AN INHIBITOR OF POLYPHENYLALANINE SYNTHESIS IN t-RNA PREPARATIONS OF SACCHAROMYCES CEREVISIAE

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Summary: An inhibitor for polyphenylalanine synthesis in a cell free system of E. coli is present in crude yeast t-RNA preparations. Its amount tends to increase during starvation and at the stationary phase of growth. Separation of the inhibitory fraction from t-RNA in preparations from starved cells was achieved by chromatography on MAK columns. Some properties of this inhibitory fraction are described.

The ability of high concentrations of yeast t-RNA to inhibit amino acid incorporation in yeast cell free systems has been reported by several authors.[1-2] No explanation was given for this phenomenon.

During our work on the fractionation of RNA from growing and nitrogen starved yeast cells, by zone centrifugation in sucrose concentration gradients, we found an increase in the low molecular weight fraction (4S) in crude preparations of RNA from starved cells.[3] The 4S fraction from starved cells had a significantly higher inhibitory effect on the poly U directed polyphenylalanine synthesis in a cell free system from E. coli than the corresponding fractions from logarithmically growing cells. An attempt has been made to elucidate the nature of this inhibition. In this communication the results of these studies are reported.

Materials and Methods

The strain of Saccharomyces cerevisiae used in these experiments was kindly supplied by Anheuser-Busch, Inc. This strain is used by them for the commercial production of bakers yeast. Cultures were grown in yeast carbon base medium (Diflo manual, 252), supplemented with 0.15% asparagine and 0.35% $(NH_4)_2SO_4$ at 30°C with aeration. To achieve nitrogen starved cells, cultures were harvested at the logarithmic phase of growth, washed with saline, suspended in unsupplemented yeast carbon base medium and incubated at 30°C with aeration under the same conditions.

Preparation of RNA

a. Bulk yeast RNA. 1 gm of packed cells was suspended in 3 ml of Crestfield solution [4] to which 10% mercaptoethanol was added and heated for 10 min at 78°C. RNA was extracted with phenol according to the method of Kirby. [5]

b. t-RNA from whole cells was prepared according to the method of Monier et al. [6] without the step of purification.

c. Cells were ground with alumina in 0.001 M tris buffer, pH 7.5; 0.01 M $MgCl_2$. RNA was prepared from washed ribosomes sedimented twice at 105,000 x g and from 105,000 x g supernatant by the method of Kirby. [5] All the preparations were purified by repeated phenolic extractions and alcohol precipitations.

Fractionation of RNA preparations was carried out on methylated albumin kieselguhn (MAK) columns according to Sueoka and Cheng. [7] Quantitative estimation of RNA was made by the orcinol method. Protein was estimated by the method of Lowry et al. [8] Polysaccharides were detected by the phenol method of Dubois et al. [9] RNase digestion was carried out with the aid of an insoluble enzyme kindly supplied by Dr. A. Riesel. [10] Pronase treatment was performed in 0.001 M tris buffer, pH 7.5, 0.01 M $MgCl_2$ at 37°C for 7 hours. After phenolic extractions and alcohol precipitation the precipitate was dissolved in water, dialyzed overnight and lyophilized. Assays of polyphenylalanine polymerization were performed as described by Littauer and Milbauer. [13]

Results

Crude yeast t-RNA preparations were found to inhibit the formation of polyphenylalanine in a cell free system of E. coli saturated with E. coli t-RNA. Preparations from nitrogen starved or stationary state cells showed a higher inhibitory activity than those prepared from logarithmically growing ones (see Table I). This inhibitory activity was independent of the method used for the preparation of the t-RNA. The possibility that the inhibitory activity might be due to contamination by RNase could be excluded since the samples used contained less than 10^{-7} µg RNase as tested with tritiated bulk E. coli RNA. [11] RNase in this concentration did not interfere with the synthetic activity of our system. The suggestion that peptidyl t-RNA may accumulate during the cessation of growth and be the interfering substance, was also considered. However, incubation of t-RNA preparations in 0.2 M glycine buffer pH 10.3 for 3 hours at 37°C did not change their activity. Purification of the RNA preparations by extraction with 2-methoxyethanol [5] or treatment with

Table I

The inhibitory effect of crude yeast t-RNA preparations on poly U directed polyphenylalanine synthesis in a cell free system of E. coli saturated with t-RNA.

