Fitzgerald, D. K., Brodbeck, U., Kiyosawa, I., Mawal, R., Colvin, B., & Ebner, K. E. (1970) *J. Biol. Chem. 245*, 2103-2108.
Geren, C. R., Magee, S. C., & Ebner, K. E. (1976) *Arch. Biochem. Biophys. 172*, 149-155.
Geren, C. R., Geren, L. M., Lee, D., & Ebner, K. E. (1977) *Biochim. Biophys. Acta 497*, 128-132.
Gill, G. N., & Garren, L. D. (1970) *Biochem. Biophys. Res. Commun. 39*, 335-343.
Hall, L. D. (1964) *Adv. Carbohydr. Chem. 19*, 51-93.
Hill, R. L., Brew, K., Vanaman, T. C., Trayer, I. P., & Mattock, P. (1968) *Brookhaven Symp. Biol. No. 21*, 139.
Horton, D. (1966) *Org. Synth. 46*, 1-5.
Hudson, C. S. (1907) *J. Am. Chem. Soc. 29*, 1572.
Ivatt, R. J., & Rosemeyer, M. S. (1972) *FEBS Lett. 28*, 195-197.
Khatra, B. S., Herries, D. G, & Brew, K. (1974) *Eur. J. Biochem. 44*, 537-560.
Klee, W. A., & Klee, C. B. (1970) *Biochem. Biophys. Res. Commun. 39*, 833-841.
Klee, W. A., & Klee, C. B. (1972) *J. Biol. Chem. 247*, 2336-2344.
Koenigs, W., & Knorr, E. (1901) *Ber. Dtsch. Chem. Ges. 34*, 957-981.
Kuhn, R., & Kirschenlohr, W. (1953) *Chem. Ber. 86*, 1331-1333.
Leaback, D. H., & Walker, P. G. (1957) *J. Chem. Soc.*, 4754-4760.
Lee, T. K., Wong, L.-J. C., & Wong, S. S. (1983) *J. Biol. Chem. 258*, 13166-13171.
Morrison, J. F., & Ebner, K. E. (1971) *J. Biol. Chem. 246*, 3992-3998.
Noller, C. R., & Rockwell, W. C. (1938) *J. Am. Chem. Soc. 60*, 2076-2077.
O'Keeffee, E. T., Mordick, T., & Bell, J. E. (1980) *Biochemistry 19*, 4962-4966.
Schanbacher, F. L., & Ebner, K. E. (1970) *J. Biol. Chem. 245*, 5057-5061.
Trayer, I. P., & Hill, R. L. (1971) *J. Biol. Chem. 246*, 6666-6675.
Wong, S. S., Malone, T. E., & Lee, T. K. (1983) *Biochim. Biophys. Acta 745*, 90-96.
Yamashita, K., Kamerling, J. P., & Kobata, A. (1983) *J. Biol. Chem. 258*, 3099-3106.

Catabolism of Bis(5′-nucleosidyl) Oligophosphates in *Escherichia coli*: Metal Requirements and Substrate Specificity of Homogeneous Diadenosine-5′,5‴-P^1,P^4-tetraphosphate Pyrophosphohydrolase

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ABSTRACT: Diadenosine-5′,5‴-P^1,P^4-tetraphosphate pyrophosphohydrolase (diadenosinetetraphosphatase) from *Escherichia coli* strain EM20031 has been purified 5000-fold from 4 kg of wet cells. It produces 2.4 mg of homogeneous enzyme with a yield of 3.1%. The enzyme activity in the reaction of ADP production from Ap_4A is 250 s^{-1} [37 °C, 50 mM tris(hydroxymethyl)aminomethane, pH 7.8, 50 μM Ap_4A, 0.5 μM ethylenediaminetetraacetic acid (EDTA), and 50 μM $CoCl_2$]. The enzyme is a single polypeptide chain of M_r 33K, as determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis analysis and high-performance gel permeation chromatography. Dinucleoside polyphosphates are substrates provided they contain more than two phosphates (Ap_4A, Ap_4G, Ap_4C, Gp_4G, Ap_3A, Ap_3G, Ap_3C, Gp_3G, Gp_3C, Ap_5A, Ap_6A, and dAp_4dA are substrates; Ap_2A, NAD, and NADP are not). Among the products, a nucleoside diphosphate is always formed. ATP, GTP, CTP, UTP, dATP, dGTP, dCTP, and dTTP are not substrates; Ap_4 is. Addition of Co^{2+} (50 μM) to the reaction buffer containing 0.5 μM EDTA strongly stimulates Ap_4A hydrolysis (stimulation 2500-fold). With 50 μM $MnCl_2$, the stimulation is 900-fold. Ca^{2+}, Fe^{2+}, and Mg^{2+} have no effect. The K_m for Ap_4A is 22 μM with Co^{2+} and 12 μM with Mn^{2+}. The added metals have similar effects on the hydrolysis of Ap_3A into ADP + AMP. However, in the latter case, the stimulation by Co^{2+} is small, and the maximum stimulation brought by Mn^{2+} is 9 times that brought by Co^{2+}. Exposure of the enzyme to Zn^{2+} (5 μM), prior to the assay or within the reaction mixture containing Co^{2+}, causes a marked inhibition of Ap_4A hydrolysis. The inhibition is relieved by prolonged incubation of the enzyme with excess EDTA.

The recent interest in bis(5′-adenosyl) tetraphosphate (Ap_4A) arose from the correlation between its intracellular concentration and cell proliferation, its ability to trigger DNA replication, and its involvement in the priming reaction of replication possibly through specific binding to DNA polymerase α [reviewed in Zamecnik (1983) and Grummt (1983)].

In pursuit of the initial observation that bis(5′-adenosyl)

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tetraphosphate (Ap_4A) occurred in vivo (Zamecnik & Stephenson, 1969) and that its intracellular level varied with the proliferation activity of the animal cells or tissues examined (Rapaport & Zamecnik, 1976), members of the family of bis(5′-nucleosidyl) oligophosphates (Ap_nN), where A stands for adenosine, N for any nucleoside, and $n = 3$ or 4, were identified as natural compounds (Gilmour & Warner, 1978; Plesner et al., 1979; Plesner & Kristiansen, 1979; Plesner & Ottesen, 1979; Nierenberg et al., 1980; Ogilvie, 1981; Flodgaard & Klenow, 1982; Grummt, 1983; Lüthje & Ogilvie, 1983; Ogilvie & Jakob, 1983; Lee et al., 1983a; McLennan & Prescott, 1984; Garrison & Barnes, 1984). The cellular concentration of these compounds is susceptible to vary with cell function, cycle, and environment (Plesner et al., 1979; Probst et al., 1983; Lee et al., 1983a,b; Weinmann-Dorsch et al., 1984; McLennan & Prescott, 1984; Bochner et al., 1984).

The unusual Ap_nN nucleotides are produced in vitro by aminoacyl-tRNA synthetases through the reversal of enzyme-bound aminoacyl adenylate by NTP (Zamecnik et al., 1966, 1967; Randerath et al., 1966; Zamecnik & Stephenson, 1968, 1969; Rapaport et al., 1975; Plateau et al., 1981; Plateau & Blanquet, 1982; Brevet et al., 1982; Goerlich et al., 1982; Blanquet et al., 1983; Traut, 1983; Jakubowski, 1983; Goerlich & Holler, 1984). In the case of a few prokaryotic as well as eukaryotic aminoacyl-tRNA synthetases, the rate of Ap_nN synthesis can be greatly enhanced upon addition of small amounts of zinc (Plateau et al., 1981; Plateau & Blanquet, 1982; Brevet et al., 1982; Goerlich et al., 1982; Blanquet et al., 1983; Jakubowski, 1983; Goerlich & Holler, 1984). This relation enabled us to propose a role of Zn^{2+} in regulating the intracellular pool of Ap_nN nucleotides (Plateau & Blanquet, 1982; Blanquet et al., 1983).

