RIBOSOME-ASSOCIATED PROTEIN KINASE FROM *SACCHAROMYCES CEREVISIAE*

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1. Introduction

Phosphorylation of animal ribosomal proteins has been demonstrated in vivo in rat liver [1], rabbit reticulocytes [2,3], tumor cells [4] as well as in vitro in the presence of [γ-^{32}P]ATP [5–9]. Incorporated phosphate has been found in ester linkages with serine or threonine residues [1,2,10]. The phosphorylation rection is catalyzed by protein kinases present in ribosome-free supernatant, high-salt ribosomal wash or on ribosomes [2,8,10–12]. Some of the kinases are stimulated by cyclic AMP. ATP serves as a phosphate donor, except with one of the kinases from rabbit reticulocytes and rat liver which uses GTP for phosphorylation of some ribosomal proteins [10,13].

We present evidence that yeast cells contain at least two distinct protein kinases; a ribosome-associated kinase and one found in the ribosome-free supernatant.

2. Material and methods

2.1. *Yeast culture*

Saccharomyces cerevisiae, strain AS, was cultured as described by Cooper et al. [14]. Yeast cells were grown aerobically and were harvested at log growth phase, centrifuged and washed twice with a standard buffer (50 mM Tris–HCl pH 7.5, 10 mM KCl, 10 mM Mg^{2+} and 6 mM mercaptoethanol). Packed cells were used immediately or stored frozen at −15°C before use.

2.2. *Preparation of ribosomes*

Cell disruption was accomplished by grinding the cells in a prechilled mortar either with three times their weight of acid-washed corundum or with twice their weight of alumina. After grinding the paste was extracted with twice the weight of standard buffer relative to the cell mass. The suspension was brought to pH 7.5 with 1.0 M Tris and was then centrifuged at 3000 rpm for 10 min to remove abrasives and cell debris and next at 30 000 *g* for 20 min. The supernatant was centrifuged for 2 hr at 150 000 *g*. The ribosomal pellets were suspended in a half volume of a standard buffer, relative to the volume of original extract, by homogenization in a Teflon-glass homogenizer. Aggregates were removed by centrifugation at 10 000 *g* for 20 min. Triton X-100 was added to the supernatant, with continuous stirring, to 1% final concentration and after 30 min additional stirring the ribosomes were pelleted for 2 hr at 150 000 *g*. These Triton X-100-treated ribosomes were in some cases additionally purified by twice washing with high ionic strength buffur (50 mM Tris–HCl, pH 7.5, 0.5 M KCl, 50 mM Mg^{2+} and 6 mM mercaptoethanol) followed by ultracentrifugation. Each time any insoluble material was removed by low speed centrifugation. Purified ribosomes were completely free of endogenous mRNA and transfer factors but exhibited very high activity in the presence of poly U and partly purified transferases [15].

2.3. *Protein kinase preparations*

Ribosome-associated protein kinase was isolated by a slightly modified procedure of Jergil [8]. Triton X-100-treated ribosomes were extracted twice with buffered 0.5 M KCl, supernatants were pooled and proteins fractionated with ammonium sulphate and then on an hydroxyapatite column. For protein elution a step-wise gradient of ammonium phosphate, pH 7.8, containing 6 mM mercaptoethanol and 10% glycerol was used. The fractions eluted with 0.3 M am-

monium phosphate contained the enzyme activity.

The protein kinase preparation from 150 000 *g* supernatant was obtained by fractionation with ammonium sulphate. The precipitate from 30–70% sat. ammonium sulphate was dissolved in a small volume of Tris–HCl buffer, pH 7.5, containing 6 mM mercaptoethanol and 10% glycerol. The protein solution was thoroughly dialyzed against the same buffer and applied onto a Whatman DE-52 column. The proteins were fractionated using a linear gradient of Tris–HCl by the procedure of Reimann et al. [16]. Details relating to the purification of both kinase preparations and some data on the activity of phosphorylated ribosomes in protein synthesis in vitro will be published elsewhere [17].

