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Catabolism of Bis(5′-Nucleosidyl) Tetraphosphates in *Saccharomyces cerevisiae*

PIERRE PLATEAU,* MICHEL FROMANT, JEAN-MARIE SCHMITTER, AND SYLVAIN BLANQUET

Laboratoire de Biochimie, Unité de Recherche Associée 240 Centre National de la Recherche Scientifique, Ecole Polytechnique, 91128 Palaiseau Cedex, France

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Bis(5′-adenosyl) tetraphosphate (Ap_4A) phosphorylase II (P. Plateau, M. Fromant, J. M. Schmitter, J. M. Buhler, and S. Blanquet, J. Bacteriol. 171:6437–6445, 1989) was obtained in a homogeneous form through a 40,000-fold purification, starting from a *Saccharomyces cerevisiae* strain devoid of Ap_4A phosphorylase I activity. The former enzyme behaves as a 36.8K monomer. As with Ap_4A phosphorylase I, the addition of divalent cations is required for the expression of activity. Mn^{2+}, Mg^{2+}, and Ca^{2+} sustain phosphorolysis by the two enzymes, whereas Co^{2+} and Cd^{2+} stimulate only phosphorylase II activity. All bis(5′-nucleosidyl) tetraphosphates assayed (Ap_4A, Ap_4C, Ap_4G, Ap_4U, Gp_4G, and Gp_4U) are substrates of the two enzymes. However, Ap_4A phosphorylase II shows a marked preference for A-containing substrates. The two enzymes catalyze adenosine 5′-phosphosulfate phosphorolysis or an exchange reaction between P_i and the β-phosphate of any nucleoside diphosphate. They can also produce Ap_4A at the expense of ATP and ADP. The gene (*APA2*) encoding Ap_4A phosphorylase II was isolated and sequenced. The deduced amino acid sequence shares 60% identity with that of Ap_4A phosphorylase I. Disruption of *APA2* and/or *APA1* shows that none of these genes is essential for the viability of *Saccharomyces cerevisiae*. The concentrations of all bis(5′-nucleosidyl) tetraphosphates are increased in an *apa1 apa2* double mutant, as compared with the parental wild-type strain. The factor of increase is 5 to 50 times, depending on the nucleotide. This observation supports the conclusion that, in vivo, Ap_4A phosphorylase II, like Ap_4A phosphorylase I, participates in the catabolism rather than the synthesis of the bis(5′-nucleosidyl) tetraphosphates.

Adenylylated bis(5′-nucleosidyl) tetraphosphates (Ap_4N, where N stands for A, C, G, or U) form a family of nucleotides found in all cell types so far examined, from bacteria to humans (12). Their intracellular concentration is usually between 0.1 and 3 μM. However, in procaryotic (6, 19) as well as eucaryotic cells (3, 9, 11), this concentration may be sharply increased in response to stimuli, such as heat shock or oxidative stress. This behavior led to the proposal that the Ap_4N nucleotides could be alarmones capable of signaling the onset of such stresses to cells (6).

In addition to Ap_4N, other bis(5′-nucleosidyl) oligophosphates, such as Bp_4B' (where B and B′ stand for C, G, or U) (10), Ap_3N (where N stands for A, C, G, or U) (25), and Gp_3G (35), have been found in living cells. The cellular concentrations of Bp_4B' and Ap_3N are also increased by the application of stresses (9, 10, 19).

An Ap_4A phosphorylase which phosphorolytically degrades bis(5′-nucleosidyl) tetraphosphates ($Np_4N' + P_i \rightarrow NDP + N'TP$ and $N'DP + NTP$, where N and N′ stand for A, C, G, or U) has been identified in extracts of the yeast *Saccharomyces cerevisiae* (14, 15). This enzyme is also capable of catalyzing (i) the synthesis of Np_4N' from $NDP + N'TP$ ($NDP + N'TP \rightarrow Np_4N' + P_i$) (8, 14), (ii) the synthesis of Ap_4A from adenosine 5′-phosphosulfate (AMPS) and ATP ($AMPS + ATP \rightarrow Ap_4A$ + sulfate) (16), (iii) the exchange between NDP and phosphate ($NDP + {}^{32}P_i \leftrightarrow [\beta\text{-}{}^{32}P]NDP + P_i$) (15), and (iv) the conversion of $AMPS + P_i$ into ADP + sulfate (15). The last property indicates a similarity between Ap_4A phophorylase and the previously described ADP sulfurylase (1, 29).

Recently, the *APA1* gene encoding Ap_4A phosphorylase I was isolated (28). Disruption of this gene was accompanied by a ca. threefold increase in the Ap_4N cellular concentration, leading to the conclusion that Ap_4A phosphorylase I was probably involved in Ap_4N catabolism rather than in Ap_4N synthesis (28). However, the study of Ap_4A degradation in crude extracts of an *apa1* strain revealed the occurrence of a second enzymatic activity also capable of phosphorolyzing Ap_4A. The corresponding enzyme was called Ap_4A phosphorylase II (28).

In this study, Ap_4A phosphorylase II was purified and its gene (*APA2*) was cloned, allowing us to design yeast cells devoid of Ap_4A phosphorylase II activity or of both Ap_4A phosphorylase I and II activities. By measuring Ap_4N and Bp_4B' in extracts of these strains, it may be concluded that Ap_4A phosphorylase II, like Ap_4A phosphorylase I, is involved in the catabolism of these nucleotides in yeast cells.

MATERIALS AND METHODS

Materials. DEAE-Sephacel, Sephacryl S200 HR, and Sephadex G75 Superfine were from Pharmacia. Hydroxylapatite, Matrex green A, TSK 3000, and Polymin-P were from BioRad, Amicon, Beckman, and Serva, respectively. Bioluminescence measurements were performed with a model 107 Nucleotimeter from CLV-Interbio (Lyon, France).