Enzymes which hydrolyze Ap_nN have been described in mammalian tissues (Vallejo et al., 1973, 1976; Lobaton et al., 1975a,b; Sillero et al., 1977; Cameselle et al., 1982, 1984; Höhn et al., 1982); Ehrlich ascites tumor cells (Ogilvie, 1981; Moreno et al., 1982), human leukemia cells (Ogilvie & Antl, 1983), yellow lupin seeds (Jakubowski & Guranowski, 1983), *Artemia salina* (Vallejo et al., 1976; Moreno et al., 1982), *Physarum polycephalum* (Barnes & Culver, 1982; Garrison et al., 1982), and *Escherichia coli* (Guranowski et al., 1983). However, from one organism to another, the hydrolases markedly vary in their substrate specificities and hydrolysis products. There appear to be distinct Ap_4N and Ap_3N hydrolases in rat liver (Sillero et al., 1977) and in lupin seeds (Jakubowski & Guranowski, 1983). On the other hand, Ap_4A is cleaved to two ADP's by the Ap_4A hydrolase isolated from *Physarum polycephalum* (Barnes & Culver, 1982) or *Escherichia coli* (Guranowski et al., 1983), while the hydrolysis products of the other enzymes are AMP + ATP (Lobaton et al., 1975; Vallejo et al., 1976; Ogilvie, 1981; Cameselle et al., 1982; Höhn et al., 1982; Ogilvie & Antl, 1983; Jakubowski & Guranowski, 1983).

Moreover, the effect of divalent metal ions on hydrolytic activity differs, depending on the origin of the hydrolase. Enzymes from rat liver, human leukemia cells, and lupin seeds strictly require the presence of Mg^{2+} (Sillero et al., 1977; Cameselle et al., 1982; Ogilvie & Antl, 1983; Jakubowski & Guranowski, 1983). Rat liver and lupin seed Ap_4A hydrolases are inhibited by Ca^{2+} (Cameselle et al., 1982; Jakubowski & Guranowski, 1983); rat liver Ap_4A hydrolase and lupin seed Ap_3A hydrolase are inhibited by Zn^{2+} (Cameselle et al., 1983; Jakubowski & Guranowski, 1983). *E. coli* enzyme is strongly stimulated by Co^{2+} and Mn^{2+} (Guranowski et al., 1983), while *P. polycephalum* enzyme is only slightly affected by the presence of Mg^{2+}, Ca^{2+}, Zn^{2+}, Co^{2+}, or Mn^{2+} (Barnes & Culver, 1982).

In the present study, we report the occurrence of a unique enzyme species in *Escherchia coli* cells capable of cleaving Ap_4N as well as Ap_3N. The specificity of this enzyme depends, however, on the nature of the metal ions in the hydrolytic assay.

Materials and Methods

EM20031 cells were cultured and harvested as described earlier (Cassio & Waller, 1971).

Blue dextran was covalently bound to CNBr-activated Sepharose by the method of Ryan & Vestling (1974). The blue dextran–Sepharose used in this study contained about 4.79 mg of blue dextran per g of dry Sepharose. Sephadex G200, DEAE A25, DEAE A50, and Sepharose 4B were from Pharmacia, TSK 3000 was from Beckman, and Chelex 100 was from Bio-Rad. Ultrafiltration was performed with a Sartorius SM 16525 apparatus.

Diadenosinetetraphosphatase was stored in 50 mM tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl) buffer (pH 7.8) containing 0.1 mM ethylenediaminetetraacetic acid (EDTA) and 0.1 mM dithioerythritol, either at 4 °C as a precipitate in an ammonium sulfate solution (2.9 M) or at −20 °C in 50% glycerol. Enzyme concentration was estimated by assuming a specific extinction coefficient at 280 nm of 1 $mg^{-1} \cdot cm^2$ (i.e., 1 optical density unit through 1-cm light path corresponded to a 1 mg/mL enzyme solution).

When necessary, buffers were freed of contaminating divalent ions by passage through a Chelex 100 column.

Assay for Diadenosinetetraphosphatase. Two different methods were used. In the first one, the incubation mixture (100 μL) in 50 mM Tris-HCl (pH 7.8) contained 150 μM $CoCl_2$ and 50 μM Ap_4A. The reaction was initiated by the addition of enzyme (20 μL) diluted in 50 mM Tris-HCl buffer (pH 7.8) containing 100 μM EDTA, 100 μM dithioerythritol, and 200 μg/mL bovine serum albumin. The incubation was monitored at 37 °C for various times and quenched by the addition of $HClO_4$ (10% w/w final concentration). The sample was then centrifuged, the supernatant was removed, neutralized (pH ~7) by the addition of a 5 M K_2CO_3 solution, and centrifuged, and the supernatant was submitted to high-performance liquid chromatography (HPLC) in order to determine the amount of the synthesized products (AMP, ADP, and ATP). This procedure was used to study the activity of homogeneous diadenosinetetraphosphatase toward different nucleotides or in the presence of various metals. The samples with Ap_2A, Ap_3A, Ap_5A, Ap_6A, Ap_4G, Ap_3G, Gp_4G, Gp_3G, Ap_4C, Ap_3C, Gp_3C, dAp_4dA, Ap_4, or NAD were analyzed by using ion-paired HPLC (see below). In this case, the reaction was terminated by freezing the sample in liquid nitrogen, instead of quenching with $HClO_4$.

The second method, designed to follow the purification of the enzyme, consisted of a radioisotopic assay of the enzyme activity using [^{3}H]Ap_4A as the substrate. Enzyme was incubated (100 μL, 37 °C) in Tris-HCl buffer (50 mM, pH 7.8) containing 150 μM $CoCl_2$, 50 μM [^{3}H]Ap_4A (20 Ci/mol, from Amersham), and 30 units/mL alkaline phosphatase (from Boehringer). This amount of phosphatase was sufficient to transform all the nucleotides formed during the incubation (AMP, ADP, and ATP) into adenosine. The reaction was stopped by adding 200 μL of a solution containing 100 μL of DEAE-Sephadex A25 resin equilibrated in 5 mM phosphate buffer (pH 6.75) and 1 mM EDTA. In these ionic strength conditions, Ap_4A binds to the resin, and adenosine does not. The solution was then centrifuged (12,000*g*, 1 min), and 100

μL of the supernatant was mixed with 2.5 mL of Picofluor scintillation cocktail (from Packard) and counted in an Intertechnique SL3 counter.

Assay of enzyme activity by both methods gave identical results, either with a crude extract or with the purified enzyme.

High-Performance Liquid Chromatography. A Model 112 pump, a Model 210 injector, and a Model 160 absorbance detector (from Beckman) were used. To follow the hydrolysis of a nucleotide by diadenosinetetraphosphatase, we applied the sample (20 or 100 μL) onto a 0.46 × 20 cm column packed with Lichrosorb RP18 (Merck). The substrates and enzyme products were isocratically eluted with one of the following buffers: 20 mM potassium phosphate, pH 5.3, in the case of Ap_4A and dATP; 35 mM potassium phosphate, pH 5.3 (AMP, ADP, ATP, GTP, dGTP, dTTP, and NADP); 50 mM potassium phosphate, pH 6.4, 0.6 mM tetrabutylammonium bromide, and 9% methanol (Gp_3C) or 14% methanol (Ap_2A, Ap_3A, Ap_4A, Ap_5A, Ap_6A, Ap_4G, Ap_3G, Gp_4G, Gp_3G, Ap_4C, Ap_3C, Ap_4, and NAD) or 18% methanol (dAp_4dA); 50 mM potassium phosphate, pH 6.4, 1.5 mM tetrabutylammonium bromide, and 11% methanol (CTP, UTP, and dCTP). The absorbance of the column effluent was followed at 254 nm. The concentrations of each nucleotide in the samples were estimated by comparison with the chromatogram of standard solutions of known nucleotide concentrations.

