, increasing the rate of phosphorylation of some protein substrates and decreasing that of others [19,20]. It is thus possible that the process of cell differentiation confers upon cells a tissue-specific pattern of protein kinase modulator distribution. Previous studies postulated that the holoenzyme, induced rapidly in mammary gland in response to prolactin, propagates the initial action of the hormone on the cell surface throughout functionally distinct compartments of the cell by phosphorylating specific protein components of plasma membrane, ribosomes and nuclei [10,21]. The observation that the modulator II produces substrate-specific inhibitory effect in vitro on the holoenzyme for the phosphorylation of multiple mammary organelle-bound phosphoproteins suggests the possibility that the modulator may have an important regulatory role to cause an altered pattern of substrate specificity for the hormonally induced protein kinase. Elucidation of the physiological significance of the modulator during cell differentiation in mammary gland is currently under investigation.

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