PHOSPHORYLATION OF SOME RAT LIVER RIBOSOMAL PROTEINS AND ITS ACTIVATION BY CYCLIC AMP

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1. Introduction

It is well known that the phosphorylation of proteins can modify their biological activity. Furthermore, the phosphorylation of some enzymes can be activated by cyclic 3′, 5′-AMP [1]. Langan [2] has recently demonstrated that phosphorylation of histones is also stimulated by this nucleotide. When studying cellular localization of histone kinase in rat liver, we investigated the possibility that this enzyme can phosphorylate other basic proteins. As one of us has already observed that *microsomal* proteins of rat liver can incorporate phosphate groups *in vivo* [3], we investigated if *ribosomal* proteins can be phosphorylated. We have shown that some rat liver ribosomal proteins may be phosphorylated *in vivo* and *in vitro*, especially on serine residues and that this phosphorylation is stimulated *in vitro* by cyclic 3′, 5′-AMP at physiological doses in the range of 10^{-9} to 10^{-5} M. Half stimulation is produced at 5×10^{-8} M.

2. Material and methods

Sprague-Dawley or Wistar rats weighing 200–250 g were fasted overnight, injected intraperitoneally with 2 mCi of carrier-free ^{32}P-orthophosphate, and sacrificed by decapitation 90 min later. Ribosomes were isolated from pooled livers according to Littlefield et al. [4] purified by resuspension in 0.25 M sucrose, 10^{-3} M Mg^{2+} and precipitated by raising the Mg^{2+} concentration to 5×10^{-2} M. This operation was repeated twice.

Ribosomal proteins were extracted from ribosomes with 66% acetic acid [5], precipitated by one volume of 30% trichloroacetic acid (TCA), washed twice with 15% TCA, then redissolved in 6 M urea and dialyzed 18 hr against several changes of this medium.

Electrophoresis was performed in 7.5% mixed agarose acrylamide gel [6] at pH 4.5 in 6 M urea [7]. Autoradiography was made by applying a Kodirex film on the dry gel after pulverization with Omny-Spray (N.E.N.).

Rat liver microsomal supernatant was obtained by centrifugation of 0.25 M sucrose homogenate at 105,000 *g* for 90 min.

Determination of alkali-labile protein phosphate was performed on ribosomes treated by a modification of Schneider technique. After extraction of acid-soluble fraction by cold 15% TCA followed by two washings with this reagent containing 10^{-3} M ATP, phospholipids were extracted by alcohol-ether (3:1) for 10 min at 60°. Nucleic acids were extracted with 5% perchloric acid (PCA) at 95° for 15 min, followed by two washings with 5% PCA at 0°. The protein precipitate was dissolved in M NaOH (18 hr at 37°). In these conditions, phosphate esterified to serine and threonine is liberated as inorganic phosphate. After precipitation of proteins by PCA (final concentration 20%) inorganic phosphate was precipitated according to Delory technique [8], and dissolved in 0.3 ml concentrated sulfuric acid. After dilution with water, radioactivity was determined in a scintillation spectrometer (Packard) and phosphorus was measured by the Macheboeuf-Delsal [9] technique.

Phosphoserine was isolated by paper ionophoresis after mild acid hydrolysis of proteins, as previously described [10].

Proteins were determined by absorption at 230 nm

Table 1
Alkali-labile phosphorus content and labelling of ribosomal proteins *in vivo*

Exp.	μg phosphorus per mg protein	cpm per μg phosphorus
a	0.47	332
b	0.56	220
c	0.42	240

Each experiment was performed with pooled livers from three rats which were injected with 2 mCi of ^{32}P and sacrificed 90 min later.

and 260 nm or by Lowry technique; nucleic acids, by absorption at 260 nm.

Carrier free ^{32}P-orthophosphate and $\gamma^{32}P$-ATP were purchased from CEA, France.

3. Results

3.1. *In vivo phosphorylation*

Table 1 shows the content and the specific radioactivity of alkali-labile phosphate determined after alkaline hydrolysis. The mean value of the phosphorus content (0.5 μg per mg of ribosomal protein) is higher than that in the microsomes of rat liver (0.2 μg per mg of protein) and that in microsomal supernatant (0.13 μg per mg of protein) [3]. These phosphate groups can be bound to serine and threonine.