Exp.	System	Yeast t-RNA added (A_{260} mμ)	C. P. M.	% Inhibition
I	Complete	-	24,988	
	" + log t-RNA	0.75	18.208	28
	+ log t-RNA	1.5	10.271	41
II	Complete	-	20.846	
	" + log t-RNA	0.42	11.868	43
	" + log t-RNA	0.84	5.590	73
III	Complete	-	22.016	
	" + starved t-RNA	0.24	7.184	68
IV	Complete	-	20.652	
	" + starved t-RNA	0.32	1.512	93
V	Complete	-	23.290	
	" + stationary t-RNA	0.2	7.290	69
	" "	0.4	4.300	81

The reaction mixture for polyphenylalanine synthesis contained (in 0.1 ml) 1.75 μmoles $MgCl_2$, 17.9 μmoles NH_4Ac, 5 μmoles Tris buffer pH 7.5, 36 mμ moles ATP, 15 mμ moles GTP, 250 mμ moles NH_4-PEP, 5 μg PK, 0.33 mμ moles (C^{14})-L-phenylalanine (300 μc/μM), 90 mμ moles mercaptoethanol, 10 μg pyromycin treated ribosomes of E. coli plus the amounts of yeast tRNA shown above 10 μg t-RNA, 4.5 μg PU and 45 μg protein E. coli BC streptomycin supernatant. The samples were reincubated at 30°C for 60 min. The reaction was stopped by addition of 5% TCA and treated as mentioned in Methods.

cetyl trimethyl ammonium bromide [12] caused a considerable decrease in the specific inhibitory activity.

The RNA was further fractionated on MAK columns. The difference in composition of bulk RNA from starved and log phase cells is shown in Fig. 1. In log phase cell preparations the fraction which emerges from the column with the front, peak 1, contains negligible amounts of materials which absorb at 260 mμ and it consists almost completely of low molecular weight dialysable components. In starved as well as in stationary state cells peak 1 is considerably increased and

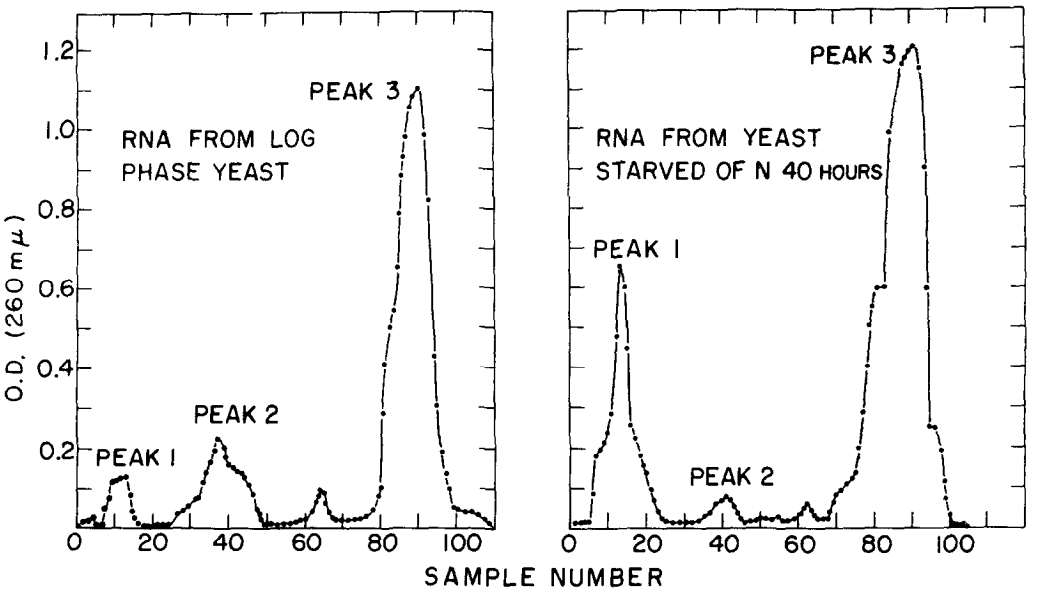

FIG. 1. Analysis of crude yeast RNA on MAK columns. A linear gradient of 0.2 to 1.2 M NaCl in 0.05 M Na phosphate pH 6.8 was applied to a column 1.8 cm in diameter and 8 cm high. 3 ml Portions were collected at the rate of flow of 1 ml per minute. Peak 1 represents material which emerges very early from the column, peak 2 is t-RNA, peak 3, r-RNA.

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around 60% of it is non-dialysable. There was a decrease in t-RNA (peak 2) during starvation relative to the amount of r-RNA. Labelling with P^{32} for 2 hours,(Fig. 2), starting 4 hours after transfer to starvation medium, revealed that the label which appears in peak 1 has a higher specific radioactivity than in t-RNA (peak 2). No label was found in r-RNA (peak 3). These results exclude the possibility that the material which absorbs at 260 mμ in peak 1 is a degradation product of t-RNA or r-RNA. Peak 1 was isolated from RNA prepared from starved ribosomes and could not be isolated in detectable amounts from preparations of log ribosomes. The fractions of starved cells bulk RNA eluted from MAK columns were desalted by filtration on Sephadex G-25 and tested for their inhibitory activity on polyphenylalanine synthesis. As shown in Table II there is almost no inhibition by addition of rather high concentrations of r-RNA (peak 3). Both t-RNA (peak 2) and peak 1 material were inhibitory but peak 1 has a much higher specific activity. Peak 1 prepared from starved washed ribosomes was highly active. Peak 1, prepared from whole starved cell contained material with a maximal adsorption of 260 mμ, gave a positive orcinal reaction, contained protein, polysaccharides and a substance highly fluorescent at neutral pH.