Purification of Ap_4A phosphorylases I and II. Ap_4A phosphorylase I was purified as described earlier (14), except that the DEAE-Sephacel column was eluted with a 0 to 500 mM KCl gradient. As previously shown (28), these chromatographic conditions enabled the separation of Ap_4A phosphorylase I and II activities.

Ap_4A phosphorylase II was purified from yeast strain YPAL16, which is devoid of Ap_4A phosphorylase I activity

* Corresponding author.

TABLE 1. Bacterial and yeast strains used in this study

Strain	Relevant genotype	Origin or reference
E. coli		
XL1-Blue	*recA1 lac endA1 gyrA96 thi hsdR17 supE44 relA1*(F′ *traD36 proAB lacI*^{q} *lacZ*ΔM15)	Stratagene
PAL2103D	F$^-$ Δ(*lac-pro*) *gyrA rpoB metB argE*(Am) *ara supF* Δ(*ksgA apaGH*)::*kan*	20
S. cerevisiae		
CMY214	*trp1-Δ1/trp1-Δ1 his3Δ200/his3Δ200 ura3-52/ura3-52 ade2-101/ade2-101 lys2-801/lys2-801 can1/CAN1*	22
YPALH	CMY214 *APA1/apa1Δ::HIS3*	28
YPAL16	*trp1-Δ1 his3Δ200 ura3-52 ade2-101 lys2-801 can1 apa1Δ::HIS3*	28
YPALHU	CMY214 *APA1/apaΔ::HIS3 APA2/apa2Δ::URA3*	This work
YPALS	*trp1-Δ1 his3Δ200 ura3-52 ade2-101 lys2-801 can1*	This work
YPALSH	YPALS *apa1Δ::HIS3*	This work
YPALSU	YPALS *apa2Δ::URA3*	This work
YPALSHU	YPALS *apa1Δ::HIS3 apa2Δ::URA3*	This work

(Table 1). Cells were grown at 28°C in 50 liters of medium containing 10 g of yeast extract per liter, 10 g of peptone per liter, 22 g of D-glucose per liter, and 50 mg of L-tryptophan per liter. After centrifugation (30 min, 15,000 × *g*), the cell pellet (850 g) was suspended in 850 ml of 100 mM potassium phosphate buffer (pH 6.75) containing 1 mM EDTA, 0.01 mM pepstatin A, 1 mM phenylmethylsulfonyl fluoride, and 1 mM diisopropyl fluorophosphate. Cells were disrupted by three successive passages through a chilled Menton-Gaulin press at 600 kg/cm^2. The extract was centrifuged for 90 min in a chilled Sharples centrifuge (15,000 × *g*). The pellet was discarded.

All of the following steps were carried out at 4 to 8°C. Nucleic acids were precipitated by the addition of Polymin-P (0.3% [wt/vol], final concentration) and centrifugation for 1 h at 15,000 × *g*. The resulting supernatant was fractionated by ammonium sulfate precipitation (35 to 65% saturation). The precipitate was dialyzed against buffer A (10 mM potassium phosphate [pH 6.75], 1 mM EDTA). The dialysate, diluted to 15 liters with buffer A, was applied to a DEAE-Sephacel column (8.5 by 25 cm; flow rate, 650 ml/h) equilibrated in buffer A. Elution was performed with a 30-liter linear gradient of 0 to 500 mM KCl in the same buffer. Active fractions were pooled and applied to a hydroxylapatite column (3.2 by 30 cm; flow rate, 60 ml/h) equilibrated in buffer A. The column was washed with 350 ml of buffer A and developed with a 3-liter linear gradient of 10 to 150 mM potassium phosphate (pH 6.75).

The enzyme preparation was successively passed through a Sephacryl S200 HR column (1.6 by 176 cm; flow rate, 5.6 ml/h) run in buffer B (20 mM Tris hydrochloride [pH 7.8], 1 mM EDTA) and through a Sephadex G75 Superfine column (1.6 by 90 cm; flow rate, 2.5 ml/h) run in the same buffer.

The pooled Sephadex G75 Superfine fractions were applied to a Matrex green column (0.95 by 10.5 cm) equilibrated in buffer A. The column was washed with 15 ml of buffer A and developed with a 180-ml linear gradient of 0 to 750 mM KCl in buffer A. Finally, the homogeneous enzyme (0.3 mg/ml) was obtained by high-performance gel filtration chromatography on a TSK 3000 column (0.7 by 30 cm) equilibrated in buffer C (10 mM potassium phosphate [pH 6.75], 150 mM NaCl, 0.1 mM EDTA). To achieve this step, samples of 100 μl of enzyme were successively applied to the column.

Nucleotides. Ap_4C, Ap_4G, Ap_4U, and Gp_4U were extracted from PAL2103D, an *Escherichia coli* strain which is devoid of Ap_4N hydrolase and in which the Ap_4N concentration is about 100-fold higher than normal (20). Bacteria were grown at 37°C in 300 ml of MOPS medium (23) supplemented with 0.2% glucose and 0.1 mg each of methionine, arginine, and proline per ml. When the optical density of the culture reached 1.0 unit at 650 nm, the culture was transferred to a water bath at 50°C. This temperature shift increases further the intracellular Ap_4N concentration (20). After 1 h at 50°C, the culture was arrested by the addition of $HClO_4$ (10% [wt/wt], final concentration). The mixture of bis(5′-nucleosidyl) tetraphosphates in the cell extract was purified by DEAE-Sephadex A25 and boronate chromatography as described previously (2, 27).

