JOURNAL OF BACTERIOLOGY, Apr. 1987, p. 1718–1723
0021-9193/87/041718-06$02.00/0

Vol. 169, No. 4

Specific Magnesium-Dependent Diadenosine 5′, 5‴-P¹,P³-Triphosphate Pyrophosphohydrolase in *Escherichia coli*

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Received 8 August 1986/Accepted 16 January 1987

A specific Mg^{2+}-dependent bis(5′-adenosyl)-triphosphatase (EC 3.6.1.29) was purified 270-fold from *Escherichia coli*. The enzyme had a strict requirement for Mg^{2+}. Other divalent cations, such as Mn^{2+}, Ca^{2+}, or Co^{2+}, were not effective. The products of the reaction with bis(5′-adenosyl) triphosphate (Ap_3A) as the substrate were ADP and AMP in stoichiometric amounts. The K_m for Ap_3A was 12 ± 5 μM. Bis(5′-adenosyl) di-, tetra-, and pentaphosphates, NAD^+, ATP, ADP, AMP, glucose 6-phosphate, *p*-nitrophenylphosphate, bis-*p*-nitrophenylphospate, and deoxyribosylthymine-5′-(4-nitrophenylphosphate) were not substrates of the reaction. The enzyme had a molecular mass of 36 kilodaltons (as determined both by gel filtration and sodium dodecyl sulfate-polyacrylamide gel electrophoresis), an isoelectric point of 4.84 ± 0.05, and a pH optimum of 8.2 to 8.5. Zn^{2+}, a known potent inhibitor of rat liver bis(5′-adenosyl)-triphosphatase and bis(5′-guanosyl)-tetraphosphatase (EC 3.6.1.17), was without effect. The enzyme differs from the *E. coli* diadenosine 5′, 5‴-P¹, P⁴-tetraphosphate pyrophosphohydrolase which, in the presence of Mn^{2+}, also hydrolyzes Ap_3A.

The metabolism and function of bis(5′-adenosyl) tetraphosphate (Ap_4A) and bis(5′-adenosyl) triphosphate (Ap_3A) are currently being studied in several biological systems (see Zamecnik [23] and Sillero et al. [21] for reviews).

Ap_4A has been implicated in the regulation of DNA replication (1, 8), in the onset of cellular stress (11), in platelet function (7, 14), and in the control of interconversion of purine nucleotides (6, 19).

A symmetrical hydrolytic activity on Ap_4A has been characterized in *Escherichia coli* (9, 15, 20). Contrary to the enzyme from animal cells, which is strictly specific for bis(5′-nucleosidyl) tetraphosphate (12, 16, 18), the Ap_4A hydrolase from *E. coli*, purified to homogeneity (20), also cleaves bis(5′-nucleosidyl) tri-, penta-, and hexaphosphates. The substrate specificity also depends on the metal (Co^{2+} or Mn^{2+}) present in the reaction mixture. On the basis of its specificity, it is thought that this enzyme accounts for the hydrolysis of both Ap_4A and Ap_3A in *E. coli* (9, 20). We report here the purification and characterization of a new enzyme from *E. coli* which, in the presence of Mg^{2+}, cleaves Ap_3A but not Ap_4A. This enzyme will be referred to as Ap_3A-Mg hydrolase.

MATERIALS AND METHODS

Materials. Molecular weight standards, NAD^+, ATP, glucose 6-phosphate, *p*-nitrophenylphosphate, and alkaline phosphatase (EC 3.1.3.1) were from Boehringer Mannheim Biochemicals. Other substrates or nucleotides were obtained from Sigma Chemical Co. Sephacryl S-200 (superfine), Sephadex G-75 (superfine), a prepacked anion exchange Mono Q HR 5/5 column, Polybuffer exchanger PBE 94, and Polybuffer 74 were obtained from Pharmacia Fine Chemicals. DEAE-SH cellulose (0.85 meq/g) was from Serva, and Minicon B15 concentrators were from Amicon Corp.

