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ADENOSINE 3′,5′-MONOPHOSPHATE DEPENDENT PHOSPHORYLATION OF RIBOSOMES AND RIBOSOMAL SUBUNITS FROM BOVINE CORPUS LUTEUM

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Summary

In a previous publication the purification and properties of two protein kinases (KI and KII) from a soluble fraction of bovine corpus luteum and the stimulation of the latter fraction by cyclic AMP and luteinizing hormone was reported (Menon, K.M.J. (1973) J. Biol. Chem. 248, 494—501). We have now studied the effects of cyclic AMP and luteinizing hormone on ribosomal protein phosphorylation of corpus luteum by protein kinase II. Protein kinase II catalyzed the phosphorylation of ribosomes by transfer of terminal phosphate of ATP to ribosomal proteins. Extraction with hot trichloroacetic acid and non-aqueous solvent revealed that about 80% of total radioactivity incorporated remain associated with the protein residue. Radioactivity was identified in the phosphoserine and phosphothreonine residues of polypeptides by high voltage paper electrophoresis. The extent of phosphorylation was stimulated by cyclic AMP but not by luteinizing hormone. At least 9 proteins of 80-S ribosomes and 12 proteins of the 60-S ribosomal subunit were phosphorylated in the presence of cyclic AMP as resolved by urea polyacrylamide gel electrophoresis. However, only one major and four minor bands were phosphorylated in the case of 40-S ribosomal subunit under the influence of cyclic AMP. The ribosomal protein phosphorylation catalyzed by protein kinase II is regulated by cyclic AMP whereas luteinizing hormone has no effect on ribosome phosphorylation.

Introduction

Adenosine 3′,5′-monophosphate (cyclic AMP) has been shown to be a

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mediator of the action of luteinizing hormone to stimulate steroidogenesis in the bovine corpus luteum [1,2]. It has also been shown that luteinizing hormone increases the endogenous cyclic AMP level prior to its effect on steroidogenesis [2] and that exogenously added cyclic AMP mimics the effect of luteinizing hormone [3]. This stimulatory effect of cyclic AMP is blocked by puromycin suggesting the involvement of a labile protein in this process [4]. The foregoing observations imply an important role of cyclic AMP in the steroidogenic process, however, the exact mechanism by which cyclic AMP brings about this effect is not clear. The presence of cyclic AMP-dependent protein kinases in tissues in which cyclic AMP acts as a second messenger has led to the proposal that the wide variety of cellular effects exhibited by cyclic AMP are mediated through the activation of protein kinases [5—16].

Previous studies from this laboratory have characterized two forms of protein kinases, KI and KII. In addition to cyclic AMP, KII was also directly stimulated by luteinizing hormone [17], suggesting that luteinizing hormone may have a direct control on the activity of KII in the corpus luteum [17].

To elucidate further the functional role of this cyclic AMP and luteinizing hormone dependent protein kinase the possible physiological substrates of this enzyme were investigated. Evidence implicating the role of cyclic AMP at the translational level in the process of progesterone synthesis [4] further suggests that ribosomal proteins may be the endogenous substrate for protein kinase. Therefore, the ability of protein kinase to phosphorylate proteins of ribosomes and ribosomal subunits was examined. The present studies demonstrate that several protein components of purified ribosomes or ribosomal subunits may serve as in vitro substrates of protein kinase II and that the phosphorylation of these proteins is enhanced by cyclic AMP, but not by luteinizing hormone.

Experimental procedures

Materials

Collection of Bovine Corpora Lutea. Bovine corpora lutea were collected from a local slaughter house and immediately dissected out to remove connective tissues. The tissues were transported to the laboratory in ice cold normal saline and then frozen on dry ice and stored at −80° C.