To separate the various bis(5′-nucleosidyl) tetraphosphates, we applied a sample after boronate chromatography to a high-pressure liquid chromatography (HPLC) column (0.46 by 20 cm) packed with Lichrosorb RP18 (Merck). Nucleotides were isocratically eluted with 50 mM potassium phosphate at pH 5.3 (27). Fractions containing a given nucleotide were pooled, desalted by boronate chromatography, and lyophilized. As judged from HPLC analysis, each nucleotide obtained was at least 90% pure. The identity of each nucleotide was controlled by complete hydrolysis with snake venom phosphodiesterase and analysis of the products by HPLC.

Other nucleotides were from Boehringer (Ap_4A, ATP, GTP, ADP, CDP, GDP, and UDP), Sigma (AMPS, Ap_3A, and Ap_4), or Pharmacia (Gp_4G and Gp_3G).

Enzymatic assays. To monitor Ap_4A phosphorylase activity in extracts and during the course of purification of Ap_4A phosphorylase II, we measured the phosphorolysis of the [^{3}H]Ap_4A substrate (28). When the enzymatic properties of homogeneous Ap_4A phosphorylases I and II were compared, the incorporation of $^{32}P_i$ into products was monitored. In this case, the reaction was stopped by the addition to the assay of 2.5 ml of a solution containing 50 mM sodium acetate, 100 mM potassium phosphate, 0.35% perchloric acid (wt/wt), and 4 g of activated charcoal (Sigma) per liter. Samples were filtered on Whatman no. 1 filter paper disks, and the radioactivity was measured in a Beckman LS1801 counter with the scintillation cocktail Picofluor (Packard). In vitro synthesis of Ap_4A from 4 mM ATP and 4 mM ADP in the presence of catalytic amounts of Ap_4A phosphorylase I or II was assayed as described previously (8).

HPLC. To analyze the products of the reactions catalyzed by Ap_4A phosphorylase, we diluted the reaction sample (50 μl) twofold into the appropriate column buffer (see below) and applied it to a column (0.46 by 20 cm) packed with Lichrosorb RP18. Nucleotides were isocratically eluted with one of the following buffers: 50 mM potassium phosphate

TABLE 2. Purification of Ap_4A phosphorylase II

Purification step	Protein (mg)	Total activity (U)[a]	Sp act (U/mg)[a]	Yield (%)	Relative purification
Extract	77,000[b]	171	0.0022		
Supernatant after Polymin-P precipitation	39,000[b]	155	0.0040	91	1.8
Ammonium sulfate fraction, 35 to 65%	16,500[b]	110	0.0067	64	3.0
DEAE-Sephacel	1,100[b]	89	0.081	52	36.4
Hydroxylapatite	140[b,c]	63	0.45	37	202
Sephacryl S200 HR	32[c]	35	1.10	20	492
Sephadex G75	4.6[c]	26	5.65	15	2,540
Matrex green	0.19[c]	14.3	75	8.4	33,900
TSK 3000	0.10[c]	9.5	95	5.6	42,800

[a] One unit defined as the amount of enzyme capable of transforming 1 μmol of Ap_4A per min (37°C; 50 mM Tris hydrochloride [pH 7.8], 120 μM Ap_4A, 1 mM potassium phosphate, 5 mM $MgCl_2$).

[b] Protein analysis was done as described by Lowry et al. (21).

[c] Protein concentration was determined from the UV absorbancy, assuming that 1 A_{280} unit corresponded to a protein concentration of 1 mg/ml.

(pH 5.3) (Ap_4A, Ap_4C, Ap_4G, and Ap_4U); 50 mM potassium phosphate (pH 6.4)–0.6 mM tetrabutylammonium bromide–8% methanol (Gp_4G and Gp_4U) or –4% methanol (Gp_3G). The absorbance of the column effluent was monitored at 254 nm and recorded on a Hitachi D-2000 integrator. The concentration of each nucleotide in the reaction samples was deduced by comparison with the chromatogram of standard solutions of known concentrations.

Cloning techniques, DNA sequencing, and peptide sequencing. The yeast and bacterial strains used in this study are listed in Table 1. Plasmid pBluescript was from Stratagene. Bacterial transformations were performed as described by Hanahan (17), while yeast transformations were performed by the lithium acetate method of Ito et al. (18). Other genetic and cloning techniques were performed as described by Sambrook et al. (31) or Sherman et al. (34). DNA sequencing was achieved by the dideoxy chain termination method (32). The N-terminal sequence of Ap_4A phosphorylase II was determined with an Applied Biosystems model 470A sequencer (4).

Bis(5′-nucleosidyl) oligophosphate measurements. For nucleotide measurements in cell extracts, a 200-ml sample of the culture was quickly filtered on a Sartorius SM11303 membrane filter. Nucleotides were extracted and purified by DEAE-Sephadex A25 and boronate chromatography (2, 27). After lyophilization, the sample was resuspended in 140 μl of water and divided into two parts. One part was used for Ap_4N and Ap_3A measurements. It was digested with alkaline phosphatase (20 mM Tris hydrochloride [pH 7.8], 1 mM $MgCl_2$, 40 U of alkaline phosphatase from calf intestine per ml; 30 min; 37°C) and analyzed on a Lichrosorb RP18 HPLC column as previously described (27). However, elution was performed in the presence of 60 mM potassium phosphate (pH 5.3) to allow separation and quantitation of Ap_3A despite the presence of a 100-fold-higher Ap_4A concentration. Measurement of Ap_4N and Ap_3A in the elution fractions was performed by bioluminescence as described previously (9, 24, 25). The other part of the above-described sample was used to estimate the total Bp_4B' concentration by a bioluminescence assay described elsewhere (10).

