Synthesis of diadenosine-5′,5‴-p¹,p³-triphosphate in yeast at heat shock

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Heat shock leads to the accumulation of diadenosine-5′,5‴-p¹,p³-triphosphate (ApppA) in *Saccharomyces cerevisiae*. Its intracellular concentration increases up to 30 μM after 45-min incubation at 41°C. I propose that ApppA can be an inducer of heat shock proteins.

Heat shock *Protein synthesis* *Alarmone* *Yeast*

1. INTRODUCTION

Heat shock is the universal protective response of pro- and eukaryotic cells to a temperature rise [2,4]. At heat shock a small set of proteins (heat shock proteins, hsps) are synthesized in cells and this is accompanied by inactivation of synthesis of other cell proteins. The production of hsps is considered to be controlled at the level of transcription and translation [3]. However, the mechanism of this control is unclear. It has been found that many chemical agents induce heat shock response [2,4].

Recently authors in [1] have shown that synthesis of hsps in *E. coli* and *S. typhimurium* at heat shock is accompanied by accumulation of adenylated nucleotides ('alarmones'). High temperature as well as some chemical heat shock inducers caused accumulation of the same set of alarmones. The authors [1] proposed that these adenylated nucleotides are the inducers of the hsps. Synthesis of alarmones in eukaryotic cells at heat shock was not shown.

We have found that ApppA is accumulated in *S. cerevisiae* during heat shock. We suggest that AppppA can be an inducer of hsps.

2. MATERIALS AND METHODS

2.1. *Cultivation, labeling and extraction of cells*

S. cerevisiae wild-type strain was used. 0.2 g of frozen yeast were suspended in 0.5 l of medium containing 0.5% peptone, 0.3% yeast extract and 1% glucose. The culture was incubated with aeration overnight at 25°C. Then 12 ml of the culture were transferred to 0.5 l fresh medium and incubation was continued for 8 h. At this time the cells reach the lag phase of growth. The suspension was centrifuged for 8 min at 1000 × *g* and the pellet was washed once in a saline containing 140 mM NaCl, 5 mM KCl, 5 mM $MgCl_2$ and 0.5% glucose. The final pellet was suspended in 15 ml of the same saline. Neutralized $H_3{}^{32}PO_4$ (1000–5000 Ci/mmol, Isotope, USSR) was added to 200 μCi/ml for labeling. After 1.5 h preincubation at 25°C, the culture was divided into two parts. The first one was incubated at 25°C and the second at 41°C for 45 min. After this, the cultures were immediately cooled. Extraction of cells with formic acid was performed as in [5].

For in vivo protein labeling, 1 μl [^{35}S]methionine (1000 Ci/mmol, 12.5 μCi/μl, Amersham, England) was added to a 100 μl aliquot of the culture. After 15 min incubation at the required temperature, the aliquot was cooled. Samples for electrophoresis were prepared as in [10].

2.2. *TLC and autoradiography*

Two-dimensional TLC on PEI-cellulose was performed as in [6].

An autoradiogram was made by taping the

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chromatogram or the electropherogram to a similar sized sheet of PM-B X-ray film (Tasma, Kazan, USSR) and exposing it at −70°C. After a sufficient period of exposure the film was developed by standard procedures. To quantitate the amount of radioactivity at a given spot, it was circled with a soft pencil, cut out, placed in a vial and counted in a Beckman LS 100C scintillation counter.

2.3. *Identification of the spot*

The spot was extracted from the chromatograms as in [5]. The extract was treated with periodate or snake venom phosphodiesterase as in [5].

2.4. *Electrophoresis of proteins*

The samples were electrophoresed on an 10–20% acrylamide gradient gel as in [9] with some modifications.

3. RESULTS

3.1. *Induction of hsps in yeast*

Shifting *S. cerevisiae* from 25 to 41°C resulted in synthesis of hsps (fig.1, see also [7,8]).