We have confirmed that some phosphate groups incorporated in ribosomal proteins are bound to serine residue: total proteins were submitted to mild acid hydrolysis and ionophoresis of hydrolysate followed by autoradiography showed the presence of radioactive phosphoserine and phosphopeptides (fig. 1).

Presence of phosphorus in ribosomal proteins was verified by the fact that after electrophoresis of these proteins followed by autoradiography, at least one radioactive band of ^{32}P appeared in the protein region (fig. 2). This radioactivity was not due to residual RNA or to nucleotides: ^{14}C-orotic acid (50 μCi) was injected to rats which were killed 18 hr later and ribosomal proteins extracted, as described above. When submitted to electrophoresis, no radioactivity was detected in the protein zone. The phosphorus so associated with ribosomal proteins can be bound to amino acids other than serine like histidine, glutamic acid.

3.2. *In vitro phosphorylation*

We have attempted to see if ribosomal proteins can be phosphorylated *in vitro* by a kinase present in the microsomal supernatant. When ribosomes were incubated with microsomal supernatant and $\gamma^{32}P$-ATP at various times, their proteins incorporated

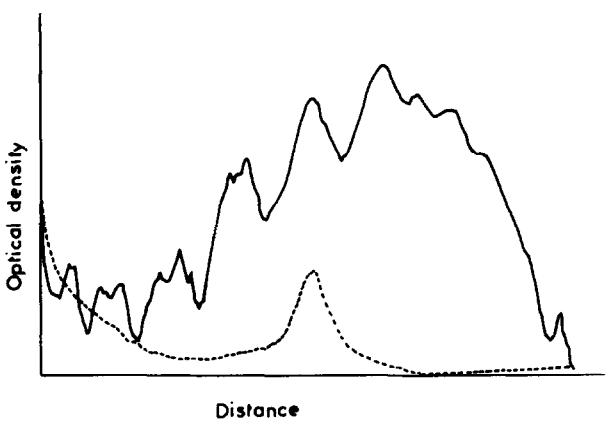

Fig. 1. Densitometry after electrophoresis and autoradiography of ribosomal proteins. ——— amidoblack; - - - - - - - - radioactivity.

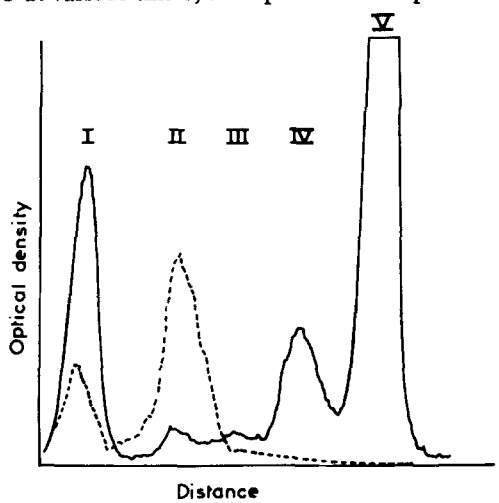

Fig. 2. Densitometry after ionophoresis of acid hydrolysate of ribosomal proteins. - - - - - - - - ninhydrin; ——— radioactivity. I, III, IV: phosphopeptide; II, phosphoserine; V, P_i.

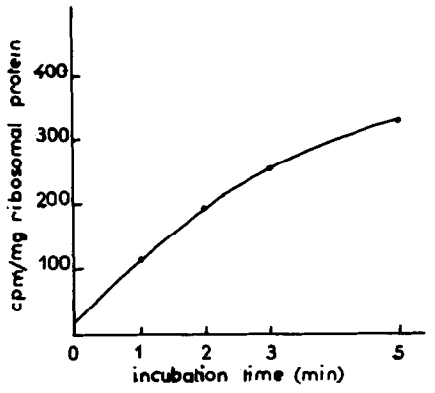

Fig. 3. Phosphorylation of ribosomal proteins by γ^{32}P-ATP and microsomal supernatant. For a final volume of 1 ml incubation mixture contained tris buffer at pH 7.5 40 μmoles; $MgCl_2$ 5 μmoles; dithiothreitol, 1 μmole; γ^{32}P-ATP 0.1 μmole ($2 \times 1\ 10^6$ cpm); microsomal supernatant 100 μg of proteins; ribosomes 2 mg of proteins. Incubation time was from 1 to 5 min at 37°. Reaction was stopped with 1 ml cold 30% TCA. After centrifugation, ribosomal proteins were extracted from the precipitate with 2 ml of 66% acetic acid, before being treated for alkali-labile ^{32}P determination.