Nucleotide sequence accession number. The *APA2* gene sequence has been submitted to the GenBank data base under accession number M34354.

RESULTS AND DISCUSSION

Purification and molecular weight of Ap_4A phosphorylase II from *S. cerevisiae*. Ap_4A phosphorylase II was purified from an *S. cerevisiae* strain lacking Ap_4A phosphorylase I (28). The crude extract for purification was obtained by disrupting cells with a Menton-Gaulin press in the presence of protease inhibitors (see Materials and Methods for details of the protocol). The purification included precipitation of nucleic acids with Polymin-P, ammonium sulfate fractionation, and successive chromatography steps on DEAE-Sephacel, hydroxylapatite, Sephacryl S200 HR, Sephadex G75, Matrex green, and TSK 3000. The enzyme obtained was at least 95% pure, as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis (data not shown). The overall purification procedure produced 0.1 mg of enzyme from 850 g of wet yeast cells, with a purification factor of about 40,000-fold over the total protein in the crude extract and an overall yield of 5.6% (Table 2).

According to sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of the purified enzyme, Ap_4A phosphorylase II is composed of a polypeptide chain with an M_r of 38,000 ± 1,000, so it is slightly larger than Ap_4A phosphorylase I (M_r, 36,000 ± 1,000) (data not shown). Cochromatography of Ap_4A phosphorylase II with proteins of known molecular weights on the TSK 3000 column in nondenaturing buffer C indicated an M_r of 37,000 ± 2,000. Together, these results showed that Ap_4A phosphorylase II behaves as a monomer with an M_r of about 38,000.

Ap_4A phosphorylases I and II require the presence of divalent ions to express activity. Ap_4A phosphorylases I and II catalyze the phosphorolytic degradation of Ap_4A: $Ap_4A + P_i \leftrightarrow ADP + ATP$ (14, 28). For each enzyme, it was verified by HPLC analysis of the products that only ADP became radioactive when $^{32}P_i$ was used as a substrate. Therefore, the mechanisms of Ap_4A degradation by the two Ap_4A phosphorylases appeared identical. It was also verified that Ap_4A could be obtained from ATP and ADP through the reverse reaction of phosphorolysis (8, 14).

The two Ap_4A phosphorylases required the addition of divalent ions to measurably catalyze Ap_4A phosphorolysis. Mn^{2+}, Mg^{2+}, and Ca^{2+} displayed the strongest stimulating effects on the activities of the two enzymes. Co^{2+} and Cd^{2+} stimulated only Ap_4A phosphorylase II activity. Ni^{2+} and Zn^{2+} had no effect. The present results slightly differ from the previous characterization of the properties of Ap_4A phosphorylase I (14); in that study significant activation by Co^{2+} and Cd^{2+} was reported. In fact, the enzyme previously characterized was probably composed of a mixture of phosphorylases I and II. This may explain why measurable activation by Co^{2+} and Cd^{2+} was observed.

TABLE 3. Initial rates of NDP-P_i exchange and AMPS phosphorolysis reactions catalyzed by Ap_4A phosphorylases I and II[a]

Reaction	Substrate	Initial rate of ^{32}P incorporation (s^{-1}) with Ap_4A phosphorylase	
		I	II
NDP-P_i exchange	ADP	40	14
	CDP	7.2	7.2
	GDP	55	11
	UDP	2.0	1.7
AMPS phosphorolysis	AMPS	130	78

[a] The reaction mixture, buffered with 50 mM Tris hydrochloride (pH 7.8), contained 5 mM $^{32}P_i$ (20 GBq/mol), 2 mM NDP or AMPS, 0.1 mM EDTA, 50 μg of bovine serum albumin per ml, and catalytic amounts of Ap_4A phosphorylase. After incubation at 37°C, the labeled nucleotide was measured as described in Materials and Methods.

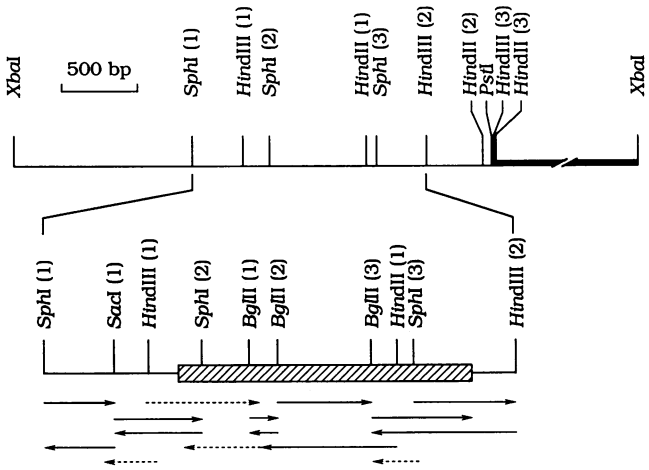

FIG. 1. Restriction map of pBS652 and strategy for sequencing the *APA2* gene. The hatched box and the heavy line correspond to the coding region for Ap_4A phosphorylase II and to pBluescript DNA, respectively. Arrows represent the length and direction of sequences obtained by the dideoxy chain termination method (32). Solid arrows indicate regions resolved through the subcloning of restriction fragments from pBS652 into M13mp18 and M13mp19 phage vectors (36). Broken arrows correspond to regions sequenced with specific primers.