Growth. *E. coli* LP136 (kindly provided by F. Baquero, Hospital La Paz, Madrid, Spain) was cultured in a medium containing, per liter: K_2HPO_4, 9 g; KH_2PO_4, 4.5 g; $(NH_4)_2SO_4$, 2 g; $MgSO_4 \cdot 7H_2O$, 0.31 g; and glucose, 2 g. Each 1.5 liter of growth medium was inoculated with 10 ml of a 12-h slant culture and shaken at 150 rpm on a gyratory shaker at 37°C for 12 to 14 h. The cells were harvested by centrifugation, washed with 20 mM Tris hydrochloride–50 mM KCl–0.5 mM EDTA (pH 7.5) (buffer A), and resuspended at 0.33 g (wet weight) per ml in buffer A.

Enzyme assays. The hydrolysis of Ap_3A, Ap_4A, bis(5′-adenosyl) pentaphosphate, and NAD^+ was assayed with an alkaline phosphatase-coupled method. The reaction mixture contained, in 0.2 ml: 50 mM Tris hydrochloride buffer (pH 8.0), divalent cation [1.5 mM $MgCl_2$, 1.5 mM $MnCl_2$, or 500 μM $Co(NO_3)_2$], 125 μM substrate, 1 μg of alkaline phosphatase, and extract. The reaction was stopped by the addition of 1.45 ml of a freshly prepared solution containing 6 volumes of 3.4 mM ammonium molybdate in 1 N H_2SO_4, 1 volume of 10% (wt/vol) ascorbic acid, and 1 volume of 3.7% (wt/vol) sodium dodecyl sulfate (SDS). After 20 min of incubation at 45°C, the A_{820} was determined. One nanomole of P_i was equivalent to an A_{820} of 0.015 to 0.020.

The hydrolysis of ATP, ADP, AMP, and glucose 6-phosphate by the enzyme, in the presence of Mg^{2+}, was measured by the liberation of P_i as described above but in the absence of alkaline phosphatase. Enzyme activity on *p*-nitrophenylphosphate, bis-*p*-nitrophenylphosphate, and deoxyribosylthymine-5′-(4-nitrophenylphosphate) was assayed in 50 mM Tris hydrochloride (pH 8.0)–1.5 mM $MgCl_2$ with 1 mM substrate and enzyme. The reaction was stopped by the addition of 1.3 ml of 0.2 N NaOH. The formation of *p*-nitrophenol was measured at 405 nm. All the assays were done at 37°C. One unit of activity was defined as the amount of enzyme which produced 1 μmol of product per min.

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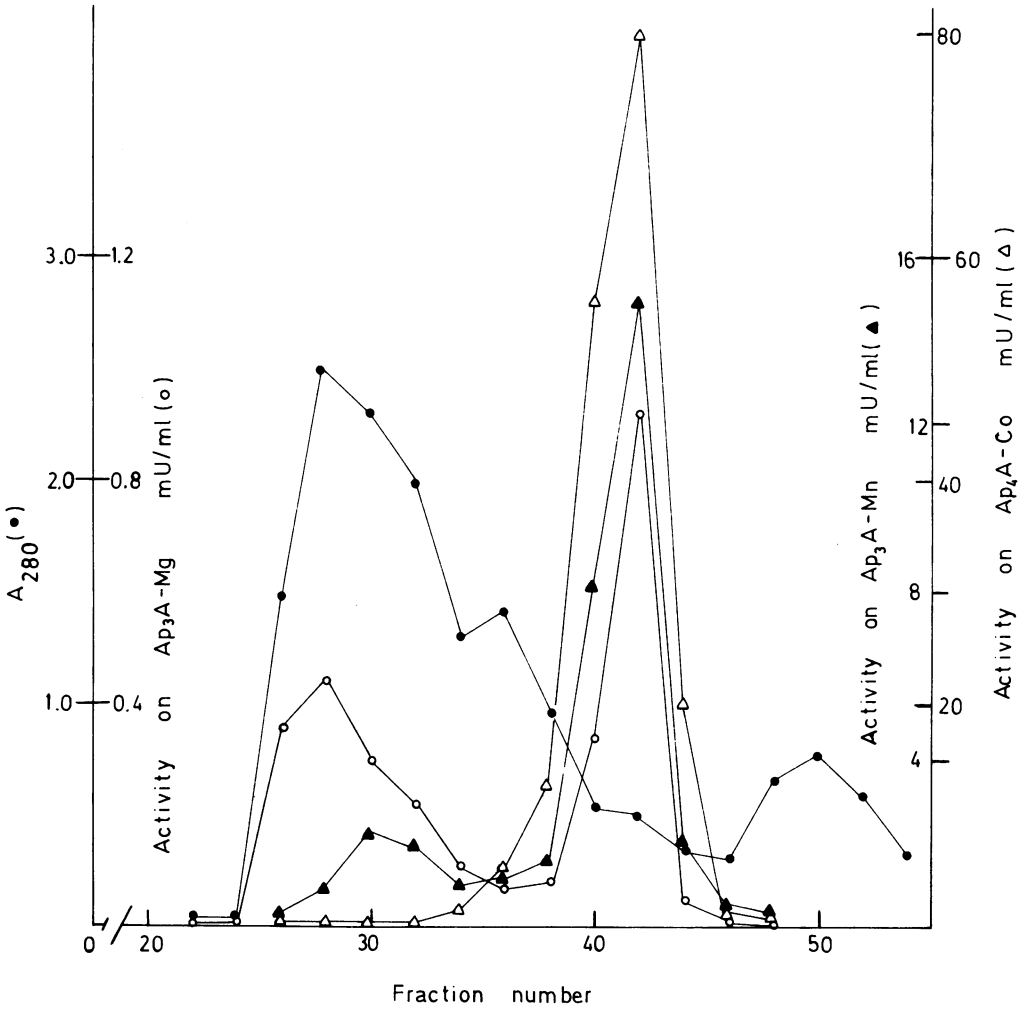