Chemicals [γ-^{32}P] ATP was prepared from carried free ^{32}P-labelled orthophosphate according to Glynn and Chappel [18]. Carrier free ^{32}P-labelled ortho-phosphate was obtained from ICN Chemical and Radioisotope Division, Irvine, California. Cyclic AMP, cyclic GMP, 5′-AMP, 5′-GMP, *O*-phospho-DL-serine, *o*-phospho-DL-threonine, mixed histones (Type IIA), pancreatic RNAase (5 times crystallized) and Triton X-100 were supplied by Sigma Chemical Company, St. Louis, Missouri. Sodium deoxycholate was a product of Difco Laboratories, Detroit, Michigan.

Methods

Isolation and purification of polyribosomes from bovine corpus luteum. Polyribosomes and stripped ribosomes (80 S) were isolated by standard procedures [19,20]. In some cases ribosomes were also purified by precipitation with $MgCl_2$ as described by Takanami [21]. Concentrations of ribosomes were determined according to a modified procedure [22] of Fleck and Munro [23].

Isolation of ribosomal subunits. Ribosomal subunits (60 S and 40 S) were prepared by treatment with puromycin and high salt concentration [24].

Purification of protein kinase II. Protein kinase II was purified according to the procedure described earlier [17].

Protein kinase assay. Protein kinase activity was determined by the previously published procedures [6,17]. The radioactivity in the aqueous protein residue was determined by liquid scintillation counting after the addition of 10 ml Herberg solution [25] and 0.05 ml formic acid as described earlier [17]. Radioactive measurements were made in a Beckman Model LS 230 Liquid Scintillation Spectrometer.

Polyacrylamide gel electrophoresis of ribosomal proteins. Ribosomes or subunits were phosphorylated (as described under each experiment) and resuspended in 0.5 ml of 10 mM sodium phosphate, pH 7.6, 10 mM β-mercaptoethanol and proteins extracted with 8 M urea/6 M LiCl at 4° C for 24 h [26]. After removal of the precipitated RNA by centrifugation, ribosomal proteins were dialyzed against three changes of 6 M urea. Polyacrylamide gel electrophoresis was performed by a slight modification of the method of Leboy et al. [26]. The intensity of the color of stained gel sections were determined by measuring the absorbance at 600 nm according to Johns [27].

Determination of phosphoserine and phosphothreonine in hydrolyzed ribosomal phosphoprotein. Ribosomal RNA from phosphorylated ribosomes or subunits was extracted by heating in 10% (w/v) trichloroacetic acid at 90° C for 30 min. The residue was spun down, washed with cold trichloroacetic acid and hydrolyzed in 6 M HCl at 105° C for 5 h in tubes sealed under nitrogen. The high voltage, paper electrophoresis was carried out as described by Langan [28] and Kabat [29] in a water cooled flat plate apparatus. The portion of the paper with authentic samples of phosphoserine, phosphothreonine and inorganic phosphate were sprayed with ninhydrin spray [30] to locate amino acids and then with a mixture of 1% (w/v) ammonium molybdate, 3% perchloric acid and 0.1 M HCl to locate orthophosphate [31]. With the aid of these markers radioactive spots were cut out and counted in a toluene-based liquid scintillation fluid [32].

Chemical estimation. Protein was measured by the method of Lowry et al. [33] using bovine serum albumin as standard and RNA by the colorimetric procedure of Ceriotti [34]. Phospholipids from ribosomes were extracted and estimated according to Chen et al. [35] and a factor of 25 was used to convert μg of inorganic phosphorus into μg of phospho-lipids. Inorganic phosphate was assayed colorimetrically [36].

Results

Characterization of ribosomes. The purified ribosomes showed average (at least 10 different determinations) $A_{260} : A_{280}$ and $A_{260} : A_{235}$ ratios of 1.82 and 1.56 respectively. The isolated ribosomes contained 45—47% RNA (Orcinol determination), 50—53% protein (Lowry determination) and less than 1% phospholipid (phosphorus determination).