Each Ap_4A phosphorylase catalyzes AMPS phosphorolysis as well as an exchange between P_i and NDP (where N stands for A, C, G, or U). Ap_4A phosphorylase I is already known to catalyze (i) AMPS phosphorolysis (AMPS + $P_i \leftrightarrow$ ADP + sulfate) and (ii) a reversible exchange reaction between inorganic phosphate and the β-phosphate of a nucleoside diphosphate (NDP + $^{32}P_i \leftrightarrow$ [β-^{32}P]NDP + P_i). To compare the specificities of the two Ap_4A phosphorylases, we systematically compared the above-listed reactions with each enzyme. AMPS and each of the four nucleoside diphosphates behaved as substrates for the two Ap_4A phosphorylases (Table 3). However, while the CDP-P_i and UDP-P_i exchanges were catalyzed by phosphorylases I and II at similar rates, the ADP-P_i exchange, the GDP-P_i exchange, and the AMPS phosphorolysis reactions were catalyzed three, five, and two times faster by phosphorylase I than by phosphorylase II.

Substrate specificity. Phosphorolysis of nucleotides structurally related to Ap_4A was assayed by monitoring $^{32}P_i$ incorporation into products. Each of the two Ap_4A phosphorylases was able to transform any bis(5′-nucleosidyl) tetraphosphate studied (Table 4). The reactions systematically yielded both a nucleoside diphosphate and a nucleoside triphosphate.

The main difference between the specificities of Ap_4A phosphorylases I and II concerned Gp_4N substrates. In the case of Ap_4A phosphorylase I, these nucleotides were as good as or even better than the corresponding Ap_4N nucle-

TABLE 4. Substrate specificities of Ap_4A phosphorylases I and II[a]

Ap_4A phosphorylase	Substrate	Initial rate of phosphorolysis (s^{-1})	Products (%)
I	Ap_4A	5.9	ATP + ADP
	Ap_4C	1.0	ATP + CDP (100)
	Ap_4G	5.9	ATP + GDP (82) or GTP + ADP (18)
	Ap_4U	1.0	ATP + UDP (100)
	Gp_4G	2.2	GTP + GDP
	Gp_4U	1.2	GTP + UDP (46) or UTP + GDP (54)
	Gp_3G	0.17	2 GDPs
	Ap_3A, Ap_3G, ATP, GTP, Ap_4	<0.03	
II	Ap_4A	33	ATP + ADP
	Ap_4C	20	ATP + CDP (100)
	Ap_4G	22	ATP + GDP (99) or GTP + ADP (1)
	Ap_4U	16	ATP + UDP (100)
	Gp_4G	4.3	GTP + GDP
	Gp_4U	2.2	GTP + UDP (24) or UTP + GDP (76)
	Ap_3A, Ap_3G, Gp_3G	<0.06	
	ATP, GTP, Ap_4	<0.06	

[a] The reaction mixture, buffered with 50 mM Tris hydrochloride (pH 7.8), contained 1 mM $MgCl_2$, 0.5 mM potassium phosphate, 50 μM substrate nucleotide under study, 0.01 mM EDTA, 50 μg of bovine serum albumin per ml, and catalytic amounts of Ap_4A phosphorylase. Incubations were carried out at 37°C. For initial rate measurements, the reactions were performed in the presence of 10^4 Bq of $^{32}P_i$ and the labeled nucleotides produced were counted as described in Materials and Methods. For detailed analysis of the various products of the reaction, the reaction was quenched by freezing the sample in liquid nitrogen before quantitative measurements by HPLC. For phosphorolysis of Gp_3G, the kinetics of $^{32}P_i$ incorporation were sigmoidal, probably because of exchange between $^{32}P_i$ and the unlabeled GDP produced through Gp_3G phosphorolysis. For this reason, the entire kinetics of Gp_3G phosphorolysis and the production of GDP were also monitored by HPLC. The products are given with percentages in parentheses. For instance, the phosphorolysis of Ap_4G produced either ATP plus GDP or GTP plus ADP. The former reaction is favored (82%) with respect to the latter reaction (18%).