Nucleotides. Ap_4G, Ap_4C, Ap_3C, and dAp_4dA were enzymatically synthesized by using purified *E. coli* lysyl-tRNA synthetase (Plateau & Blanquet, 1982). The reaction mixture (1 mL) contained 20 mM Tris-HCl (pH 7.8), 10 mM $MgCl_2$, 150 μM $ZnCl_2$, 2 mM L-lysine, 0.01 mg/mL yeast pyrophosphatase (Boehringer), and 500 nM lysyl-tRNA synthetase; 5 mM ATP and 10 mM GTP were added for Ap_4G synthesis, 5 mM ATP and 10 mM CTP for Ap_4C synthesis, 3 mM ATP and 5 mM CDP for Ap_3C synthesis, and 5 mM dATP for dAp_4dA synthesis. After consumption of ~90% of the initial ATP or dATP, the nucleotides present in the sample were separated by HPLC on a Lichrosorb RP18 column. Ap_4G was isocratically eluted with 30 mM ammonium acetate (pH 6.2), Ap_4C with 20 mM potassium phosphate (pH 6.4), 0.6 mM tetrabutylammonium bromide, and 12.5% methanol, Ap_3C with 35 mM potassium phosphate (pH 6.4), 1.5 mM tetrabutylammonium bromide, and 11% methanol, and dAp_4dA with 50 mM potassium phosphate (pH 6.4), 3 mM tetrabutylammonium bromide, and 17% methanol. Fractions containing the desired nucleotide were pooled and lyophilized. For removal of salts, 30 mL of NH_4HCO_3 (10 mM, pH 8.2) was added, and the sample was applied onto a 2.5 × 9 mm column of DEAE-Sephadex A25 equilibrated with 10 mM NH_4HCO_3 (pH 8.2). The bound nucleotide was recovered from the resin with a linear gradient (2 × 20 mL) of 10–1000 mM NH_4HCO_3 (pH 8.2). Fractions containing the nucleotide were pooled and lyophilized. The identity and the concentration of the final products were checked by complete hydrolysis with *E. coli* diadenosinetetraphosphatase, analysis of the formed compounds by HPLC, and measurement of their concentrations as described above. Other nucleotides were obtained from P-L Biochemicals (Gp_4G, Gp_3G, Gp_3C, and Ap_3G), Sigma (Ap_2A, Ap_3A, Ap_6A, and Ap_4), and Boehringer (Ap_4A, Ap_5A, NAD, NADP, AMP, ADP, ATP, GTP, CTP, UTP, dATP, dGTP, dCTP, and dTTP). All nucleotides were at least 95% pure, as analyzed by HPLC, except for GTP which was found to contain 10% GDP, dGTP (10% dGDP), Ap_4 (10% ATP), and Ap_6A (25% unidentified compound). Ap_6A was purified by HPLC (Lichrosorb RP18 column, 50 mM potassium phosphate, pH 6.4, 0.6 mM tetrabutylammonium bromide, and 14% methanol) and desalted as described above for Ap_4C. Other nucleotides were used without further purification.

Results

Diadenosinetetraphosphatase Activity in E. coli Extracts. The diadenosinetetraphosphatase activity in *E. coli* extracts was demonstrated by adding cellular extracts to 50 μM [^{3}H]Ap_4A at 37 °C in 50 mM Tris-HCl (pH 7.8). Analysis of the products by HPLC revealed the formation of ^{3}H-labeled AMP, ADP, and ATP. As shown further below, the homogeneous enzyme cleaved Ap_4A into 2 mol of ADP, and the products above were likely to reflect an adenylate kinase activity in the extracts. Under the above assay conditions, radioactive Ap_4A had to be used because of the high contaminating nucleotide concentrations in the cellular extracts. The hydrolyzing activity of the *E. coli* extract was stimulated 100-fold by the addition of 150 μM Co^{2+}, as already observed by Guranowski et al. (1983). With Co^{2+}, diluted extracts could be assayed with nonradioactive Ap_4A as a substrate.

In a preliminary attempt to purify the hydrolase, a marked lability of the activity (4 °C) in 50 mM Tris-HCl (pH 7.8) was observed with a half-life of 3 days. Enzyme activity was not protected by the addition of either 10% glycerol, 10 mM 2-mercaptoethanol, 100 μM dithioerythritol, or 150 μM Co^{2+}. It was weakly protected by EDTA (half-life of 20 days). The combination of 100 μM EDTA and 100 μM dithioerythritol fully protected the activity, and 100% of the initial activity remained after 60 days storage at 4 °C. These conditions were therefore adopted to monitor the purification of the *E. coli* diadenosinetetraphosphatase.

Purification of Diadenosinetetraphosphatase. Diadenosinetetraphosphatase was purified from *Escherichia coli* EM20031 (F32). All buffers contained 0.1 mM EDTA and 0.1 mM dithioerythritol.

(*A*) *Step 1*: *Preparation of Crude Extract.* Four kilograms of wet cells was suspended in 4 L of 20 mM potassium phosphate (pH 8.0) at 0 °C. Cells were disrupted by two successive passages through a refrigerated Menton Gaulin apparatus (500 kg/cm^2). Then the extract was centrifuged for 3 h in a refrigerated Sharples centrifuge (15,000*g*). The pellet was discarded. All the following steps were carried out at 4–8 °C.

(*B*) *Step 2*: *Streptomycin Precipitation.* Nucleic acids and ribosomes were precipitated by addition of a streptomycin solution to the supernatant from step 1 (final streptomycin concentration 57 g/L), which was then centrifuged for 2 h (15,000*g*).

(*C*) *Step 3*: *Ammonium Sulfate Fractionation.* The supernatant from step 2 was brought to 50% ammonium sulfate saturation, left to stand overnight, and centrifuged 2 h (8000*g*). The pellet was dissolved in a minimal volume of 20 mM potassium phosphate (pH 8.0) and dialyzed against 40 L of the same buffer.

(*D*) *Step 4*: *Sephadex G200 Column Chromatography.* The solution (2.1 L from step 3) was applied upwards at a flow rate of 0.2 L/h on two Sephadex G200 columns (25 × 41 cm) coupled in series. Phosphate buffer (20 mM) (pH 8.0) was pumped upwards at a flow rate of 0.7 L/h, and 0.7-L fractions of the eluate were collected and assayed for diadenosinetetraphosphatase activity.

(*E*) *Step 5*: *DEAE-Sephadex Chromatography, pH 8.0.* The fractions from the elution profile of the chromatography in step 4 were pooled (19.5 L) and applied on a column of DEAE-Sephadex A50 (15 × 35 cm) equilibrated in 20 mM potassium phosphate (pH 8.0). Enzyme activity was eluted

Table I: Purification of *E. coli* Diadenosinetetraphosphatase

purification step	protein (mg)	total act. ($\times 10^6$ units)[c]	sp act. (units/mg)	yield (%)	rel purification
extract	420000[a]	19.1	45.5		
supernatant after streptomycin pptn	395000[a]	35.1	88.9	100	1
ammonium sulfate fraction, 0–50%	290000[a]	31.6	109	90	1.2
Sephadex G200	133000[a]	28.4	213	81	2.4
DEAE-Sephadex A50 (pH 8.0)	27700[a]	26.7	928	76	10.4
DEAE-Sephadex A50 (pH 6.75)	8200[a,b]	16.8	2050	48	23.1
Sephadex G200	3700[b]	15.2	4100	43	46.1
blue dextran–Sepharose	115[b]	9.9	86000	28	967
TSK 3000	5.4[b]	2.1	430000	6.0	4840
TSK 3000	2.4[b]	1.1	458000	3.1	5150

[a] Protein analysis according to Lowry et al. (1951). [b] Protein amount determined from UV absorbancy, assuming $A_{280} = 1$ mg^{-1}·cm^2. [c] 1 unit = enzyme activity capable of transforming 1 nmol of Ap_4A per min (37 °C, 50 mM Tris, pH 7.8, 50 μM Ap_4A, and 150 μM $CoCl_2$).

from the column with a 2 × 50 L linear gradient of 0.05–0.45 M NaCl in the same buffer.

(*F*) *Step 6*: *DEAE-Sephadex Chromatography, pH 6.75.* Fractions containing activity in step 5 were pooled (8.6 L), concentrated to 1.45 L by ultrafiltration, and dialyzed overnight against 40 L of 20 mM potassium phosphate (pH 6.75). Enzyme was then applied on a column of DEAE-Sephadex A50 (8 × 20 cm) equilibrated in the same buffer. Fractions containing enzyme activity and recovered from the column by using a 2 × 10 L linear gradient of 0.1–0.4 M NaCl were pooled (2.2 L) and brought to 70% ammonium sulfate saturation.

(*G*) *Step 7*: *Sephadex G200 Column Chromatography.* Proteins precipitated in step 6 were centrifuged 2 h (8000*g*). The pellet was dissolved in a minimal volume of 20 mM potassium phosphate, pH 8.0, dialyzed, and applied upwards on two Sephadex G200 columns (4.4 × 72 cm) coupled in series. Fractions containing diadenosinetetraphosphatase activity were pooled and concentrated with ammonium sulfate as in step 6.