Since dissociation of ribosomes into 60 S and 40 S subunit is dependent on the levels of Mg^{2+} as well as K^+ [24,39—41], optimal conditions for the

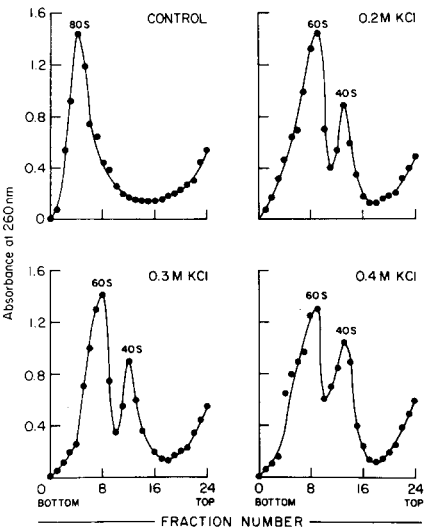

Fig. 1. Sucrose density gradient centrifugation of KCl and puromycin-treated polyribosomes from bovine corpus luteum. An aliquot of ribosomes (4.0 A_{260} units) was combined with 1 M KCl and 0.1 mM puromycin to give the final KCl concentration as indicated and 0.1 M puromycin. After incubation at 37° C for 10 min, samples were layered on a 10—30% linear sucrose gradient containing 10 mM Tris · HCl, pH 7.8, 2 mM $MgCl_2$ and the KCl concentrations as indicated. Centrifugation was carried out at 38 000 rev./min for 3 h at 4° C using a SW 50.1 rotor. Simultaneously, a sample of ribosomes was treated with RNAase to obtain the position of monosomes (80 S).

isolation of corpus luteum ribosomal subunits were determined. Polyribosomes purified discontinuous sucrose density gradient centrifugation were treated with indicated concentrations of KCl containing 0.1 mM puromycin. Nearly complete dissociation into subunits was observed at 0.3 M KCl (Fig. 1). Below this level dissociation into subunits was incomplete, while above 0.3 M KCl the 60-S subunit showed a significant reduction in sedimentation due to the loss of proteins (Fig. 1). The isolated subunits did not contain any cross contamination detectable by resedimentation in a sucrose density gradient.

Phosphorylation of ribosomal proteins by protein kinase II. Ribosomes or ribosomal subunits isolated from bovine corpus luteum were incubated with [γ-^{32}P] ATP and protein kinase II in the presence or absence of cyclic AMP at 30° C. As can be seen in Table I purified 80-S ribosomes and 60-S and 40-S ribosomal subunits were phosphorylated by protein kinase II and this phosphorylation was stimulated by cyclic AMP. Under identical experimental conditions maximum phosphorylation was observed with 80-S ribosomes followed by 60-S and 40-S subunits.

More conclusive evidence that ribosomal proteins are phosphorylated by protein kinase in the presence of [γ-^{32}P] ATP was obtained by sucrose density gradient analysis of the incubation mixture. From Fig. 2 it is evident that most of the incorporated radioactivity co-sedimented with the ribosomal subunits. A small portion of radioactivity associated with the top of the gradient was due to contamination from [γ-^{32}P] ATP.

Solubility characteristics of phosphorylated ribosomal constituents. Ribosomes which had been phosphorylated by protein kinase II and [γ-^{32}P] ATP in

TABLE I

PHOSPHORYLATION OF RIBOSOMAL PROTEINS BY PARTIALLY PURIFIED PROTEIN KINASE II FROM BOVINE CORPUS LUTEUM

The incubation medium in a final volume of 0.2 ml contained 3.9 nmol [γ-^{32}P]ATP ($1.3 \cdot 10^6$ cpm), 2 μmol KF, 0.5 μmol theophylline, 2 μmol $MgCl_2$, 10 μmol α-glycerophosphate buffer, pH 6.0, 5 A_{260} units of ribosomes or their subunits, 10 μg of protein kinase II. The contents were incubated for 15 min at 30°C and then reaction was stopped by the addition of 2 ml 7.5% (w/v) trichloroacetic acid. The samples were then assayed for precipitated radioactivity as described under Experimental Procedures.