FIG. 1. Elution profiles of the Ap_3A-Mg, Ap_4A-Co, and Ap_3A-Mn activities on a Sephacryl S-200 column. Portions (10 ml) from step 2 (Table 1) were applied to the column and eluted with buffer A as described in the text.

Products of Ap_3A hydrolysis. The products of Ap_3A hydrolysis were analyzed by high-pressure liquid chromatography. Ap_3A (12.5 nmol) in a final volume of 0.1 ml was incubated for different times in the presence of 50 mM Tris hydrochloride (pH 8.0)–1.5 mM $MgCl_2$ and enzyme from step 5 (see Table 1) at 37°C. To stop the reaction, we added 0.25 ml of distilled water; the sample was then heated for 1 min at 100°C and filtered through a membrane filter (Millipore Corp.). Portions of the filtrate were injected into a Nova Pak C_{18} column (3.9 by 150 mm; Waters Associates, Inc.) and eluted with a 10-ml linear gradient of 5 to 100 mM sodium phosphate (pH 7.0) in 10 mM tetrabutylammonium–20% methanol. The absorbance was monitored with a 250-nm filter. An HP 1090 chromatograph, an HP-85B computer, and an HP 3390 A integrator were used.

Protein determination. Protein concentration was determined by the methods of Lowry et al. (13) and Bradford (2) with bovine serum albumin as a standard.

RESULTS

Purification of the enzyme. Unless otherwise stated, all operations were carried out at 4°C. *E. coli* LP136 (21.5 g [wet weight]) was suspended in 64.5 ml of buffer A and disrupted by sonification. Cell debris was removed by centrifugation at 27,000 × *g* for 30 min. The supernatant fluid was further centrifuged at 215,000 × *g* for 100 min. The 35 to 60% ammonium sulfate fraction, which contained most of the Ap_3A-Mg hydrolytic activity, was dissolved in buffer A and dialyzed overnight. The enzyme solution was adjusted to a protein concentration of 15 mg/ml; 10-ml portions were applied to a Sephacryl S-200 column (2.5 by 90 cm) and eluted with buffer A at a flow rate of 0.2 ml/min (Fig. 1). Fractions of 6.3 ml were collected. A major peak containing Ap_4A-Co, Ap_3A-Mn, and Ap_3A-Mg hydrolytic activities, fractions 39 to 43, and two minor peaks containing Ap_3A-Mg and Ap_3A-Mn hydrolytic activities were observed. This chromatographic step was repeated four times. Fractions 39 to 43 were pooled and applied to a DEAE-cellulose column (1.9 by 6.1 cm). The column was washed with 100 ml of buffer A containing 0.1 M KCl. The Ap_3A-Mg activity was eluted with a linear KCl gradient (0.1 to 0.5 M) in 20 mM Tris hydrochloride buffer (pH 7.5)–0.5 mM EDTA. The profile of Ap_3A-Mg activity was different from those of the coincident Ap_4A-Co and Ap_3A-Mn activities (Fig. 2). This was the first clear indication that the Ap_3A-Mg activity was unrelated to the Ap_4A hydrolase activity previously described (9, 20). Fractions 85 to 102 were pooled and precipitated with