(*H*) *Step 8*: *Blue Dextran–Sepharose Column Chromatography.* The enzyme from step 7 was dissolved and dialyzed overnight against 50 mM Tris-HCl buffer (pH 7.8). After dialysis, the volume of the enzyme pool was adjusted to 0.4 L, and $MnCl_2$ was added at a final concentration of 0.2 mM. Fractions of 0.2 L were applied on a column (4.4 × 10 cm) of blue dextran–Sepharose equilibrated in 50 mM Tris-HCl buffer containing 0.2 mM $MnCl_2$. After application of the sample, each column was washed with 0.8 L of the equilibrating buffer, and the enzyme was then eluted with a 2 × 1 L linear gradient of 0–0.8 M NaCl in the same buffer. Fractions containing enzyme activity were pooled and concentrated by ammonium sulfate precipitation.

(*I*) *Step 9*: *High-Performance Gel Filtration Chromatography.* The enzyme was further purified by high-performance gel filtration chromatography onto a Beckman TSK 3000 column (0.7 × 30 cm) equilibrated in 50 mM Tris-HCl (pH 7.8) and 150 mM NaCl (Figure 1). The buffer flow rate was 0.5 mL/min. After dialysis in the same buffer, samples of 100 μL containing 20 mg of protein per mL were successively applied on the column. After this step, the purity of the enzyme was close to 90% as assessed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis analysis. To reach homogeneity, enzyme activity was pooled, concentrated by ammonium sulfate precipitation, and dialyzed against 50 mM Tris-HCl buffer (pH 7.8) containing 150 mM NaCl, and a second high-performance gel filtration chromatography was performed under conditions identical with those above.

The purification procedure gave 2.4 mg of homogeneous enzyme as evidenced by sodium dodecyl sulfate–polyacrylamide gel electrophoresis analysis (Figure 2), with a yield of

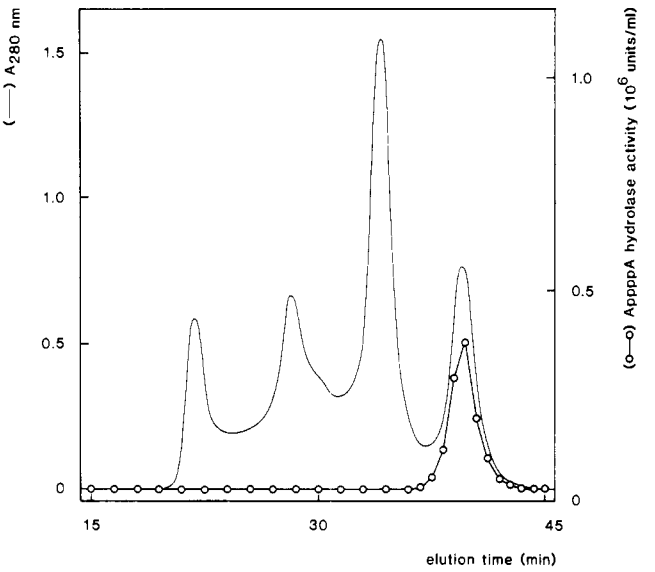

FIGURE 1: High-pressure gel filtration chromatography of *E. coli* diadenosinetetraphosphatase on TSK 3000. After blue dextran–Sepharose chromatography, the fractions containing enzyme activity were concentrated by ammonium sulfate precipitation and dialyzed against 50 mM Tris-HCl (pH 7.8), 0.1 mM EDTA, 0.1 mM dithioerythritol, and 150 mM NaCl. Elution in the same buffer was performed at 0.5 mL/min. The sample was applied in 100 μL at a protein concentration of 20 mg/mL. The absorbance of the column effluent was followed at 280 nm. Diadenosinetetraphosphatase activity (O) was measured by the standard assay described under Materials and Methods.

3.1% (Table I). Homogeneous diadenosinetetraphosphatase migrated as a unique polypeptide chain of M_r 34K ± 1K.

On the other hand, the molecular ratio of the native enzyme could be estimated by high-performance gel filtration chromatography. It was 33 ± 1K, as deduced from the cochromatography with proteins of known molecular weight.

The following comments on the purification procedure can be made: after streptomycin precipitation, the total activity increased markedly (2-fold), if compared to the activity in the cell extract. This behavior can be explained by an inhibition effect of nucleic acids on the enzyme activity, as observed by Guranowski et al. (1983). In Table I, the yield of the purification was estimated with respect to 100% activity in the supernatant after streptomycin precipitation, rather than with respect to the lower activity in the cell extract. A 967-fold purification factor was reached after blue dextran–Sepharose chromatography, with a yield of 28% [to be compared to 390-fold and 37% in the work of Guranowski et al. (1983)]. Among the various chromatographic procedures assayed to obtain homogeneous enzyme, hydroxylapatite and aminohexyl-Sepharose appeared inefficacious at this stage. Two

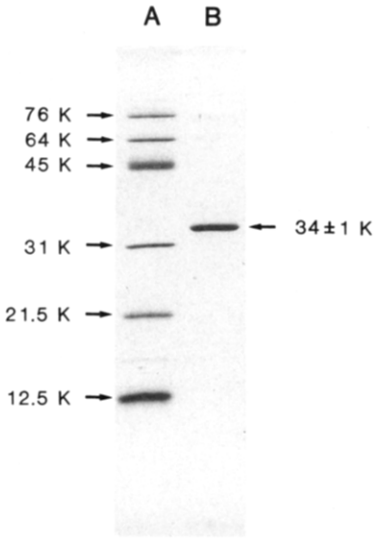

FIGURE 2: Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of *E. coli* diadenosinetetraphosphatase. Electrophoresis was performed according to Laemmli (1970), using a 12.5% polyacrylamide gel. The gel was stained with Coomassie blue R-250 (Sigma). (Lane A) Molecular weight markers: *E. coli* methionyl-tRNA synthetase (M_r 76K); trypsin-modified *E. coli* methionyl-tRNA synthetase (M_r 64K); ovalbumin (M_r 45K); bovine erythrocyte carbonic anhydrase (M_r 31K); soybean trypsin inhibitor (M_r 21.5K); horse heart cytochrome *c* (M_r 12.5K). (Lane B) Purified *E. coli* diadenosinetetraphosphatase.

successive HPLC steps on TSK 3000 separated the enzyme from the ultimate contaminating proteins. The relative yields of these chromatographic steps were both of the order of 90%. However, marked losses of activity were observed during the concentration of the samples by ammonium sulfate precipitation before each HPLC step.

Metal Ion Requirements of Diadenosinetetraphosphatase. Homogeneous diadenosinetetraphosphatase converted Ap_4A into ADP, whatever the assay conditions used here.

The rate of production of ADP in 50 mM Tris-HCl buffer (pH 7.8) containing 50 μM Ap_4A was reduced from 10 to 0.1 s^{-1} (37 °C) by adding EDTA to the reaction mixture. This reduction was completed with the addition of 0.5 μM EDTA and remained unchanged in the presence of up to 50 μM EDTA. A similar effect on the rate of the hydrolysis (1.2 s^{-1}) was observed upon treatment of the assay solution with Chelex 100 prior to the reaction. These results indicated the importance of divalent ions in sustaining the maximal rate of Ap_4A hydrolysis by the *E. coli* enzyme.

In a second set of experiments, the effect of various divalent metal ions on the enzyme activity was investigated by adding a 50 μM concentration of each metal to the assay solution systematically pretreated by Chelex 100 chromatography. The results are shown in Table II. While most of the assayed metals had no effect on the Ap_4A hydrolytic activity, clearly two metal ions, Co^{2+} and Mn^{2+}, strongly stimulated the activity (250 and 90 s^{-1}, respectively). The stimulation factors remained unchanged upon doubling the concentration of these metals. The stimulating effects of these two metal ions were not additive; i.e., adding 50 μM Mn^{2+} to an assay solution containing 50 μM Co^{2+} did not lead to further increase of the enzyme hydrolytic activity.