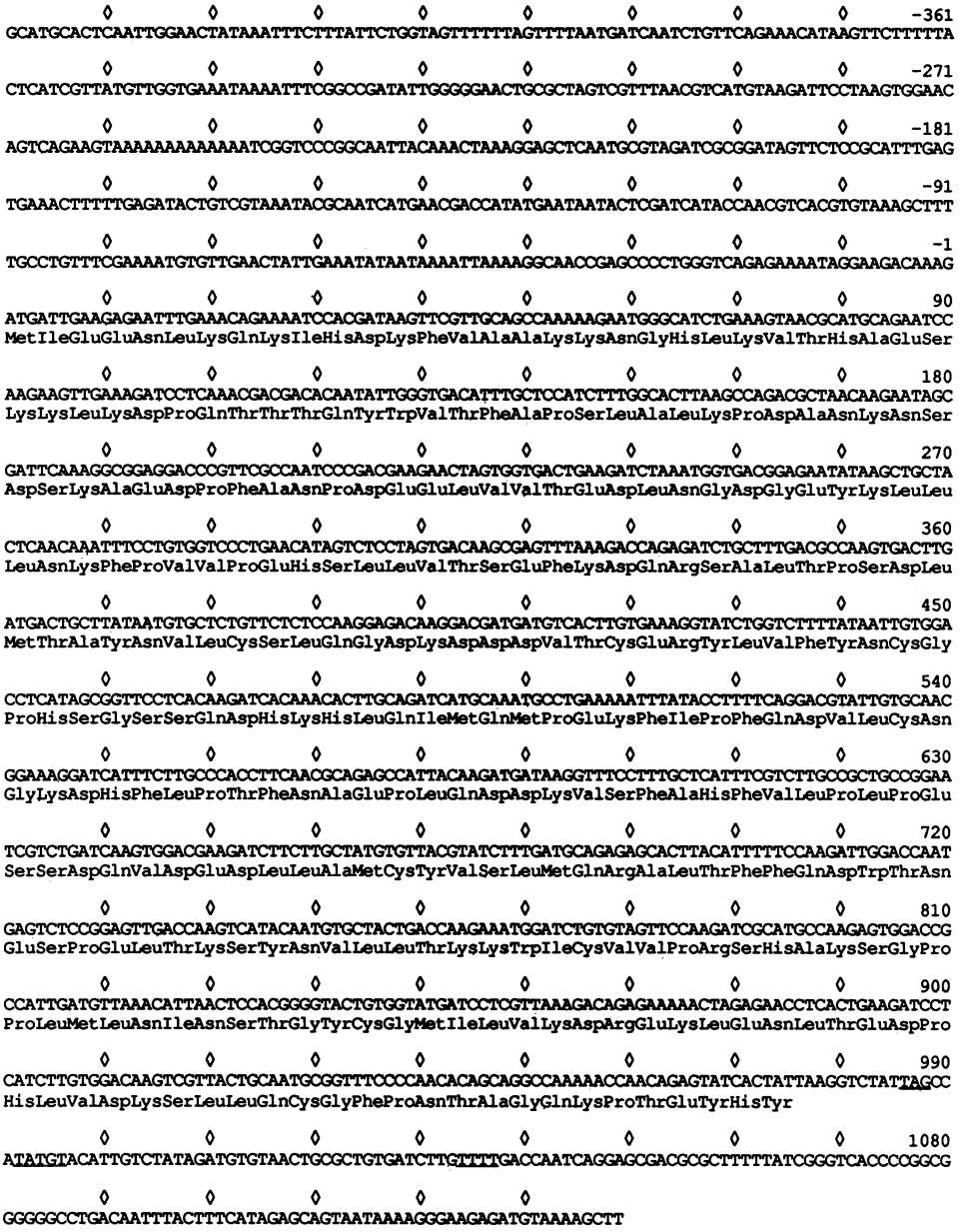

FIG. 2. Nucleotide and deduced amino acid sequences of the *APA2* gene. A stretch of 1,589 nucleotides encompassing the coding region of the *APA2* gene is shown. The sequence extends from the *Sph*I(1) site to the *Hin*dIII(2) site (Fig. 1). The underlined DNA sequence located at nucleotides 986 to 1037 conforms to the tripartite consensus sequence for transcriptional termination and polyadenylation of yeast transcripts (37).

otides as substrates. On the other hand, Ap_4A phosphorylase II preferred the A-containing substrates: Gp_4G and Gp_4U were split by Ap_4A phosphorylase II at rates about sixfold lower than those observed for Ap_4G or Ap_4U phosphorolysis.

None of the other assayed nucleotides (ATP, GTP, Ap_4, Ap_3A, Ap_3G, or Gp_3G) was a substrate for the Ap_4A phosphorylases, except for Gp_3G, which was found to be transformed into 2 GDP molecules in the presence of Ap_4A phosphorylase I.

Cloning of the *APA2* gene encoding Ap_4A phosphorylase II. As indicated above, the differences between the kinetic properties of Ap_4A phosphorylases I and II appeared more quantitative than qualitative. Therefore, it was likely that Ap_4A phosphorylase II, like Ap_4A phosphorylase I (28), was involved in Ap_4N catabolism in vivo. To clarify this point, we undertook the cloning of the *APA2* gene encoding Ap_4A phosphorylase II. A mixture of 32-mer oligonucleotides corresponding to the DNA strand coding for the N-terminal peptide of Ap_4A phosphorylase II, Met-Ile-Glu-Glu-Asn-Leu-Lys-Gln-Lys-Ile-His, was synthesized. To decrease the complexity of the DNA probe, we synthesized only the codons frequently used in *S. cerevisiae* (13): TGRATYTTT TGYTTYAARTTTTCTTCAATCAT (in which R stands for A or G and Y stands for C or T). Southern blot experiments with this probe indicated that the *APA2* gene was located on a 3.3-kbp *Xba*I-*Pst*I genomic DNA fragment devoid of any *Bam*HI site. To clone this fragment, we ligated chromosomal

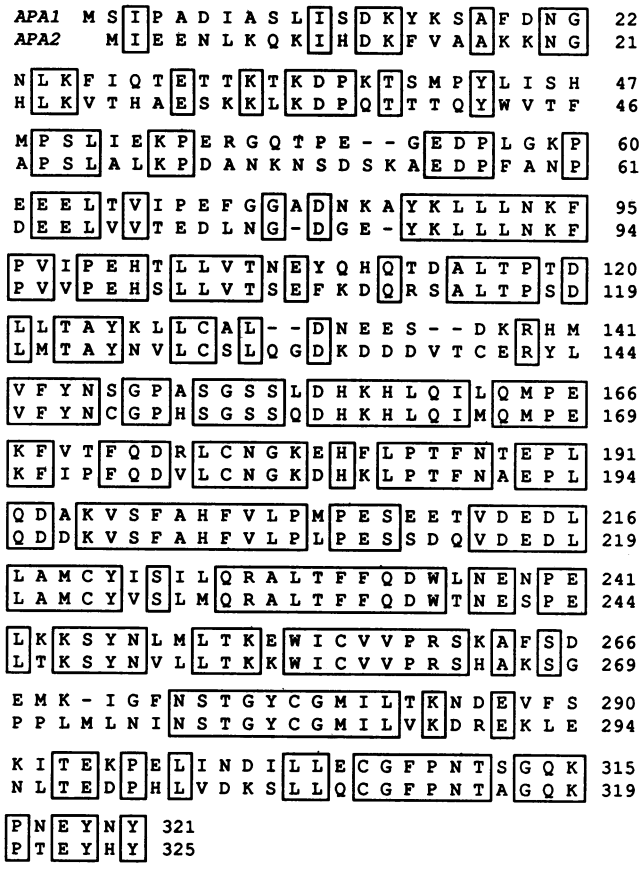

FIG. 3. Similarities between Ap_4A phosphorylase I and II primary sequences. The entire sequences of the two proteins are superimposed. Identical residues are boxed.

