BBA 66606

A NEW MACROMOLECULAR INHIBITOR OF YEAST RIBONUCLEASE MEDIATED THROUGH PURINE NUCLEOTIDES

AKIRA IMADA, ANTHONY J. SINSKEY AND STEVEN R. TANNENBAUM
Department of Nutrition and Food Science, Massachusetts Institute of Technology, Cambridge, Mass. 02139 (U.S.A.)

(Received January 17th, 1972)

SUMMARY

Ribonuclease activity in cell-free extracts of *Candida lipolytica* is strongly inhibited by purine nucleoside di-, tri- and tetraphosphates and only slightly by the corresponding pyrimidine nucleoside phosphates. The inhibition by the purine nucleotides is noncompetitive, and K_i values for ATP and GTP are approximately $2 \cdot 10^{-5}$ M. The inhibitory principle is nondialyzable and heat-labile. Purified ribonuclease from the same organism is slightly inhibited by nucleotides in buffer solution but greatly inhibited by nucleotides in the presence of the crude cell-free extracts. These facts suggest that a macromolecular ribonuclease inhibitor exists in *C. lipolytica*, and its activity is mediated by purine nucleoside polyphosphates.

INTRODUCTION

Degradation of endogenous RNA is initiated by heating *Candida* yeasts[1-3]. Also, while developing a method of extracting ribonuclease from *Candida lipolytica*, we found that ribonuclease in the cell-free extracts was activated 1.6- to 2.0-fold by heating at either 37 °C for 15 h or at 45 °C for 2 h (ref. 2).

One of the possible biochemical events brought about by heat treatment prior to visible RNA degradation is the activation of ribonuclease. The activation may occur by changes in cellular location of ribonuclease, by conformational changes in ribonuclease itself or *via* the denaturation of ribonucleases inhibitors. Liberation of latent ribonuclease from ribosomes exemplifies the first possibility[4,5]. The same may not be true for *Candida* yeasts, however, since most of the ribonuclease activity is found in the soluble fraction of cell-free extracts and is not bound to ribosomes. The second and the third possibilities may occur in *Aspergillus oryzae*; latent ribonuclease present as an enzyme–inhibitor complex in conidia could be activated by heat treatment[6], while in mycelia, its activation was induced by autolysis[7].

We have postulated[3] that a repressive mechanism controls ribonuclease activity in living cells and is destroyed by a heat treatment. After conducting a series of experiments, we found that ribonuclease activity in unheated cell-free extracts was

completely inhibited by purine nucleoside polyphosphates, while that in heated preparations was less inhibited. These observations suggest that the denaturation of this repressive mechanism for ribonuclease is an initiating reaction in the thermal process of endogenous RNA degradation in *Candida* yeasts. This paper deals with the the features of the nucleotide inhibition of ribonuclease in cell-free extracts of *C. lipolytica*.

MATERIALS AND METHODS

C. lipolytica (No. 60-26) was cultivated, harvested and mechanically disintegrated according to a previous report[2]. The disintegrated cells were centrifuged at 4000 × *g* for 5 min to remove cell debris and glass beads, and then at 27 000 × *g* for 30 min to remove cell fragments. The supernatant fluid was then centrifuged at 105 000 × *g* for 2 h. This is the "cell-free extract" used in most of our experiments. The extract was then dialyzed against deionized water.

The ribonuclease preparation was purified by extracting ribonuclease from acetone-dehydrated cells according to a previous paper[2], fractionating the extract by acetone (42–67%) and by ammonium sulfate (55–75% saturation), followed by chromatography on DEAE-cellulose. The yield and purification were approximately 50% and 100-fold, respectively. Details will be described in a future publication.

The ribonuclease assay was carried out by incubating a mixture of 0.2 ml of 0.5 M sodium acetate buffer (pH 5.0), 0.1 ml of 2% RNA, enzyme preparation and nucleotides (if added) in a total volume of 0.5 ml at 37 °C for 30 min. The preparation was immersed in an icebath for 2 min to stop the reaction before the addition of 0.5 ml of 1 M HCl. Blank tests were run without added RNA. RNA was added after 2 min cooling and the immediate addition of HCl followed. The tubes were cooled for 10 min in an icebath and centrifuged at 1500 × *g* for 5 min at 0 °C; absorbance at 260 nm of the supernatant fluid was measured after 40 times dilution with deionized water. One unit of ribonuclease causes 0.1 absorbance unit increase under the above described conditions. Protein was measured by the Folin-Ciocalteu reagent[8]. All assays were conducted in duplicate.