Additions	[^{32}P]phosphate transferred (pmol)
Ribosomes (80 S) alone	2
+ cyclic AMP (5 μM)	3
+ protein kinase II (10 μg)	41
+ protein kinase II (10 μg) + cyclic AMP (5 μM)	83
60 S Subunit alone	2
+ cyclic AMP (5 μM)	2
+ protein kinase II (10 μg)	36
+ protein kinase II (10 μg) + cyclic AMP (5 μM)	79
40 S Subunit alone	2
+ cyclic AMP (5 μM)	2
+ protein kinase II (10 μg)	24
+ protein kinase II (10 μg) + cyclic AMP (5 μM)	33

the presence of cyclic AMP were subjected to hot trichloroacetic acid and non-aqueous solvent treatments to determine radioactivity into nucleic acids and phospholipids. The results showed that only 11% of the incorporated radioactivity was extracted into soluble material by hot acid treatment. On the contrary, 95—98% of the RNA was extracted by this procedure. Likewise, extraction of the remaining precipitate with a series of non-aqueous solvents [40] resulted in release of only a small percentage (8%) of radioactivity and

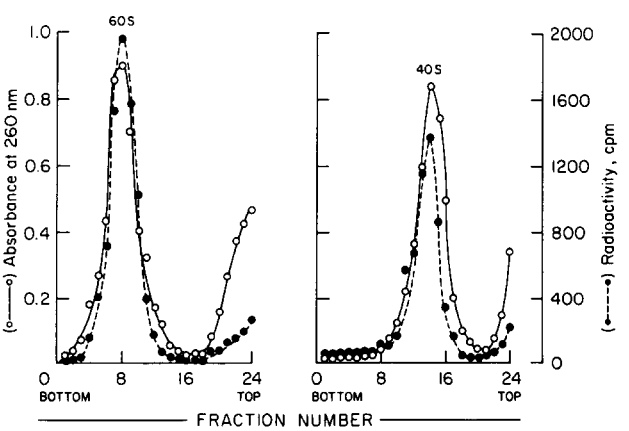

Fig. 2. Sucrose gradient analysis of phosphorylated 60-S and 40-S subunits. Phosphorylation was performed as described under Table I in the presence of cyclic AMP and incubation was carried out for 60 min. After incubation, the reaction mixture was cooled, adjusted to 0.3 M KCl with 1 M KCl and centrifuged in a 10—30% sucrose gradient in 10 mM Tris · HCl, pH 7.8, 300 mM KCl, 2 mM $MgCl_2$ and 0.5 mM β-mercaptoethanol. Centrifugation was carried out at 38 000 rev./min for 5 h at 5° C in a SW 50.1 rotor. Fractions (200 μl) were collected and hot trichloroacetic acid insoluble radioactivity and absorbancy at 260 nm were determined.

about 80% ^{32}P-labelled orthophosphate incorporated into ribosomes remain associated with the residual protein fraction. Incubation of residual proteins with 1 M NaOH at 37° C for 18 h released almost 90 percent of radioactivity into the medium as ^{32}P-labelled orthophosphate.

Effect of protein kinase II concentration on ribosomal protein phosphorylation. The incorporation of ^{32}P from [γ-^{32}P] ATP into ribosomal proteins with increasing enzyme concentration is shown in Fig. 3. The rate of incorporation was linear with increasing enzyme concentration up to 25 μg enzyme protein.

Effect of cyclic AMP concentration. The extent of phosphorylation was dependent on the concentration of cyclic AMP and maximum effect was observed at a cyclic AMP concentration of $5 \cdot 10^{-6}$ M (Fig. 4). Other nucleotides such as cyclic GMP, 5'-AMP and 5'-GMP were ineffective in this concentration range.