alkali-labile phosphorus (fig. 3). In this experiment reaction was stopped by TCA, ribosomal proteins were extracted by 66% acetic acid then treated for determination of alkali-labile ^{32}P radioactivity. The same quantity of supernatant incubated with yeast RNA (1 mg/ml) as a carrier and treated in the same conditions, showed practically no radioactivity.

We have also examined if cyclic AMP stimulates this incorporation. Fig. 4 shows the results obtained when ribosomes were incubated with microsomal supernatant in presence of increased concentrations of cyclic AMP. In this experiment, the ribosomes were precipitated from the incubation medium by raising the concentration of Mg^{2+} to 10^{-1} M, then treated for determination of alkali-labile ^{32}P radioactivity. Cyclic AMP produced a 3-fold increase in this phosphorylation. Half-maximum stimulation of ribosomal protein phosphorylation was produced by approximately 5×10^{-8} M cyclic AMP. We have observed that the weak endogenous phosphorylation of the microsomal supernatant was pactically not stimulated by cyclic AMP. Similar results were obtained with purified ribosomal proteins in place of ribosomes (to be published).

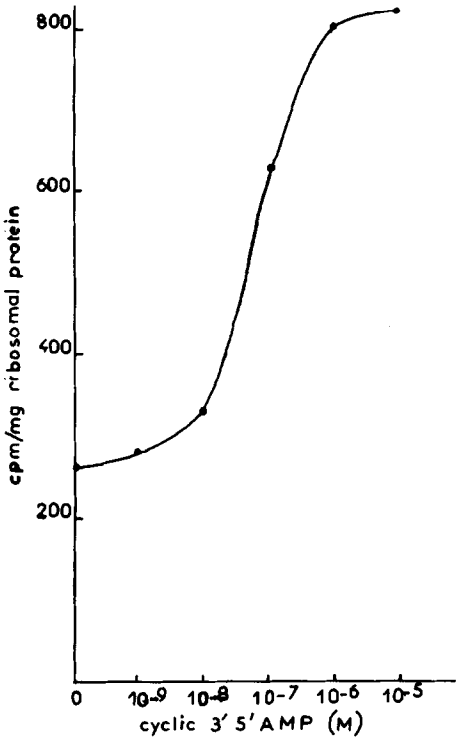

Fig. 4. Stimulation of *in vitro* protein phosphorylation by cyclic 3′, 5′-AMP. Same incubation mixture as for fig. 3 was used, plus 1 μmole theophylline and various concentrations of cyclic AMP were added. After 3 min of incubation at 37°, ribosomes were precipitated by addition of 0.1 ml of M $MgCl_2$ and were extracted for alkali-labile ^{32}P as indicated.

4. Discussion

We have shown that some ribosomal proteins of rat liver can incorporate phosphate on serine residues *in vivo*. They are also phosphorylated *in vitro* by ATP and a kinase present in rat liver microsomal supernatant. Although phosphorylation of microsomal proteins in some tissues has already been reported, to our knowledge, it is the first time that phosphorylation of ribosomal proteins has been shown.

Ribosomal proteins play an important role in the mechanism of ribosome functioning. It may be possible

that phosphorylation of some residues of ribosomal proteins can change their enzymatic activity or binding with other macromolecules. The isolation of ribosomal proteins which are phosphorylated would help to elucidate these possible mechanisms.

We have also shown that this phosphorylation is activated *in vitro* by physiological doses of cyclic AMP. There are some indications that cyclic AMP can intervene in mammalian tissues at the translational level [11–15]. A possible action of this hormonal effector could be the stimulation of phosphorylation of some ribosomal proteins.

Acknowledgments

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