Highly polymerized RNA (*Saccharomyces cerevisiae*), *Torula* yeast RNA (grade VI), nucleosides, and nucleotides were purchased from Sigma Chemical Company, St. Louis, Mo.

RESULTS AND DISCUSSION

Ribonuclease inhibition by nucleotides

Ribonuclease activity of the extracts was assayed with and without added nucleotides at various concentrations and the inhibition percentage was calculated (Table I). Among the nucleotides tested, purine nucleoside di-, tri- and tetraphosphates at $2 \cdot 10^{-4}$ M inhibited ribonuclease activity from 50 to 88%. Inhibition by purine nucleotides increased as increasing numbers of phosphates were added to the 5′-position of the nucleosides. The inability of adenosine, 3′-AMP, or 3′,5′-cyclic AMP to inhibit ribonuclease suggests the necessity of a 5′-phosphate moiety for binding or reactivity.

Pyrimidine nucleotides were far less inhibitory than purine nucleotides. This

tendency was observed with both 5′-monophosphate and 5′-triphosphate nucleotides at all concentrations examined. Sodium pyrophosphate was not inhibitory and thus the base moiety is also necessary for inhibition.

The mode of inhibition of ribonuclease by purine nucleoside 5′-tri- and 5′-tetraphosphates was found to be noncompetitive as illustrated by the Lineweaver–Burk plot[9] in Fig. 1.

K_i values for ATP and GTP calculated by plotting $1/v$ *versus* inhibitor concentration were $1.6 \cdot 10^{-5}$ M and $1.7 \cdot 10^{-5}$–$2.5 \cdot 10^{-5}$ M, respectively.

TABLE I

INHIBITION OF RIBONUCLEASE BY VARIOUS NUCLEOTIDES

Cell-free extracts (Expt I) and dialyzed extracts (Expt II) were used. All assays were done in duplicate.

Expt No.	*Addition*	*Ribonuclease inhibition (%) at*		
		$2 \cdot 10^{-5}$ *M*	$2 \cdot 10^{-4}$ *M*	$2 \cdot 10^{-3}$ *M*
I	Adenosine		4.0	0
	3′,5′-Cyclic AMP		0	8.0
	3′(2′)-AMP		0.3	37
	5′-AMP		1.0	45
	5′-GMP		5.0	50
	5′-CMP		0	4
	5′-UMP		0	0
	ATP		78	96
	GTP		82	92
	CTP		27	62
	UTP		9	41
	Sodium pyrophosphate		0	0
II	5′-AMP		4	24
	ADP		50	85
	ATP	38	80	90
	ATPP*	65	86	94
	5′-GMP		9	38
	GDP		48	75
	GTP	32	76	89
	GTPP**	68	88	92

* Adenosine 5′-tetraphosphate at $4 \cdot 10^{-5}$ M, $4 \cdot 10^{-4}$ M and $4 \cdot 10^{-3}$ M, respectively.
** Guanosine 5′-tetraphosphate at $3 \cdot 10^{-5}$ M, $3 \cdot 10^{-4}$ M and $3 \cdot 10^{-3}$ M, respectively.

Several investigators have reported the inhibition of ribonucleases by nucleic acid derivatives. Ukita *et al.*[10] and Gamble and Wright[11] demonstrated weak inhibition of purified pancreatic ribonuclease by several nucleotides. Yamasaki and Arima[12–15] found that intracellular ribonuclease of *Bacillus subtilis* was strongly inhibited by ATP and ADP with K_i values of approximately 10^{-7} M. Irie[16,17] carried out extensive studies on the competitive inhibition by various nucleosides and nucleotides of ribonuclease M from *Asp. saitoi*. 2′-AMP was the strongest inhibitor followed by 3′- or 5′-AMP.

The observed pattern of nucleotide inhibition of *C. lipolytica* ribonuclease differs from all of the previously described results.