DNA digested by *Xba*I and *Pst*I with pBluescript DNA previously digested by the same enzymes. The mixture was digested by *Bam*HI and used to transform *E. coli* XL1-Blue. A set of 1,200 transformants were screened by colony hybridization with the labeled probe. One clone, containing plasmid pBS652, gave a signal much stronger than the other clones.

Restriction map of pBS652 plasmid DNA and sequence of the *APA2* gene. The restriction map of pBS652 DNA is shown in Fig. 1. Southern blot experiments demonstrated that the probe hybridized with the *Sph*I(2)-*Hin*dIII(1) restriction fragment. The DNA sequence around these sites contained a region which corresponded exactly to the N terminus of Ap_4A phosphorylase II. This region was followed by an open reading frame of 975 nucleotides (Fig. 2). At the 3′ end of the open reading frame, the sequence TAG...TATGT...TTTT was found (Fig. 2); this sequence conforms to the consensus sequence for termination and polyadenylation of yeast transcripts (37).

The M_r of the protein deduced from the DNA sequence (36,817) was close to that of the purified enzyme (38,000). The protein sequence deduced from the DNA sequence showed 60% identity with that of Ap_4A phosphorylase I (Fig. 3). No significant identity could be found between the two phosphorylases and the protein sequences stored in the GenBank 63 data base.

Disruption of the *APA2* gene. To determine the role of Ap_4A phosphorylase II in Ap_4N metabolism, we constructed yeast strains devoid of Ap_4A phosphorylase II and/or Ap_4A phosphorylase I activities. For this purpose, the *APA2* gene was disrupted in the diploid *apa1/APA1* strain YPALH by the one-step procedure of Rothstein (30). In this strain, one copy of *APA1* has been disrupted by deletion of a 595-bp DNA fragment internal to the gene and insertion of the *HIS3* marker (28). For disruption of the *APA2* gene, a 414-bp fragment of plasmid pBS652 between the *Bgl*II(1) and *Bgl*II(3) sites was deleted and replaced by the *URA3* marker (26). The corresponding plasmid was linearized and used to transform strain YPALH. Southern blot analysis of one of the *ura*$^+$ transformants confirmed the integration of *URA3* within the *APA2* locus (data not shown). These diploid cells were sporulated, and haploids were selected as canavanine-resistant clones (YPALH is *can*^{r}/*CAN*^{s}). Among the *can*^{r} cells obtained, the distributions of the *his*$^+$ *ura*$^-$, *his*$^-$ *ura*$^+$, *his*$^+$ *ura*$^+$, and *his*$^-$ *ura*$^-$ cell types were 22, 23, 34, and 21%, respectively. The corresponding mutant strains were named YPALSH (*apa1*), YPALSU (*apa2*), and YPALSHU (*apa1 apa2*) (Table 1). A control *his*$^-$ *ura*$^-$ strain (YPALS) was also selected.

The above results demonstrated that it was possible to disrupt *APA1* alone, *APA2* alone, or both genes without affecting cell viability. It was verified that the *apa1*, *apa2*, or *apa1 apa2* mutant strains could grow on minimal medium and remained capable of normally mating and sporulating (data not shown).

Dinucleoside polyphosphate concentrations in *apa1*, *apa2*,

TABLE 5. Ap_4A phosphorolysis activity and dinucleotide concentrations in various yeast strains[a]

Strain	Ap_4A phosphorolysis (U/mg of protein)[b]	Dinucleotide concn (μM)[c]													
		During exponential growth							1 h after cadmium addition						
		Ap_4N	Ap_4A	Ap_4C	Ap_4G	Ap_4U	Ap_3A	Bp_4B'	Ap_4N	Ap_4A	Ap_4C	Ap_4G	Ap_4U	Ap_3A	Bp_4B'
YPALS (control)	31	1.6	0.34	0.27	0.24	0.73	0.16	0.48	9.0	1.4	1.1	0.57	5.9	3.4	0.90
YPALSH (*apa1*)	6.5	3.7	1.1	0.51	0.52	1.6	0.15	1.7	85	9.9	5.7	3.3	66	3.8	5.0
YPALSU (*apa2*)	26	1.9	0.31	0.43	0.22	0.92	0.17	0.87	22	1.4	1.7	0.62	18	4.4	1.3
YPALSHU (*apa1 apa2*)	0.5	53	13	13	1.2	26	0.13	4.5	444	68	64	12	300	5.2	9.4

[a] Yeast cells were grown in rich YPD medium (34). When the optical density of the culture (500 ml) at 650 nm reached 1.2, two samples of the culture (200 ml each) were removed for Ap_4A phosphorylase and dinucleotide measurements. The remaining portion of the culture (100 ml) was immediately supplemented with 5 mM $CdSO_4$, and the dinucleotides were measured 1 h later.

[b] Ap_4A phosphorolysis was measured in crude extracts obtained by sonication (28). One unit is defined as the enzyme activity capable of transforming 1 pmol of Ap_4A per s under standard assay conditions.