Effect of luteinizing hormone on the ribosomal protein phosphorylation. As luteinizing hormone was shown to stimulate the phosphorylation of histone in vitro [17], the effect of luteinizing hormone on the phosphorylation of ribosomal proteins by protein kinase II was tested. The results presented in Table II show that no stimulatory effect was observed when ribosomal proteins were used as substrate. Even at higher luteinizing hormone concentrations, there was no stimulation of ribosomal protein phosphorylation by protein kinase II (data not shown).

Polyacrylamide gel electrophoresis of ^{32}P-phosphorylated ribosomal proteins. Although we have observed that 80-S ribosomes, 60-S and 40-S ribosomal subunits were phosphorylated by protein kinase II without much substrate

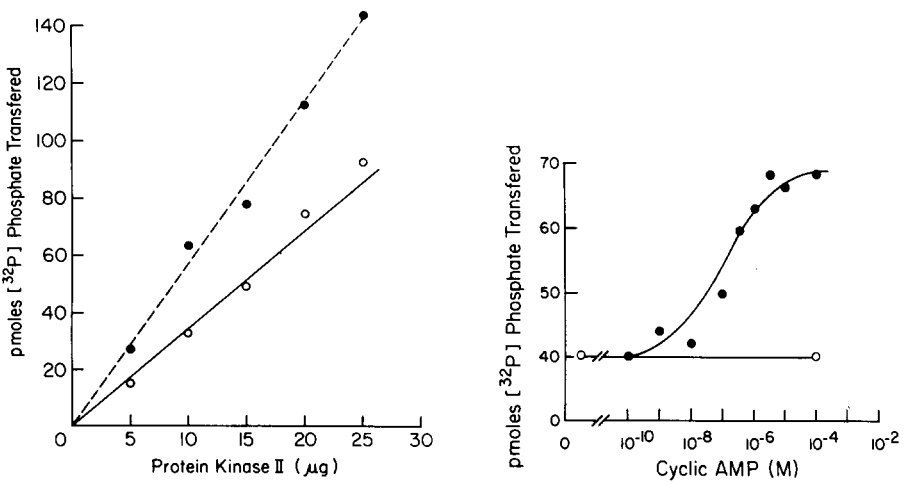

Fig. 3. Effect of protein kinase II concentration on the phosphorylation of 80-S ribosomes from bovine corpus luteum. Incubation medium was similar to that described in Table I except that the indicated concentrations of enzyme was included in the reaction mixtures. ○———○, without cyclic AMP; ●– – – –●, with cyclic AMP ($5 \cdot 10^{-6}$ M).

Fig. 4. Effect of cyclic AMP concentrations on the phosphorylation of 80-S ribosomes by protein kinase II. Incubation conditions were identical to Table I. ○———○, without cyclic AMP; ●———●, with cyclic AMP ($5 \cdot 10^{-6}$ M).

TABLE II

EFFECT OF LUTEINIZING HORMONE ON THE PHOSPHORYLATION OF RIBOSOMES AND HISTONES BY PROTEIN KINASE II

In Experiment 1, the incubation medium (0.2 ml) contained 3 nmol [γ-^{32}P] ATP ($8.9 \cdot 10^5$ cpm), 2 μmol KF, 0.5 μmol theophylline, 2 μmol $MgCl_2$, 10 μmol α-glycerophosphate, pH 6.0, 5 A_{260} units of ribosomes, 25 μg protein kinase II. In Experiment 2, ribosomes were replaced by 200 μg mixed histone. Other details are similar to those shown under Table I.

Additions	[^{32}P] phosphate transferred (pmol)
Experiment 1	
Ribosomes (80 S) + protein Kinase II (25 μg)	143
+ cyclic AMP (5 μM)	212
+ NIH-LH-B7 (5 μg)	110
+ NIH-LH-B7 (5 μg) + cyclic AMP (5 μM)	194
Experiment 2	
Histone + protein kinase II (25 μg)	68
+ cyclic AMP (5 μM)	394
+ NIH-LH-B7 (5 μg)	119
+ NIH-LH-B7 (5 μg) + cyclic AMP (1 μM)	308