Finally, for evaluation of the report by Guranowski et al. (1983) of a synergistic activity of Mg^{2+} on the *E. coli* diadenosinetetraphosphatase activity sustained by cobalt, 50 μM Mg^{2+} was added to the assay solution containing either 2 μM Co^{2+} or 2 μM Mn^{2+}. As shown in Figure 3, this treatment had no significant effect on the activity of the homogeneous enzyme. At the highest Mg^{2+} concentrations, the activity

Table II: Initial Rates of AppppA Hydrolysis in the Presence of Various Divalent Cations[a]

metal ion present	rate (s^{-1})	metal ion present	rate (s^{-1})
none	1.2	Mg^{2+}	1.3
Ca^{2+}	1.1	Mn^{2+}	90
Co^{2+}	250	Ni^{2+}	3.8
Fe^{2+}	1.1	Zn^{2+}	0.5

[a] The sample (0.1 mL), buffered with 50 mM Tris-HCl (pH 7.8), contained 50 μM Ap_4A, 40 μg/mL bovine serum albumin, and catalytic amounts of *E. coli* diadenosinetetraphosphatase. For removal of divalent cations, each of the buffer and nucleotide solutions was passed through a Chelex 100 column (0.5 × 1 cm) previous to the incubation. The metal under study was added at a final concentration of 50 μM. The reaction (10 min, 37 °C) was stopped by adding $HClO_4$ (10% w/w final volume). Rates were obtained under conditions of initial velocity. Products were measured by high-performance liquid chromatography as described under Materials and Methods.

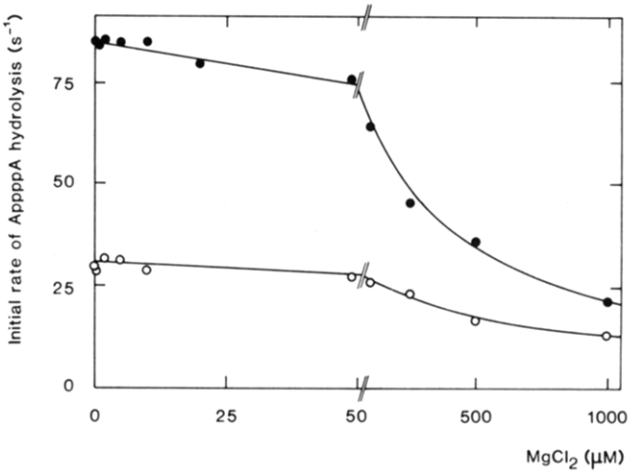

FIGURE 3: Dependence on $MgCl_2$ concentration of the initial rate of Ap_4A hydrolysis catalyzed by *E. coli* diadenosinetetraphosphatase. The sample (100 μL), buffered with 50 mM Tris-HCl (pH 7.8), contained 25 μM Ap_4A, 40 μg/mL bovine serum albumin, 2 μM $CoCl_2$ (●) or 2 μM $MnCl_2$ (○), catalytic amounts of *E. coli* diadenosinetetraphosphatase, and various $MgCl_2$ concentrations. The reaction (10 min, 37 °C) was stopped by adding $HClO_4$ (10% w/w final concentration). Products were measured by high-pressure liquid chromatography as described under Materials and Methods.

decreased, probably reflecting the competition between Co^{2+} (or Mn^{2+}) and the marked excess of Mg^{2+} toward a common target, Ap_4A or the hydrolase itself, as will be discussed further below. The discrepancy between our result and that of Guranowski et al. (1983) can be explained by the fact that these authors followed the effect of Mg^{2+} under conditions where most of the cobalt might have been complexed with the nucleotide. Magnesium addition could have stimulated the enzyme hydrolysis rate by displacing part of the cobalt complexed with Ap_4A.

Effect of Zinc Ions on Diadenosinetetraphosphatase Activity. The activity of the enzyme in the presence of 150 μM Co^{2+} (250 s^{-1}) decreased to 50 s^{-1} upon the addition of 2 μM Zn^{2+} in the assay solution. Since the possibility existed that this inhibition resulted from a time-dependent effect of the zinc ion during the incubation (10 min) of the assay, another experiment was designed where the enzyme was first incubated for 1 min in the presence of 5 μM Zn^{2+}, then diluted 10-fold in 50 mM Tris-HCl buffer (pH 7.8) with 100 μM EDTA and 200 μg/mL bovine serum albumin, and immediately assayed for 10 min after a further 5-fold dilution in the assay solution containing 150 μM $CoCl_2$ and 50 μM Ap_4A. These conditions showed an enzyme activity of 80 s^{-1}, while the control experiment with the enzyme directly assayed in the presence of 0.1 μM Zn^{2+} and 20 μM EDTA gave the normal rate of 250

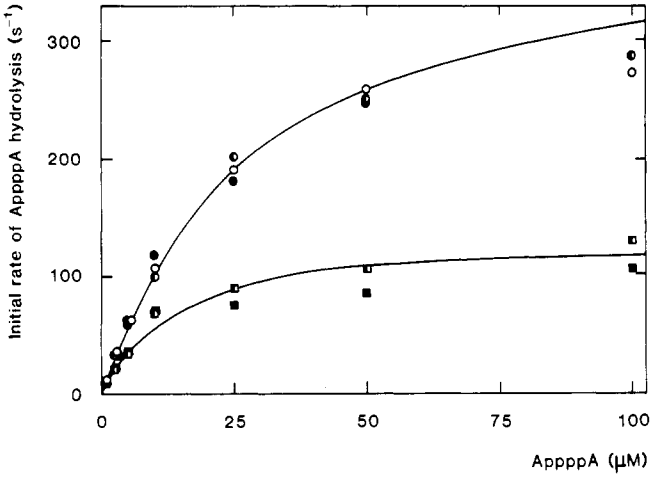

FIGURE 4: K_m value for Ap_4A. The sample (100 μL), buffered with 50 mM Tris-HCl (pH 7.8), contained 40 μg/mL bovine serum albumin, catalytic amounts of *E. coli* diadenosinetetraphosphatase, various Ap_4A concentrations, and a cobalt concentration fixed to 20 μM (●), or equal to the Ap_4A concentration plus 20 μM (◐), or equal to the double of the Ap_4A concentration plus 20 μM (○), or a manganese concentration fixed to 20 μM (■) or equal to the Ap_4A concentration plus 20 μM (◧). The reaction (10 min, 37 °C) was stopped by adding $HClO_4$ (10% w/w final concentration). Rates were obtained under conditions of initial velocity. Products were measured by high-pressure liquid chromatography as described under Materials and Methods.

s^{-1}. It could be therefore concluded that the observed inhibitory effect of zinc was not instantaneously reversible. The above effect of the incubation of the enzyme with 5 μM Zn^{2+} was eliminated, however, upon prolonged incubation (6 h) in the presence of EDTA and bovine serum albumin prior to assaying the enzyme activity.

K_m Value for Ap_4A. The initial rate of Ap_4A hydrolysis was followed as a function of Ap_4A concentration in the assay mixture (Figure 4). Since the possibility existed that the stimulating effect of cobalt resulted from its complexation to Ap_4A, Ap_4A concentration was varied from 1 to 100 μM in the presence of various cobalt conditions. In the first experiment, total cobalt concentration was fixed at 20 μM. In the others, total cobalt concentration was equal to the Ap_4A concentration plus 20 μM, or to double the Ap_4A concentration plus 20 μM. As shown in Figure 4, the rates of catalysis were the same in the three experiments, giving a K_m value for Ap_4A of 22 ± 2 μM and a maximal rate of 390 s^{-1}. The fact that the enzyme rate in the presence of a fixed 20 μM Co^{2+} concentration (close to the Ap_4A K_m value above) followed the same variation suggests that the cobalt ion acts directly on the enzyme or on the enzyme–Ap_4A complex rather than through the formation of an Ap_4A–cobalt complex. Similar results were obtained in the presence of manganese instead of cobalt. In this case, the K_m value for Ap_4A was 12 ± 2 μM and the maximal rate 130 s^{-1}.

Substrate Specificity. The initial rates of hydrolysis of nucleotides structurally related to Ap_4A were measured in the presence of either 10 μM $CoCl_2$ or 10 μM $MnCl_2$. In each case, the nucleotide concentration was 50 μM, and the products (<5 μM) were identified and quantified by high-performance liquid chromatography, as described under Materials and Methods.