2.4. *Assay of in vitro phosphorylation of protein*

Incubation mixture contained: 20 mM Tris–HCl, pH 7.5, 10 mM Mg^{2+}, 6 mM mercaptoethanol, 0.1–0.5 mg of ribosomes (dialyzed previously against standard buffer), or another protein substrate (see legends to the figures and tables), 5–15 μg protein phosphokinase preparation and 20 nmoles of [γ-^{32}P] ATP, spec.act. 3–12 × 10^4 cpm/nmole. The samples in a total volume of 0.5 ml were incubated at 30°C for 10 min, unless otherwise stated. The reaction was stopped by the addition of 3 ml of 10% trichloroacetic acid and the samples heated for 20 min at 90–95°C. Protein precipitates were collected by centrifugation, suspended in 5% trichloroacetic acid, filtered onto 0.45 μM Millipore filters or glass-fiber filters and thoroughly washed with 5% trichloroacetic acid and then with a methanol–chloroform mixture (3:1 v/v) [18]. Radioactivity was measured at a window G-M counter.

2.5. *Chemicals*

[γ-^{32}P] ATP was from Amersham (Searle) spec.act. 13–16 Ci/mmol, Triton X-100 and cyclic AMP from Serva, Feinbiochemica, N^6,2′-*O*-dibutyryl cyclic AMP from Schuchardt. Histones, types IIA and III were from Sigma and lysinerich and arginine-rich histones were supplied by POCH, Gliwice, Poland. Hydroxyapatite was from Bio-Rad Laboratories and DEAE-cellulose (DE-52) from Whatman.

3. Results and discussion

Fig. 1 shows the phosphorylation of ribosomal proteins by endogenous ribosomal protein kinase. As can be seen Triton X-100-treated ribosomes (open circles) exhibit higher phosphorylation than a crude ribosomal preparation (closed circles). This may be due to the unmasking of the kinase or substrate protein by the detergent treatment. On the other hand purified ribosomes (open squares) are only slightly phosphorylated by endogenous enzyme. This might mean that the washing of ribosomes removes the ribosome-associated protein kinase.

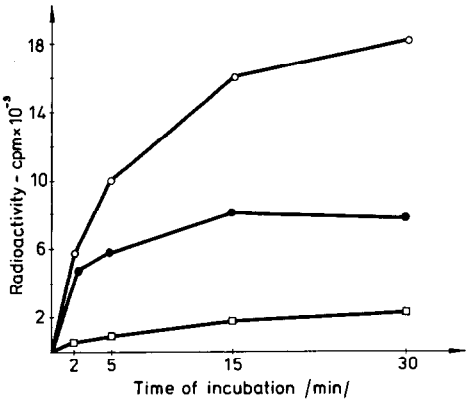

Fig. 1. Activity of ribosome-associated protein kinase of various ribosomal preparations. Each sample contained 0.5 mg ribosomes. Zero time incorporations have been subtracted. (○—○—○) Triton X-100-treated ribosomes; (●—●—●) crude ribosomes; (□—□—□) twice washed ribosomes.

The enzyme is rather firmly bound to ribosomes and cannot be removed by extraction with low concentrations of buffered KCl (table 1). These findings are consistent with the observations of Jergil [8] and Fontana et al. [11] who have also shown ribosome-bound kinases in trout testis and reticulocytes ribosomes respectively.

The kinase activity can be recovered from yeast ribosomal wash. A partly purified kinase preparation has been obtained by us using ammonium sulphate fractionation of ribosomal wash proteins and hydroxyapatite column chromatography according to Jergil [8]. We have also succeeded [17] in isolating the kinase activity from ribosome-free supernatant following the slightly modified procedure of Reimann et al. [16]. Comparison of both kinase activities has shown that they differ in several respects. Ribosome-associated kinase preferentially phosphorylates ribo-

Table 1
Protein kinase activity of ribosomal preparations washed with different concentrations of KCl

Ribosomal preparation	Incorporation of ^{32}P into ribosomal proteins, counts/min.
Triton X-100 treated ribosomes – control	2990
Ribosomes washed with 0.2 M KCl	2440
Ribosomes washed with 0.3 M KCl	1250
Ribosomes washed with 0.4 M KCl	700
Ribosomes washed with 0.5 M KCl	600

5–10 mg portions of Triton X-100-treated ribosomes were washed with buffered KCl of different concentrations. The pellets obtained after centrifuging for 2 hr at 150 000 *g* were suspended in standard buffer and dialyzed overnight against the same buffer. 100 μg of each ribosome preparation was tested separately. Incubation, 10 minutes at 30°C, and assay as described under Materials and methods.