[c] Dinucleotide concentrations were measured by bioluminescence as described in Materials and Methods. The experimental errors associated with the concentrations are estimated to be ±10% for Ap_4N and Bp_4B' and ±20% for Ap_3A. The Ap_4N concentration is the sum of the Ap_4A, Ap_4C, Ap_4G, and Ap_4U concentrations.

and *apa1 apa2* yeast strains. As expected, inactivation of both *APA1* and *APA2* in YPALSHU led to a great decrease in Ap_4A degradation activity in a crude extract of this strain (Table 5). The residual activity was less than 2% of that measured in an extract of YPALS.

Dinucleoside polyphosphates were quantified in the above-listed strains grown in rich YPD medium (34). Upon *APA1* inactivation, the concentrations of the various Ap_4N species increased from 1.9-fold to 3.1-fold (Table 5). This result extends the previous observation that inactivation of the Ap_4A phosphorylase I gene is accompanied by a ca. threefold increase in the total Ap_4N concentration (28). The concentrations of the nonadenylylated bis(5'-nucleosidyl) tetraphosphates (Bp_4B') also increased. They were 3.5-fold higher in the *apa1* strain than in the control *APA1*$^+$ strain (Table 5).

Inactivation of *APA2* in an *APA1*$^+$ strain resulted in minor increases in bis(5'-nucleosidyl) tetraphosphate concentrations. The effect mainly concerned Ap_4C (1.6-fold), Ap_4U (1.3-fold), and Bp_4B' (1.8-fold), while Ap_4A and Ap_4G concentrations were unchanged (Table 5). In contrast, inactivation of *APA2* in an *apa1* strain caused great increases (from 2.3-fold to 26-fold) in the concentrations of all bis(5'-nucleosidyl) tetraphosphate species, as compared with those in the *apa1* strain. The concentrations of the dinucleotides were, therefore, 5- to 50-fold higher in the *apa1 apa2* strain than in the control *APA1*$^+$ *APA2*$^+$ strain.

A striking feature of these data is that inactivation of both of the genes induces a greater increase in bis(5'-nucleosidyl) tetraphosphate concentrations than does the additive inactivation of each gene. For instance, the difference between Ap_4C concentrations in the *apa1 APA2* and *APA1 APA2* strains is 0.24 μM (0.51 − 0.27). Similarly, the difference between Ap_4C concentrations in the *APA1 apa2* and *APA1 APA2* strains is 0.16 μM. In the *apa1 apa2* strain, the Ap_4C concentration is 13 μM, a value markedly larger than the value expected (0.51 + 0.16 = 0.67 μM) if one assumes that the two species of Ap_4A phosphorylase are in distinct cell compartments and that Ap_4C cannot diffuse from one compartment to another. Therefore, it is reasonable to conclude that the two characterized Ap_4A phosphorylases act in the same cell compartment or on a common Ap_4C pool.

In agreement with the observation that Ap_3A was not an in vitro substrate of the two Ap_4A phosphorylases, the cellular Ap_3A concentration was not changed upon *APA1* and/or *APA2* inactivation (Table 5). However, this measurement establishes, for the first time, the presence of the Ap_3A nucleotide in exponentially growing yeast cells.

The bis(5'-nucleosidyl) tetra- and triphosphate concentrations can be increased by the addition of cadmium to a yeast culture medium (6, 10). Therefore, it was of interest to compare the effects of this metal on the various strains studied here. For all strains, the bis(5'-nucleosidyl) tetra- and triphosphate concentrations increased upon 1 h of exposure to 5 mM cadmium (Table 5). The Ap_4N concentrations reached strongly depended on the presence or absence of the phosphorylases. Thus, the Ap_4A concentration in the presence of cadmium was 50-fold higher in the *apa1 apa2* strain than in the *APA1 APA2* strain. In contrast, the increase in the Ap_3A concentration caused by the metal was independent of the expression of Ap_4A phosphorylase activity (Table 5). These results indicate that (i) each of the two phosphorylases counterreacts against the induction of the dinucleoside tetraphosphates caused by a stress such as cadmium addition and (ii) the cadmium-induced accumulation of Np_4N' does not originate from an in vivo regulation of Ap_4A phosphorylase activity, since Np_4N' accumulation occurs in the *apa1 apa2* strain.

Conclusions. Inactivation of both the *APA1* and the *APA2* genes promotes a great increase in the cellular concentration of bis(5'-nucleosidyl) tetraphosphate nucleotides. This result indicates that, in yeast cells, the catabolism of these nucleotides is mainly sustained by Ap_4A phosphorylase I and II activities. Consequently, the capacity of Ap_4A phosphorylase to synthesize Ap_4N or Bp_4B', as revealed in vitro (8, 16), seems not to apply in vivo, at least under the growth conditions assayed here. This conclusion is reminiscent of the case for *E. coli*, in which an Ap_4N hydrolase ensures the catabolism of the Np_4N' nucleotides during exponential growth as well as during stress adaptation (20). Recently, aminoacyl-tRNA synthetases were shown to be responsible for Ap_4N biosynthesis in *E. coli* (7). Yeast and *E. coli* aminoacyl-tRNA synthetases are highly similar (33). They also share the property that their Ap_4N synthetase activities can be stimulated in vitro in the presence of trace amounts of zinc (5). Therefore, it is likely that, in yeast cells, these enzymes play a role similar to that in *E. coli* cells for the biosynthesis of Ap_4N nucleotides. Consequently, at this stage, we may consider that the metabolism of Ap_4N in *S. cerevisiae* cells as well as in *E. coli* cells obeys the same pathway, with the difference that, in *S. cerevisiae* cells, the products of Ap_4A catabolism are ATP plus ADP, whereas in *E. coli*, Ap_4A produces 2 ADP molecules.

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