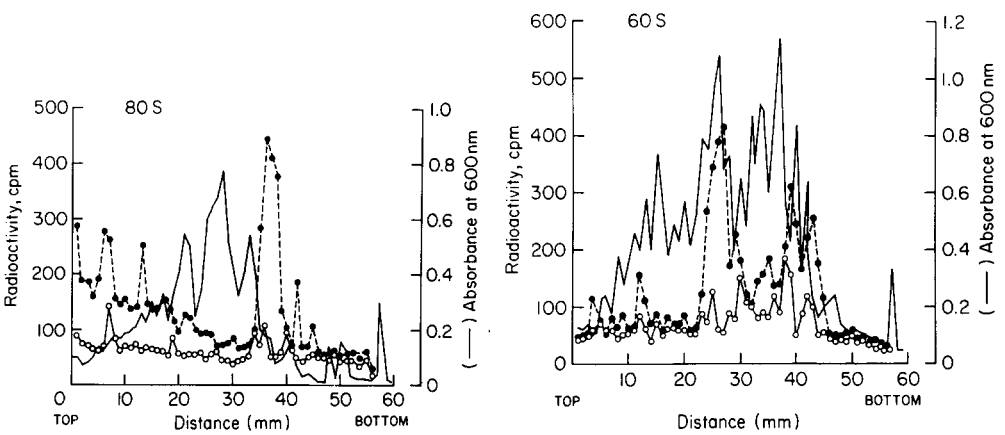

Fig. 5. Polyacrylamide gel electrophoresis of phosphorylated ribosomal proteins by protein kinase II. Phosphorylation was carried out in a final volume of 2 ml containing 50 mM glycerophosphate buffer, pH 6.0, 10 mM $MgCl_2$, 10 mM KF, 2.5 mM theophylline, $5 \cdot 10^{-6}$ M cyclic AMP, 250 μg protein kinase, 0.15 mM ATP ($3.8 \cdot 10^5$ cpm per nmol) and 65 A_{260} units of 80 S ribosomes. Incubation time was 60 min. After incubation, reaction mixtures were cooled and discontinuous sucrose gradient analysis was performed by layering 1 ml reaction mixture over 3 ml of 0.5 M sucrose in Buffer (10 mM Tris · HCl, pH 7.8, 2 mM $MgCl_2$, 0.5 mM β-mercaptoethanol) and centrifuged at 40 000 rev./min for 18 h at 4° C in SW 50.1 rotor. Protein extractions and gel electrophoresis were carried out as described under Experimental Procedures. To each gel 80 μg protein was applied. ○———○, without cyclic AMP; ●– – – –●, with cyclic AMP ($5 \cdot 10^{-6}$ M).

Fig. 6. Polyacrylamide gel electrophoresis of 60-S subunit ribosomal proteins phosphorylated by protein kinase II. A total of 55.0 A_{260} units of 60-S subunits were phosphorylated with 0.15 mM [γ-^{32}P] ATP ($4.2 \cdot 10^6$ cpm per mol) with 250 μg protein kinase in the incubation medium as described in Fig. 5. The time of incubation was 1.5 h. To each gel 100 μg protein was applied. Other details were identical to Fig. 5. ○———○, without cyclic AMP; ●– – – –●, with cyclic AMP ($5 \cdot 10^{-6}$ M).