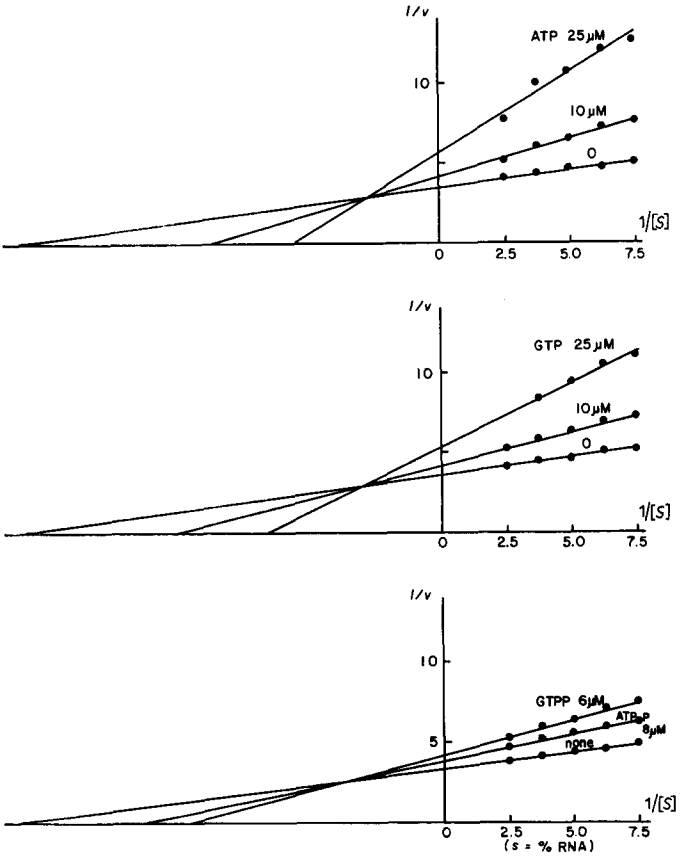

Fig. 1. Lineweaver–Burk[9] plot of ribonuclease inhibition by nucleotides. GTPP = guanosine 5′-tetraphosphate; ATPP = adenosine 5′-tetraphosphate.

Heat-induced reduction of nucleotide inhibition

Nucleotide inhibition of ribonuclease was studied using ATP as an effector. Heat treatment of the cell-free extract at 37 °C for 15 h or at 45 °C for 2 h greatly reduced the sensitivity of ribonuclease to ATP (Table II). Purified ribonuclease obtained through a heat-treatment process[2] was also insensitive to ATP (Table II). Two hypothetical explanations for the effect of heat treatment are: (1) ribonuclease itself is changed from a sensitive to a resistant structure, or (2) a ribonuclease inhibitor, which is active in the presence of ATP, is denatured or modified by the heat treatment. The following experiments were conducted to differentiate between these two possibilities.

Inhibition of purified ribonuclease by cell-free extracts

Cell-free extracts and purified ribonuclease preparations were diluted to similar ribonuclease activities; activities were measured on the separate and combined extracts (Table III). Ribonuclease activity was then determined in the presence and absence of 1 mM ATP. When the cell-free extract and purified ribonuclease prepa-

TABLE II

HEAT DESENSITIZATION OF NUCLEOTIDE INHIBITION OF RIBONUCLEASE

Preparation	*Inhibition by 1 mM ATP (%)*
Heat-treated cell-free extract	
2 °C, 15 h (control)	82.0
37 °C, 15 h	25
45 °C, 2 h	20.0
Purified preparation	20

ration were combined, the observed ribonuclease activities were far less than the sum of the individual activities. Assuming the reduced ribonuclease activity was due to the inhibition of purified ribonuclease by some factor in cell-free extract, the inhibition of ribonuclease in the purified preparation by the cell-free extract was calculated according to the equation footnoted in Table III. In the absence of ATP, the calculated inhibition of purified ribonuclease by cell-free extract was 52%, while the addition of 1 mM ATP increased the inhibition of purified ribonuclease by the cell-free extract to 70%.

The ATP inhibition of ribonuclease in each preparation is indicated in the last column of Table III. It is again demonstrated that the ribonuclease is sensitive to ATP only in the presence of the cell-free extract. Ribonuclease in the heat-treated cell-free extract, as well as ribonuclease in the purified preparation, was inhibited by the fresh cell-free extract in the presence of ATP.

From the above observations, two conclusions can be drawn: (1) a ribonuclease inhibitor is present in fresh cell-free extracts, and (2) the inhibition is promoted by the presence of ATP or other purine nucleotides.

Moreover, the inhibitor is a heat-labile (Table II) macromolecule as revealed by the inhibition of ribonuclease in the dialyzed cell-free extract by several purine nucleotides (Experiment II, Table I).

TABLE III

INHIBITION OF PURIFIED RIBONUCLEASE BY CELL-FREE EXTRACT IN THE PRESENCE AND ABSENCE OF ATP

Ribonuclease activity was assayed at pH 5.5. Cell-free extract and purified ribonuclease preparation contained 3.34 and 0.03 mg protein per ml, respectively. Ribonuclease activity in units/ml.