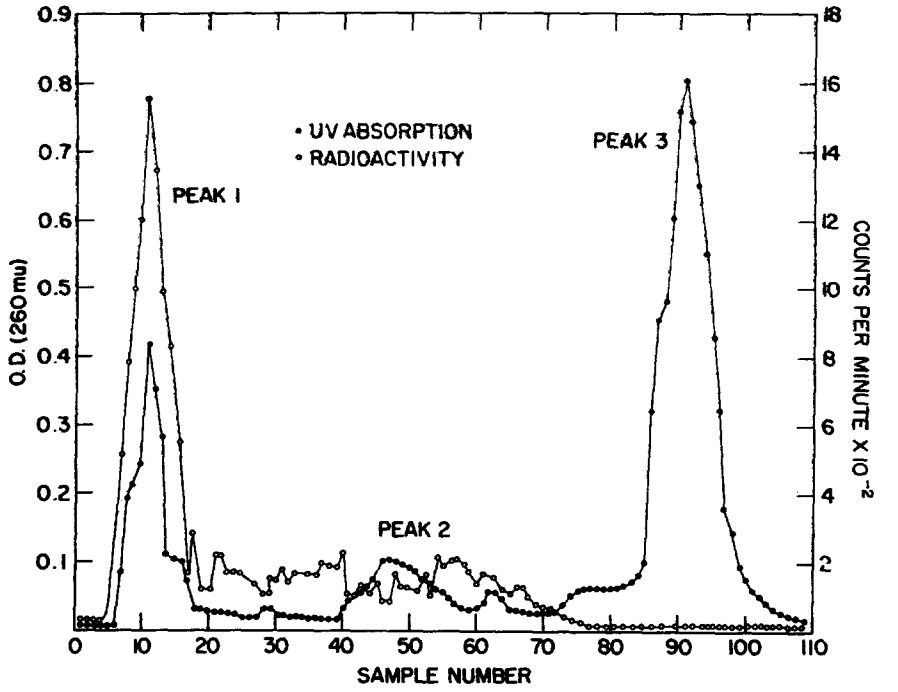

FIG. 2. Incorporation of P^{32} into crude yeast RNA during starvation. Washed log phase yeast cells were incubated in carbon base medium for 4 hours. P^{32} was added and the incubation was continued for an additional 2 hours. RNA was prepared and fractionated on MAK column as described in Fig. 1. OD at 260 mμ and radioactivity were recorded.

Table II

Inhibition of poly U directed polyphenyl alanine synthesis in a cell free system of E. coli by fraction of yeast RNA eluted from MAK column.

System	Fractions of RNA added (A_{260} mμ)	C. P. M.	% Inhibition
Complete		20.625	
" + r-RNA	1.75	19.404	6
" + t-RNA	0.25	13.652	44
" + PiK I fraction	0.028	15.902	23
	0.056	11.952	42
	0.084	7.012	66

Conditions of experiment as described in Table I.

Polysaccharides could not be detected in peak 1 prepared from starved ribosomes.

There was no change in the inhibitory activity after extraction of peak 1 with organic solvents such as ethanol-ether or methanol-chloroform. The inhibitory activity decreased when the sample was treated with RNase and completely disappeared after prolonged exposure. Pronase treatment did not change the biological activity. However, pronase does not seem to attack the protein of this fraction, since there was no difference in the RNA-protein ratio before the digestion and after it.

Discussion

An inhibitor for protein synthesis is present in cell free extracts of S. cerevisiae. This inhibitor is extracted and precipitated together with t-RNA. However, since the inhibitory activity decreases with the purification of t-RNA, this inhibitor seems to differ from t-RNA. The fact that a fraction with a high inhibitory activity could be separated on MAK columns from t-RNA only in the case of starved and stationary state cells may be due to accumulation of the inhibitor under conditions of growth cessation.

Loss of the inhibitory ability after subjection of this fraction (peak 1) to RNase treatment and its insensitivity to pronase may be an indicator for its polynucleotide rather than protein nature. However, the fact that the ratio of RNA to protein remains constant after pronase digestion points to the possibility that the inhibitor might be a polynucleotide-polypeptide, or even a more complex substance where the polypeptide moiety is protected from pronase digestion.

A more detailed study of the chemical and biological nature of the inhibitor as well as its presence in other types of cells is in progress.

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