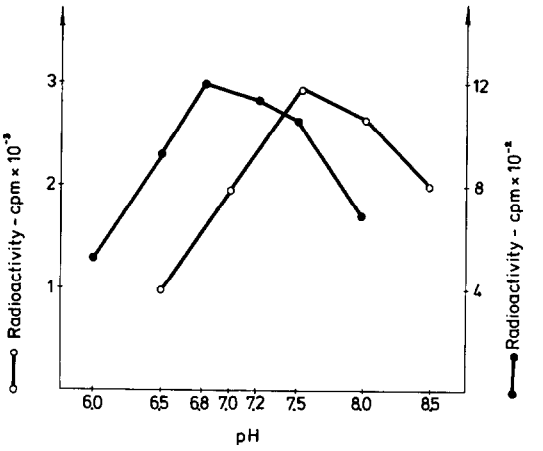

Fig. 2. Effect of pH on activity of protein kinases. Buffers used for incubation: pH range: 6.0 to 7.0–10 mM Tris–maleate; 7.2 to 8.5–20 mM Tris–HCl. 200 μg of purified ribosomes and 100 μg histone type IIA were phosphorylated by 10 μg hydroxylapatite fraction of ribosome-associated kinase and 15 μg of supernatant kinase respectively. (○—○—○) ribosome–associated enzyme; (●—●—●) supernatant enzyme.

Table 2
Substrate specificity of protein kinase preparations

Substrate	^{32}P incorporated, pmol/mg protein	
	ribosome-bound kinase	supernatant kinase
Ribosomal proteins	213	9
Histone type IIA	5	30
Histone type III	22	8
Lysine-rich histone	42	12
Arginine-rich histone	3	24

Incubation mixture contained: 100 μg of each protein substrate, 7 μg of hydroxyapatite fraction of ribosome-associated kinase or 15 μg of supernatant kinase (DE-52 eluate), 10 nmoles of [γ-^{32}P]ATP spec. act. 190 cpm/pmole. Incubation, 15 min at optimal pH for each kinase. All other details as under Material and methods.

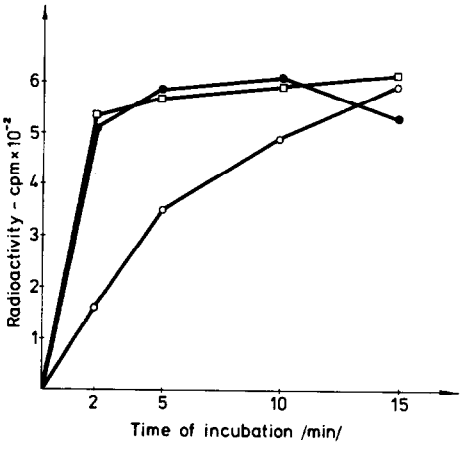

Fig. 3. The effect of cAMP and dibutyryl-cAMP on activity of supernatant protein kinase. The samples were incubated as described under Materials and methods in the absence of cAMP (○—○—○) or in the presence of 10^{-6} M cAMP (●—●—●) and 10^{-6} M dibutyryl-cAMP (□—□—□). Zero time incorporations have been substracted.

somal proteins (table 2) whereas supernatant enzyme phosphorylates histone type IIA. The highly ribosome-protein-specific kinase, observed first in our laboratory, may play a specific role in the modification of ribosomes. Fig. 2 shows that the pH optima are different for the two enzyme preparations.

It appears, however, that the optimal concentration of Mg^{2+} and the requirement for SH compounds (data not shown) are the same for both enzymes.

The supernatant enzyme appears to contain a regulatory site. After a short incubation time, the stimulatory effect of cAMP is readily observable (fig. 3). On the other hand ribosome-associated kinase is cAMP-independent. Using different concentrations of cAMP and dibutyryl-cAMP we have not been able to show any effect of these nucleotides on the phosphorylating activity, of the latter enzyme. It is worth noting that, in contrast to the supernatant enzyme, the ribosome-bound enzyme is extremely labile even on storage in a deep freeze and in the presence of 50% glycerol.

These observations suggest that *Saccharomyces cerevisiae* cells contain two distinct protein kinase activities and one of them seems to be highly ribosome-protein-specific.

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