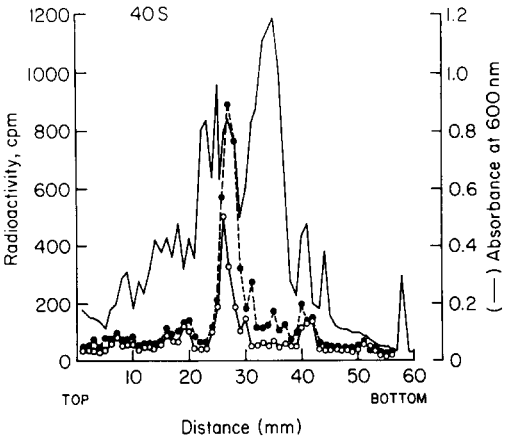

Fig. 7. Polyacrylamide gel electrophoresis of 40-S subunit ribosomal proteins after phosphorylation by protein kinase II. 60 A_{260} units of 40-S ribosomal subunits were phosphorylated with 0.15 mM [γ-^{32}P]-ATP [4.8 · 10^6 cpm per nmol) and 250 μg protein kinase as described in Fig. 5. Other details were the same as described under Fig. 5. To each gel, 110 μg protein was applied. ○———○, without cyclic AMP; ●———●, with cyclic AMP (5 · 10^{-6} M).

specificity, the effect of protein kinase II to differentially phosphorylate proteins in different subunits was investigated. To test this possibility, ribosomes (80 S) or ribosomal subunits were phosphorylated both in the presence and absence of cyclic AMP, the proteins were extracted and separated by electrophoresis on urea/polyacrylamide gels and finally radioactivity measurements were made. Radioactivity measurements made on the gels indicated that covalently bound radioactive phosphate migrated on electrophoresis with ribosomal proteins (Figs 5—7). We have been able to detect a quantitative difference in the pattern of the phosphorylated proteins of 80-S ribosomes and 60-S and 40-S subunits. One major band of 40-S and 11 distinct bands in 60-S

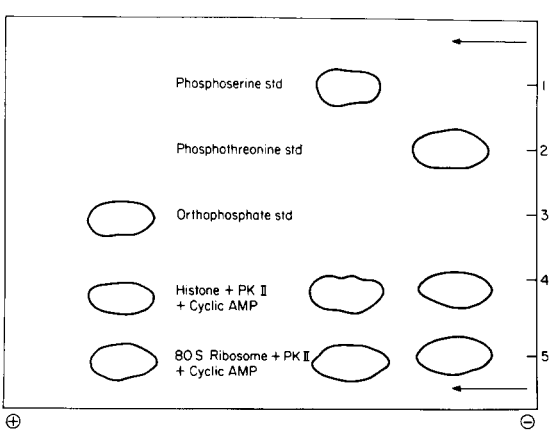

Fig. 8. Separation of phosphothreonine and phosphoserine from acid hydrolysates of phosphorylated ribosomal proteins by high voltage paper electrophoresis. Ribosomes were phosphorylated with [γ-^{32}P]-ATP in the presence of protein kinase II as shown under Table III. After removal of RNA by 10% trichloroacetic acid at 90° C for 30 min, the residue was hydrolyzed in 6 M HCl at 105° C for 5 h and high voltage paper electrophoresis was carried out as described in the text.

TABLE III

RELATIVE LABELLING OF PHOSPHOSERINE AND PHOSPHOTHREONINE RESIDUES OF RIBOSOMES, RIBOSOMAL SUBUNITS AND MIXED HISTONES BY PROTEIN KINASE II FROM BOVINE CORPUS LUTEUM

Ribosomes, ribosomal subunits or mixed histones were phosphorylated by 50 μg protein kinase II in the presence of $5 \cdot 10^{-6}$ M cyclic AMP as described under Table I and hydrolyzed under acidic conditions. The hydrolysates were subjected to electrophoresis on paper and the radioactive phosphoserine and phosphothreonine were determined by direct paper strip counting in a Beckman LS-230 Liquid Scintillation Spectrometer. The ratio is expressed as the radioactivity of phosphoserine or phosphothreonine. In these experiments, 10, 8 and 7.3 A_{260} units of 80-S, 60-S, 40-S ribosomes, respectively, were used.

Additions	cpm · 10^{-2}		Phosphoserine: phosphothreonine ratio
	Phosphoserine	Phosphothreonine	
80 S Ribosomes	132	4.6	28.6
60 S Ribosomal subunits	100	3.9	25.6
40 S Ribosomal subnits	67	3.0	22.3
Mixed histone (Type IIA) (100 μg)	194	6.0	32.3

subunits as separated on polyacrylamide gels were phosphorylated by protein kinase II and the incorporation of ^{32}P was increased by cyclic AMP. About 7—8 protein bands were phosphorylated when the 80-S ribosomes were the substrate.