Enzyme preparation	*Without ATP*		*With 1 mM ATP*		*Inhibition by ATP (%)*
	Ribonuclease activity	*Inhibition of (B) by (A) (%)**	*Ribonuclease activtiy*	*Inhibition of (B) by (A) (%)**	
(A) Cell-free extract	31		5		85
(B) Purified ribonuclease	36		29		20
(C) Cell-free extract *plus* purified ribonuclease	49	52	14	70	72

* $[1 - \frac{(C) - (A)}{(B)}] \cdot 100$.

Apart from the inhibition of ribonuclease by nucleotides, there are a number of reports on mammalian[18–22], and a few microbial[6,7,23] ribonuclease inhibitors. There are, however, no indications concerning the effect of nucleotides on the action of those ribonuclease inhibitors.

The ribonuclease inhibitor system in *C. lipolytica* is more sophisticated than those previously reported, since it requires both the inhibitory principle and purine nucleoside polyphosphates. Thus, it could participate in the regulation of ribonuclease activity in *C. lipolytica* cells. When the purine nucleoside polyphosphate level is lowered due to reduced anabolic activities, cells would no longer have to maintain a high level of ribosomal RNA, and the RNA degradative system would be activated.

The presence of a heat-labile macromolecular ribonuclease inhibitor suggests that the degradation of endogenous RNA in *Candida* yeast by the thermal process[1–3] might be initiated by the denaturation of the ribonuclease inhibitor, and the activation of ribonuclease during the solvent dehydration buffer extraction of ribonuclease[2] may also be due to the denaturation of the inhibitor system.

Purification of the inhibitor principle and the examination of its biological role in connection with the mechanism of heat-initiated RNA degradation in *Candida* yeast[1–3] are in progress.

ACKNOWLEDGMENTS

This study was supported by the Alfred P. Sloan Foundation under grant No. 68-4-6. The paper is Contribution No. 1892 from the Department of Nutrition and Food Science, M.I.T.

REFERENCES

1 S. B. Maul, A. J. Sinskey and S. R. Tannenbaum, *Nature*, 228 (1970) 181.
2 A. Imada, A. J. Sinskey and S. R. Tannenbaum, *Biotech. Bioeng.*, 14 (1972) 103.
3 S. Ohta, S. B. Maul, A. J. Sinskey and S. R. Tannenbaum, *Appl. Microbiol.*, 22 (1971) 415.
4 D. Elson, in D. B. Roodyn, *Enzyme Cytology*, Academic Press, London and New York, 1970, p. 407.
5 Y. Ohtaka and K. Uchida, *Biochim. Biophys. Acta*, 76 (1963) 94.
6 K. Horikoshi, *Biochim. Biophys. Acta*, 240 (1971) 532.
7 T. Uozumi, G. Tamura and K. Arima, *Agric. Biol. Chem. Tokyo*, 32 (1968) 963.
8 O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.*, 193 (1951) 265.
9 H. Lineweaver and D. Burk, *J. Am. Chem. Soc.*, 56 (1934) 658.
10 T. Ukita, K. Waku, M. Irie and O. Hoshino, *J. Biochem. Tokyo*, 50 (1961) 405.
11 W. Gamble and L. D. Wright, *Biochem. J.*, 97 (1965) 340.
12 M. Yamasaki and K. Arima, *Biochim. Biophys. Acta*, 139 (1967) 202.
13 M. Yamasaki and K. Arima, *Biochem. Biophys. Res. Commun.*, 37 (1969) 430.
14 M. Yamasaki, K. Yoshida and K. Arima, *Biochim. Biophys. Acta*, 209 (1970) 463.
15 M. Yamasaki and K. Arima, *Biochim. Biophys. Acta*, 209 (1970) 475.
16 M. Irie, *J. Biochem. Tokyo*, 65 (1969) 133.
17 M. Irie, *J. Biochem. Tokyo*, 65 (1969) 907.
18 J. S. Roth, *Biochim. Biophys. Acta*, 21 (1956) 34.
19 J. S. Roth, *J. Biol. Chem.*, 231 (1958) 1085.
20 K. Shortman, *Biochim. Biophys. Acta*, 55 (1962) 88.
21 M. R. Robinovitz, L. M. Sreebny and E. A. Smuckler, *J. Biol. Chem.*, 243 (1968) 3441.
22 Y. Takahashi, K. Mase and Y. Suzuki, *J. Neurochem.*, 17 (1970) 1433.
23 J. R. Smeaton and W. H. Elliott, *Biochim. Biophys. Acta*, 145 (1967) 547.