As summarized in Table III, diadenosinetetraphosphatase catalyzed the cleavage of all assayed dinucleoside polyphosphates, provided the phosphate chain contained at least three phosphates. Ap_3N (for N = A, C, or G), Ap_4N, Gp_3G, Gp_3C, Gp_4G, Ap_5A, and Ap_6A were substrates, whereas Ap_2A,

Table III: Substrate Specificity of *E. coli* Diadenosinetetraphosphatase[a]

substrate	initial rate of hydrolysis (s^{-1}) Co^{2+}	Mn^{2+}	products
Ap_4A	250	89	ADP
Ap_5A	126	36	ADP + ATP
Ap_6A	50	15	ADP + Ap_4
Ap_4G	84	33	ADP + GDP
Ap_4C	85	21	ADP + CDP
Gp_4G	43	24	GDP
dAp_4dA	200	58	dADP
Ap_3A	5.2	13	ADP + AMP
Gp_3G	27	43	GDP + GMP
Ap_3C	5.1	7.4	ADP + CMP
	4.2	7.1	CDP + AMP
Ap_3G	62	31	ADP + GMP
	<1	<1	GDP + AMP
Gp_3C	50	15	CDP + GMP
	<1	<1	GDP + CMP
Ap_4	14	3.7	ADP + PP_i[b]
Ap_2A, NAD, NADP	<1	<1	
ATP, ADP, AMP			
GTP, CTP, UTP			
dATP, dGTP, dCTP, dTTP			
ATP–Mg^{2+}, GTP–Mg^{2+}[c]			
CTP–Mg^{2+}, UTP–Mg^{2+}[c]			

[a] The reaction mixture (20 or 100 μL), buffered with 50 mM Tris-HCl (pH 7.8), contained 10 μM $CoCl_2$ or 10 μM $MnCl_2$, 50 μM nucleotide, 40 μg/mL bovine serum albumin, and catalytic amounts of *E. coli* diadenosinetetraphosphatase. The reaction (5–20 min, 37 °C) was stopped by freezing the sample in liquid nitrogen. Rates were obtained under conditions of initial velocity. Products were measured by high-performance liquid chromatography as described under Materials and Methods. It was systematically verified that hydrolysis of the nucleotide was negligible (less than 1%) in a control experiment without enzyme. [b] PP_i is supposed to be the other product of Ap_4 cleavage. [c] Magnesium was added at a 50 μM concentration, stoichiometric to that of the nucleotide in the assay.

NAD, and NADP were not. A nucleoside diphosphate was always one of the products. Ap_4N was hydrolyzed into equimolar concentrations of ADP and NDP, Ap_5A into ADP plus ATP, etc. Since dAp_4dA was also a substrate, the replacement of Ap_4A riboses by deoxyriboses apparently did not change the enzyme action.

The case of Ap_3N was particular. The expected products were ADP, NDP, AMP, and NMP. This was the case with Ap_3C, where ADP, CDP, AMP, and CMP appeared at similar rates. In contrast, Ap_3G and Gp_3C were cleaved asymmetrically, the only products being ADP + GMP and CDP + GMP, respectively.

None of the assayed nucleoside triphosphates (ATP, GTP, CTP, UTP, dATP, dGTP, dCTP, and dTTP) was hydrolyzed either in the absence or in the presence of Mg^{2+}. However, Ap_4 was cleaved into ADP and, presumably, PP_i.

Finally, a possible inhibitory effect of ATP on Ap_4A hydrolysis was searched for by adding ATP to an assay containing 25 μM Ap_4A, 50 μM Co^{2+}, and 2 mM $MgCl_2$. Magnesium was added to minimize a displacement of free Co^{2+} by the possible formation of ATP–Co^{2+}, and also to mimic the probable in vivo conditions. Under such conditions, the addition of 500 μM ATP decreased the rate of Ap_4A hydrolysis by less than 20%.

With Ap_4N as well as with Gp_4G, Ap_5A, and Ap_6A, 10 μM Co^{2+} systematically sustained better rates of hydrolysis than 10 μM Mn^{2+} (Table III). In contrast, 10 μM $MnCl_2$ displayed the strongest stimulatory effect on Ap_3A or Gp_3G hydrolysis. For further comparison of the roles of these two metals toward the substrate specificity of the hydrolase, cobalt and manganese

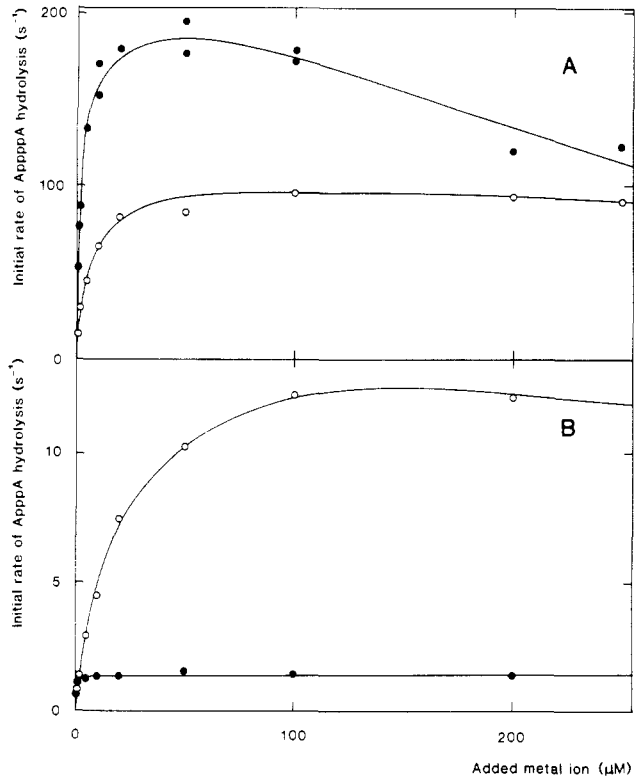

FIGURE 5: Dependence on $CoCl_2$ or $MnCl_2$ concentration of the initial rate of Ap_4A (A) and Ap_3A (B) hydrolysis by *E. coli* diadenosinetetraphosphatase. The sample (100 μL), buffered with 50 mM Tris-HCl (pH 7.8), contained 25 μM Ap_4A or Ap_3A, 0.5 μM EDTA, 40 μg/mL bovine serum albumin, catalytic amounts of *E. coli* diadenosinetetraphosphatase, and various $CoCl_2$ (●) or $MnCl_2$ (○) concentrations. The reaction (10 min, 37 °C) was stopped by adding $HClO_4$ (10% w/w final volume). Products were measured by high-performance liquid chromatography as described under Materials and Methods. The rate of Ap_4A cleavage in the absence of metal was 0.07 s^{-1}.

concentrations were varied from 0 to 250 μM in an assay solution containing 0.5 μM EDTA, and the rates of Ap_4A and Ap_3A hydrolysis were compared. As shown in Figure 5A, Ap_4A hydrolysis was stimulated better by cobalt than by manganese, whatever the concentration of the metal ion. A maximum stimulation of 2500-fold was observed at 50 μM Co^{2+} (900-fold with Mn^{2+}). Half-maximum stimulations were reached at 2.5 μM Co^{2+} and 5 μM Mn^{2+}, respectively. In contrast, the maximum rate of Ap_3A hydrolysis brought by Mn^{2+} was 9 times greater than that brought about by Co^{2+}, although Co^{2+} stimulated the hydrolysis better than Mn^{2+} at concentrations less than 1 μM (Figure 5B).

Discussion

In this study, the *E. coli* Ap_4A-hydrolyzing activity was followed in order to purify the enzyme and to describe its properties. A 5000-fold purification factor was necessary to obtain the homogeneous hydrolase. The enzyme behaves as a monomer of M_r 33K, slightly different from the molecular weight of 27K estimated by Guranowski et al. (1983) after a 400-fold purification. However, some of the properties already described by these authors, such as the mode of cleavage of dinucleoside oligophosphates and the stimulatory effect of cobalt, are also displayed by the homogeneous enzyme studied here.