Analysis of hydrolysates of phosphorylated ribosomal proteins by high voltage paper electrophoresis. Results presented in Fig. 8 show that acid hydrolysates of phosphorylated 80-S ribosomes, 40-S and 60-S ribosomal subunits contained radioactive materials which coelectrophoreted with phosphoserine and phosphothreonine residues. The hydrolysates also contained free ^{32}P-labelled orthophosphate which may arise from the hydrolysis of phosphate ester bonds [29]. The result presented in Table III shows the relative labelling and the ratio of phosphoserine to phosphothreonine residues. In all the instances phosphoserine residues were phosphorylated to a greater extent than phosphothreonine. However, a larger proportion of ^{32}P-labelled orthophosphate was incorporated into phosphothreonine residues of ribosomes and ribosomal subunit than histone.

Discussion

The present studies demonstrate that ribosomes and ribosomal subunits from bovine corpus luteum are phosphorylated by cyclic AMP dependent cytosol protein kinase II. The enzyme catalyzes the transfer of the terminal phosphate [γ-^{32}P] ATP into serine and threonine residues in a number of proteins in the 80-S ribosomes and 60-S and 40-S ribosomal subunits. Although cyclic AMP stimulated the above phosphorylation, under identical experimental conditions luteinizing hormone was ineffective. These results are in contrast to earlier observations [17] in which it was shown that luteinizing hormone stimulated the phosphorylation of histone by protein kinase II. These differences with respect to substrates are not currently understood.

The phosphorylation of eukaryotic ribosomal proteins in vitro by ribosomes associated protein kinase has been reported in several system [29,40—50] although it is not evident whether the observed phosphorylation represents phosphorylation of ribosomal proteins or the loosely attached proteins such as initiation and elongation factors. Recently Jergil and Ohlsson [51] have described the phosphorylation of proteins that are loosely attached to smooth and rough endoplasmic reticulum by associated kinases which can be released by mild salt treatment. In the present studies we have attempted the phosphorylation of salt-washed, stripped ribosomes and ribosomal subunits and also established the criteria necessary for phosphorylation of ribosomes [49]. That is, (a) the phosphate incorporated into the ribosomes survived repeated centrifugal washings of the particles in buffer containing high ionic strength which remove loosely attached proteins to ribosomes, (b) the phosphate was present covalently bound to serine and phosphothreonine residues in ribosomal proteins and (c) the covalently bound phosphate migrated with the ribosomal proteins on electrophoresis in polyacrylamide gels. Such criteria are particularly important for experiments under in vitro conditions where the possibility of ^{32}P contamination of ribosomal constituents other than proteins exists. It is also evident that a larger proportion of proteins which act as a substrate of protein kinase were integrated with ribosomal structure since they can not be solubilized by high ionic strength. Although the protein pattern of ribosomes and ribosomal subunits did not differ greatly, their phosphoprotein pattern showed interesting variations. Such differences in the properties of phosphorylation may be due to conformational differences and the differences in the metabolic functions between ribosomes and ribosomal subunits. Thus, phosphorylation and dephosphorylation reactions may be playing an important role in the association and dissociation of ribosomal subunits during the process of protein synthesis.

The role of cyclic AMP stimulated phosphorylation of ribosomes in bovine corpus luteum in luteinizing hormone-induced progesterone synthesis is not understood at the present time. Stansfield et al. [52] have recently postulated a role of protein kinase and phosphorylated proteins as possible regulatory factors in the luteal steroidogenesis by luteinizing hormone and cyclic AMP. The present cyclic AMP mediated phosphorylation of ribosomal proteins and involvement of a labile protein in luteinizing hormone-induced steroidogenesis [4] further support this concept.

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