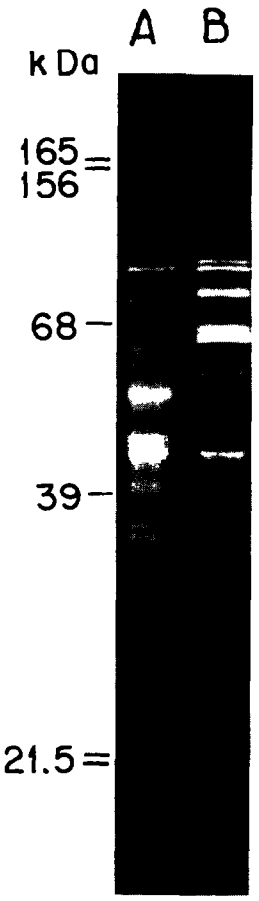

Fig.1. Pulse-labeled yeast proteins. Cells were incubated with [^{35}S]methionine for 15 min at 25°C (A) or cells were incubated for 45 min at 41°C and then labeled for 15 min at 41°C (B). The cells were disrupted and the cytoplasmic proteins were electrophoresed on a 10–20% gradient acrylamide gel (each slot contained an equal amount of proteins). The gel was dried and autoradiographed.

3.2. *Phosphorylated compound accumulates during heat shock*

We used the method of two-dimensional TLC on PEI-cellulose as in [6]. The compounds are separated according to their phosphate content in the first dimension and to their base content in the second dimension. Assuming the intracellular concentration of ATP to be 3 mM, we can detect phosphorylated metabolites with intracellular concentrations down to 5 μM (normalized to phosphorus).

A standard resolution of some metabolites by the two-dimensional system is shown in fig.2A. The set of ^{32}P-labeled cellular compounds is shown in fig.2B. Heat shock leads to the appearance of an additional spot (fig.2C) (heat shock spot, hss). After 45 min incubation at 41°C, its intracellular concentration rises to 100 μM (normalized to phosphorus). We did not detect hss in normal cells.

3.3. *Identification of the hss*

In the first dimension hss migrates close to ADP. This means that both compounds possess a similar net negative charge, i.e., −3. The authors who developed this two-dimensional TLC [6] have shown that ApppA comigrates with ADP in the first dimension while in the second dimension it migrates slower than ADP. On these grounds, we suggest that hss might be ApppA. To verify its identity, hss was purified and digested by venom phosphodiesterase. The results are shown in fig.3. It is seen that 5′-ADP and 5′-AMP are the products of partial digestion while 5′-AMP and P_i are the products of complete digestion. hss is sensitive to the periodate treatment (fig.4).

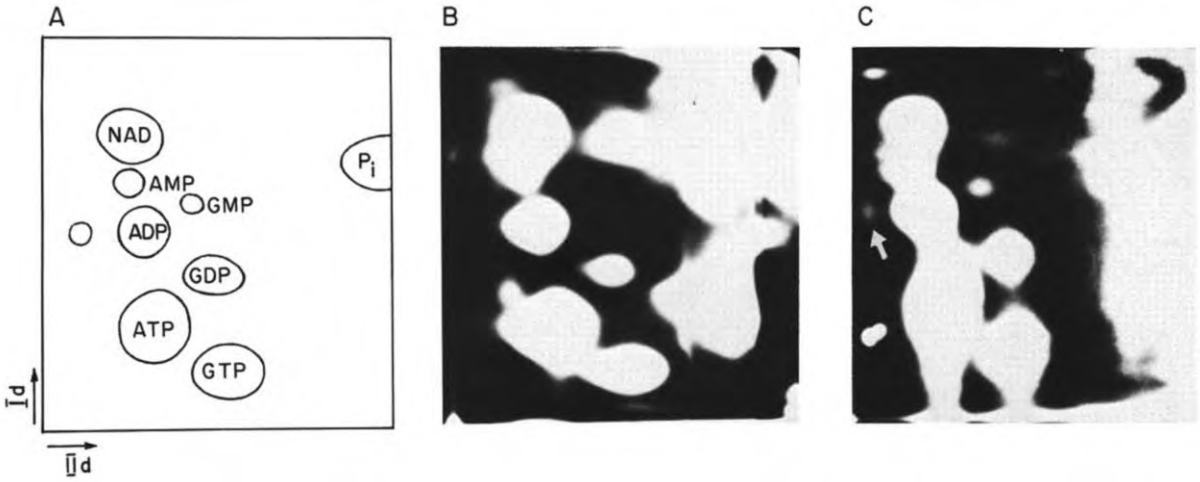

Fig.2. Accumulation of the phosphorylated compound in yeast at heat shock. (A) Standard separation of some cellular metabolites on two-dimensional TLC. (B,C) Autoradiograms exposed from two-dimensional separation of a ^{32}P-labeled extract of yeast after incubation at 25°C (B) and at 41°C during 45 min (C). The arrow indicates the appearance of an induced phosphorylated metabolite.