As shown in this paper, the stimulation of Ap_4A hydrolysis brought about by the cobalt ion is considerable. A variation of cobalt concentration from 0 to 50 μM is capable of increasing the initial rate of hydrolysis by the enzyme from 0.1 to 250 s^{-1}. The purification enables us to estimate a diadenosinetetraphosphatase concentration in *E. coli* of 0.58 nmol/g of wet cells. By assuming 10^{12} cells per g, a cell volume of 10^{-15} L, and a water space of the *E. coli* cell of 72% (Roberts et al., 1957), the cellular Ap_4A hydrolase would be capable of hydrolyzing 200 μM Ap_4A per s provided Ap_4A and Co^{2+} are nearly saturating. In the absence of metal cofactor, it could hydrolyze 0.08 μM Ap_4A per s.

Little information is available concerning cobalt concentration in bacteria. In *E. coli* cells harvested by centrifugation late in the exponential phase of growth, Rouf (1964) was not able to detect this metal by spectrochemical analysis and excluded a cobalt concentration greater than 0.5 ppm/g dry weight cells [3 μM cellular cobalt concentration assuming that 1 g dry weight accounts for 2.9 mL of cell water, according to Roberts et al. (1957)]. However, in various archaebacteria, Scherer et al. (1983) found 20–120 ppm of cobalt per g dry weight cells (120–700 μM), using inductively coupled plasma emission spectrometry. Clearly, in the case of *E. coli*, further measurements of cobalt should be performed, particularly as a function of growth stage and of environmental stresses in order to possibly compare the cellular concentration of this metal and the Ap_4A variations observed by Bochner et al. (1984) in response to oxidizing stresses. In this context, it is tempting to relate the high concentration of cobalt in archaebacteria to the strict anaerobic conditions under which these cells were cultivated.

As shown in this study, Mn^{2+} is also capable of sustaining a marked activity of diadenosinetetraphosphatase. The concentration of this metal in *E. coli* was estimated by Rouf (1964), who measured 20 ppm of manganese per g dry weight cells (125 μM intracellular concentration). This value is in reasonable agreement with the estimation of Jones et al. (1979) for *E. coli* cultivated in basal seawater medium at 20 °C to late exponential growth. Their value (2.7 ppm/g) enables us to deduce a cellular manganese concentration of 17 μM. On the other hand, from Scherer et al. (1983), we calculate 30–250 μM manganese in a variety of archaebacteria. By comparison of this set of values to the metal requirements of the diadenosinetetraphosphatase in this study, an in vivo role of manganese in regulating the catabolism of dinucleoside tri- and tetraphosphates can be proposed, although it remains to be determined whether, in the cell, the metal is free or not.

The study of the substrate specificity of diadenosine-5′,5‴-P^1,P^4-tetraphosphate pyrophosphohydrolase from *E. coli* has revealed some properties similar to those exhibited by the corresponding enzyme from *Physarum polycephalum* (Garrison et al., 1982). Ap_4A and Gp_4G are symmetrically hydrolyzed to 2 mol of ADP and GDP, respectively. In contrast, the cleavage of Ap_4A in higher eukaryotic organisms yields ATP and AMP. Other substrates of the *E. coli* enzyme are Ap_4, Ap_3A, Ap_5A, and Ap_6A; in each case, ADP is one of the products. The enzyme also hydrolyzes all of the dinucleoside triphospates that we have examined: Ap_3A, Ap_3C, Ap_3G, Gp_3G, and Gp_3C. Ap_3G and Gp_3C are cleaved asymmetrically to ADP + GMP and CDP + GMP, respectively, whereas cleavage of Ap_3C is symmetric, giving rise to comparable concentrations of ADP + CMP and AMP + CDP. Thus, the adenosine and cytosine moieties of dinucleoside triphosphates appear to be attacked similarly, due maybe to matters of conformation.

The substrate specificity of *E. coli* diadenosinetetraphosphatase was examined in the presence of Co^{2+} or Mn^{2+}, the two metals identified as potent stimulators of the enzymic Ap_4A cleavage. This systematic comparison reveals that Co^{2+}

favors the hydrolysis of the Ap_4N series, while Mn^{2+} favors that of Ap_3A, Ap_3C, and Gp_3G. The cases of Ap_4A and Ap_3A were studied thoroughly by varying Co^{2+} and Mn^{2+} concentrations and measuring initial rates of hydrolysis. The experiment shows that the hydrolysis of Ap_3A is strongly disfavored by the absence of manganese. This point raises the possibility that the in vivo balance of the two metal ions, Co^{2+} and Mn^{2+}, may control the expression of the specificity of the hydrolase toward the set of substrates identified here. Bochner et al. (1984) have characterized different oxidizing stress conditions resulting in the marked increase in *S. typhimurium* of either Ap_4A or Ap_3A. Such responses to various stresses may reflect distinct cellular metallic contents causing the activation of different catabolic pathways of dinucleoside polyphosphates.

Another aspect of the regulation of the catabolic pathway of Ap_4A and of the related nucleotides concerns the role of zinc. A marked inhibition of Ap_4A or Ap_3A hydrolases from several eukaryotic organisms has been observed in the presence of micromolar concentrations of zinc (Cameselle et al., 1983; Jakubowski & Guranowski, 1983). This opened the possibility that the same coenzyme factor, zinc, could serve to stimulate the synthesis of Ap_4A by aminoacyl-tRNA synthetases and to block its degradation. In this study, the observation of the effect of zinc is extended to the *E. coli* hydrolase. However, it is shown that the effect of zinc on the enzyme is not simply reversible and that a prolonged incubation in the presence of excess EDTA must be applied in order to recover the initial enzyme activity. This may reflect a zinc-induced enzyme aggregation which causes an inactivation such as that described for other proteins by Mayaux & Blanquet (1981) and Goerlich & Holler (1984). Such an effect of zinc on the diadenosinetetraphosphatase activity in vivo, particularly under oxidizing conditions, deserves further investigation.

In view of the proposal of a biosynthetic pathway of Ap_4A controlled by the binding of zinc to a few aminoacyl-tRNA synthetases (Blanquet et al., 1983), the demonstration of the occurrence in cell extracts of specific Ap_4A catabolizing activities justifies the present interest for the biological function of this unusual nucleotide and the related ones. The characterization in *E. coli* of a single enzyme molecule capable of hydrolyzing all dinucleoside polyphosphates should help in the search for mutants, which are expected to have a modified Ap_4A cellular pool. Such mutants should be invaluable in the deciphering of the biological role of this nucleotide.

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References

Barnes, L. D., & Culver, C. A. (1982) *Biochemistry 21*, 6123–6128.

Blanquet, S., Plateau, P., & Brevet, A. (1983) *Mol. Cell. Biochem. 52*, 3–11.

Bochner, B. R., Lee, P. C., Wilson, S. W., Cutler, C. W., & Ames, B. N. (1984) *Cell (Cambridge, Mass.) 37*, 225–237.

Brevet, A., Plateau, P., Cirakoglu, B., Pailliez, J. P., & Blanquet, S. (1982) *J. Biol. Chem. 257*, 14613–14615.

Cameselle, J. C., Costas, M. J., Sillero, M. A. G., & Sillero, A. (1982) *Biochem. J. 201*, 405–410.

Cameselle, J. C., Costas, M. J., Sillero, M. A. G., & Sillero, A. (1983) *Biochem. Biophys. Res. Commun. 113*, 717–722.

Cameselle, J. C., Costas, M. J., Sillero, M. A. G., & Sillero, A. (1984) *J. Biol. Chem. 259*, 2879–2885.

Cassio, D., & Waller, J. P. (1971) *FEBS Lett. 12*, 309–312.

Flodgaard, H., & Klenow, H. (1982) *Biochem. J. 208*, 737–742.

Garrison, P. N., & Barnes, L. D. (1984) *Biochem. J. 217*, 805–811.

Garrison, P. N., Roberson, G. M., Culver, C. A., & Barnes, L. D. (1982) *Biochemistry 21*, 6129–6133.

Goerlich, O., & Holler, E. (1984) *Biochemistry 23*, 182–190.

Goerlich, O., Foeckler, R., & Holler, E. (1982) *Eur. J. Biochem. 126*, 135–142.

Gilmour, S. J., & Warner, A. H. (1978) *J. Biol. Chem. 253*, 4960–4965.

Grummt, F. (1983) *Plant Mol. Biol. 2*, 41–44.