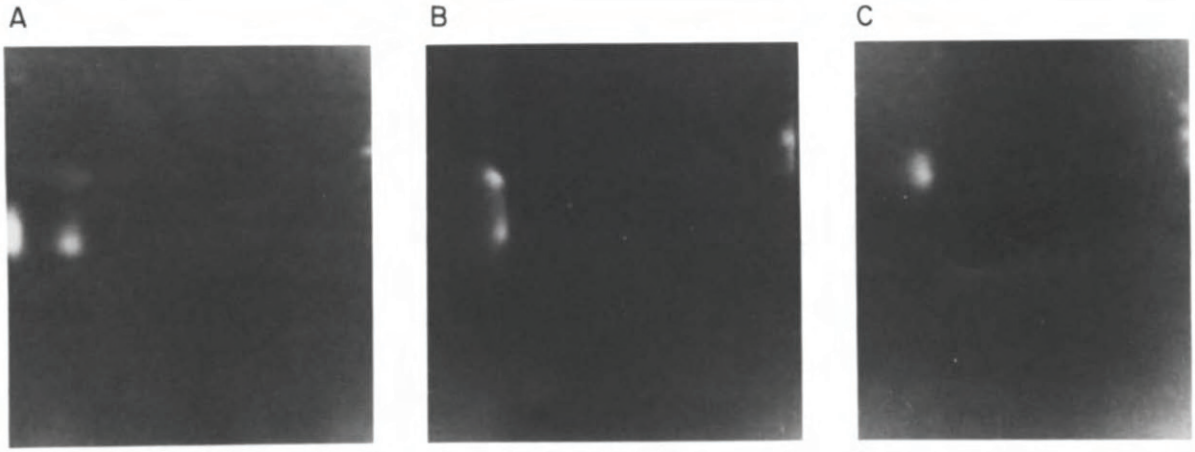

Fig.3. Two-dimensional resolution of venom phosphodiesterase digestion of hss. The mixture was: 10 μl extracted spot, 0.5 μl enzyme (Koch-Light, 10 μg/ml), 0.5 μl of 200 mM $MgCl_2$. After incubation at 37°C for 0.5 h (A), 4 h (B) and 20 h (C), 3-μl aliquots were separated by two-dimensional TLC and autoradiographed.

These facts support our suggestion. We can now calculate that after 45 min of heat shock the intracellular concentration of ApppA in yeast rises to 30 μM.

4. DISCUSSION

We have shown that in addition to the induction of hsps, heat shock causes the accumulation of ApppA in yeast. Some questions arise. Firstly, which enzyme systems produce ApppA? Secondly, is there any relation between ApppA and the induction of hsps?

Authors in [11] showed the synthesis of AppppA in a system containing lys-aminoacyl tRNA synthetase (from *E. coli*), Mg_2^+, L-lysine. In this reaction, ATP displaces the aminoacyl moiety of aapA to form AppppA. Other ribonucleoside or

Fig.4. Two-dimensional resolution of periodate treatment of hss. ^{32}P-labeled extract of heat-shocked cells was treated with periodate and urea as in [5]. 5′-Phosphorylated ribonucleotides were destroyed by this treatment; their $^{32}P_i$ was converted primarily to products which run off in the margin of the chromatogram. Compare this chromatogram with the chromatogram of untreated extract (fig.3C).

deoxyribonucleoside di- and triphosphates can also perform this displacement [12]. Not all aminoacyl synthetases form AppppA [12,13].

AppppA and ApppA were found in living cells. Their concentrations were low, 1–0.01 μM [12,15].

AppppA is a pleiotropic metabolic effector. AppppA stimulates DNA-polymerase α and can act as a primer for the nascent DNA chain [12]. AppppA inhibits the activity of terminal deoxynucleotidyl transferase and protein kinase pp60^{src} [12]. AppppA in vitro can serve as a primer for (2′–5′)-oligoadenylate synthetase [12]. The metabolic action of ApppA has been studied to a lesser extent. ApppA inhibits the stimulating effect of AppppA on DNA synthesis [14]. ApppA accumulated in human platelets is released during aggregation [15]. No other adenylated nucleotide functions are known.

Authors in [1] have shown that heat shock induces accumulation of ApppA, AppppA, ApppG, AppppG and ApppGpp. This accumulation did not depend on the synthesis of hsps. The authors [1] suggest that these adenylated nucleotides alert the cell to the onset of certain metabolic stresses and so can induce synthesis of hsps. Only ApppA is synthesized in yeast during heat shock. This fact simplifies the detection of the hsp inducer among alarmones.

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