Guranowski, A., Jakubowski, H., & Holler, E. (1983) *J. Biol. Chem. 258*, 14784–14789.

Höhn, M., Albert, W., & Grummt, F. (1982) *J. Biol. Chem. 257*, 3003–3006.

Jakubowski, H. (1983) *Acta Biochim. Pol. 30*, 51–69.

Jakubowski, H., & Guranowski, A. (1983) *J. Biol. Chem. 258*, 9982–9989.

Jones, G. E., Royle, L. G., & Murray, L. (1979) *Appl. Environ. Microbiol. 38*, 800–805.

Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.

Lee, P. C., Bochner, B. R., & Ames, B. N. (1983a) *J. Biol. Chem. 258*, 6827–6834.

Lee, P. C., Bochner, B. R., & Ames, B. N. (1983b) *Proc. Natl. Acad. Sci. U.S.A. 80*, 7496–7500.

Lobaton, C. D., Sillero, M. A. G., & Sillero, A. (1975a) *Biochem. Biophys. Res. Commun. 67*, 276–279.

Lobaton, C. D., Vallejo, C. G., Sillero, A., & Sillero, M. A. G. (1975b) *Eur. J. Biochem. 50*, 495–501.

Lowry, O. H., Rosenbrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.

Lüthje, J., & Ogilvie, A. (1983) *Biochem. Biophys. Res. Commun. 115*, 253–260.

McLennan, A. G., & Prescott, M. (1984) *Nucleic Acids Res. 12*, 1609–1619.

Moreno, A., Lobaton, C. D., Sillero, M. A. G., & Sillero, A. (1982) *Int. J. Biochem. 14*, 629–634.

Nierenberg, D. W., Pogolotti, A. L., & Santi, D. V. (1980) *J. Chromatogr. 190*, 212–216.

Ogilvie, A. (1981) *Anal. Biochem. 115*, 302–307.

Ogilvie, A., & Antl, W. (1983) *J. Biol. Chem. 58*, 4105–4109.

Ogilvie, A., & Jakob, P. (1983) *Anal. Biochem. 134*, 382–392.

Plateau, P., & Blanquet, S. (1982) *Biochemistry 21*, 5273–5279.

Plateau, P., Mayaux, J. F., & Blanquet, S. (1981) *Biochemistry 20*, 4654–4662.

Plesner, P., & Kristiansen, K. (1979) in *The Regulation of Macromolecular Synthesis by Low Molecular Weight Mediators* (Koch, G., & Richter, D., Eds.) pp 233–248, Academic Press, New York.

Plesner, P., & Ottesen, M. (1980) *Carlsberg Res. Commun. 45*, 1–8.

Plesner, P., Stephenson, M. L., Zamecnik, P. C., & Bucher, N. L. R. (1979) in *Specific Eukaryotic Genes* (Engberg, J., Leick, V., Klenow, H., & Thuysen, J. H., Eds.) pp 383–393, Munksgaard, Copenhagen.

Probst, H., Hamprecht, K., & Gekeler, V. (1983) *Biochem. Biophys. Res. Commun. 110*, 688–693.

Randerath, K., Janeway, C. M., Stephenson, M. L., & Zamecnik, P. C. (1966) *Biochem. Biophys. Res. Commun. 24*, 98-105.

Rapaport, E., & Zamecnik, P. C. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 3984-3988.

Rapaport, E., Svihovec, S. K., & Zamecnik, P. C. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 2653-2657.

Roberts, R. B., Cowie, D. B., Abelson, P. H., Bolton, E. T., & Britten, R. J. (1957) in *Studies of Biosynthesis in Escherichia coli*, Carnegie Institution of Washington Publication, Washington, DC.

Rouf, M. A. (1964) *J. Bacteriol. 88*, 1545-1549.

Ryan, L. D., & Vestling, C. S. (1974) *Arch. Biochem. Biophys. 160*, 279-284.

Scherer, P., Lippert, H., & Wolff, G. (1983) *Biol. Trace Elem. Res. 5*, 149-163.

Sillero, M. A. G., Villalba, R., Moreno, A., Quintanilla, M., Lobaton, C. D., & Sillero, A. (1977) *Eur. J. Biochem. 76*, 331-337.

Traut, T. (1983) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 42*, 2809.

Vallejo, C. G., Infante, J. M., Renart, J., & Sillero, A. (1973) *Biochem. Biophys. Res. Commun. 51*, 113-118.

Vallejo, C. G., Lobaton, C. D., Quintanilla, M., Sillero, A., & Sillero, M. A. G. (1976) *Biochim. Biophys. Acta 438*, 304-309.

Weinmann-Dorsch, C., Hedl, A., Grummt, I., Albert, W., Ferdinand, F. J., Friis, R. R., Pierron, G., Moll, W., & Grummt, F. (1984) *Eur. J. Biochem. 138*, 179-185.

Zamecnik, P. C. (1983) *Anal. Biochem. 134*, 1-10.

Zamecnik, P. C., & Stephenson, M. L. (1968) in *Regulatory Mechanisms for Protein Synthesis in Mammalian Cells* (San Pietro, A., Lambory, M. R., & Kenney, P. T., Eds.) pp 3-16, Academic Press, New York.

Zamecnik, P. C., & Stephenson, M. L. (1969) in *The Role of Nucleotides for the Function and Conformation of Enzymes* (Kalckar, H. M., Klenow, H., Munch-Petersen, G., Ottesen, M., & Thuysen, J. M., Eds.) pp 276-291, Alfred Benzon Symposium I, Munksgaard Copenhagen.

Zamecnik, P. C., Stephenson, M. L., Janeway, C. M., & Randerath, K. (1966) *Biochem. Biophys. Res. Commun. 24*, 91-97.

Zamecnik, P. C., Janeway, C. M., Randerath, K., & Stephenson, M. L. (1967) in *Regulation of Nucleic Acid and Protein Biosynthesis* (Koningsberger, V. V., & Bosch, L., Eds.) pp 169-176, Elsevier, Amsterdam.

Enzyme Phosphorylation with Inorganic Phosphate Causes Ca^{2+} Dissociation from Sarcoplasmic Reticulum Adenosinetriphosphatase†

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ABSTRACT: Sarcoplasmic reticulum ATPase is phosphorylated by ATP in the presence of calcium, with a consequent reduction of the affinity of the binding sites for calcium and dissociation of the divalent cation from the enzyme. ATPase phosphorylation with P_i, on the other hand, requires prior removal of calcium from the enzyme, indicating that the energy requirement for phosphorylation of the enzyme–calcium complex can be met by ATP but not by P_i. We find that when the energy yield of the P_i reaction with the enzyme is increased by the addition of dimethyl sulfoxide to the medium, ATPase phosphorylation with P_i occurs even in the presence of calcium, and the binding sites undergo a reduction in affinity with consequent dissociation of Ca^{2+} from the enzyme, in analogy to the effect of ATP. It is thereby demonstrated experimentally that an essential step in the coupling of catalytic and transport activities is an interdependence and mutual ligand exclusion of the phosphorylation and calcium sites, in which ATP does not play a direct role. An important difference between the effects of ATP and P_i is that the former produces dissociation of Ca^{2+} inside the vesicles as the result of advancement of the catalytic cycle in the forward direction, while P_i produces dissociation of calcium into the outer medium as a consequence of equilibration of enzyme states producing a shift in the reverse direction of the enzyme cycle. These observations demonstrate how equilibration of intermediate enzyme states determines extent and direction of overall reaction flow. It is also found that in the presence of dimethyl sulfoxide ATP can undergo hydrolysis (at very low rates) even in the absence of calcium, indicating that the coupling rules are not absolute, and it is possible for the reaction flow to proceed through shunted pathways depending on the experimental conditions.

It is well established that active transport of Ca^{2+} in sarcoplasmic reticulum (SR) vesicles requires a change in orientation and a reduction of the affinity of the ATPase sites for Ca^{2+} (Inesi et al., 1978a; de Meis & Vianna, 1979). This transformation occurs as a consequence of phosphoryl transfer from ATP to an aspartyl residue of the ATPase catalytic site (Yamamoto & Tonomura, 1967; Makinose, 1969; Bastide et al., 1973; Degani & Boyer, 1973), from which the free energy required for this transformation is derived. The phosphory-

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