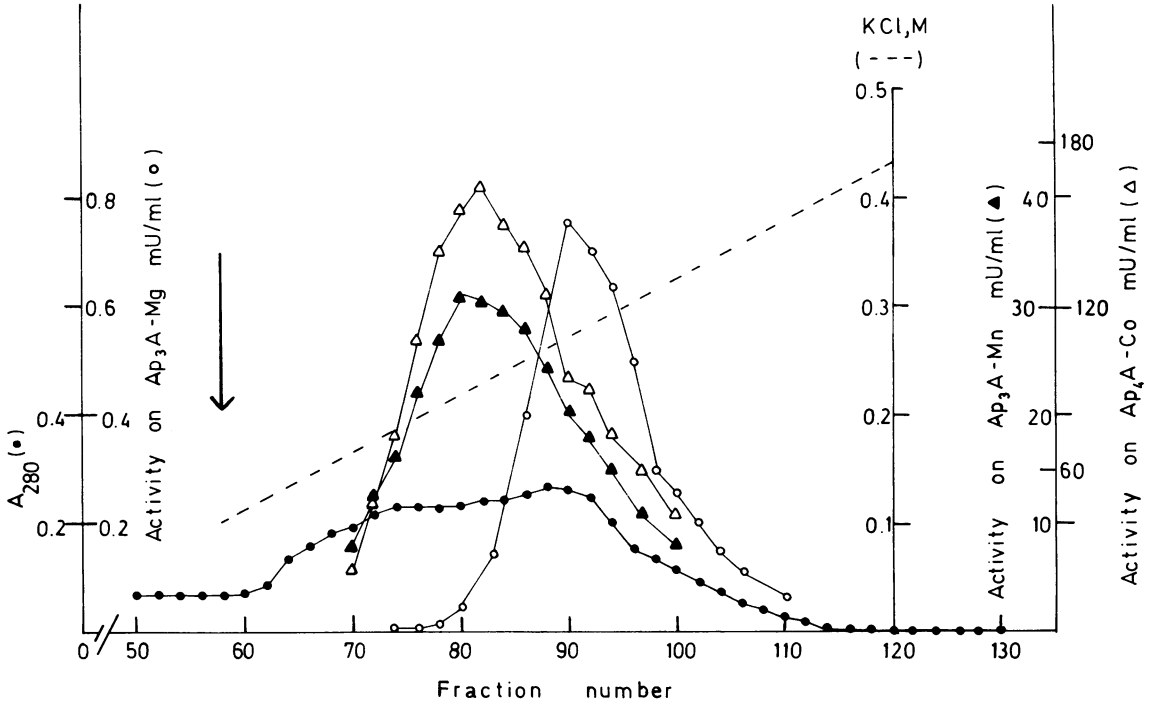

FIG. 2. Chromatography on DEAE-cellulose (step 4 of purification). A 126-ml sample from the previous step was applied to a DEAE-cellulose column equilibrated with buffer A. Unretained protein was eluted with buffer A supplemented with KCl up to 0.1 M (not shown). Ap_3A-Mg, Ap_4A-Co, and Ap_3A-Mn activities were eluted with a 200-ml linear KCl gradient (0.1 to 0.5 M) in 20 mM Tris hydrochloride buffer (pH 7.5)–0.5 mM EDTA. Fractions of 2.3 ml were collected. The arrow indicates the start of the gradient.

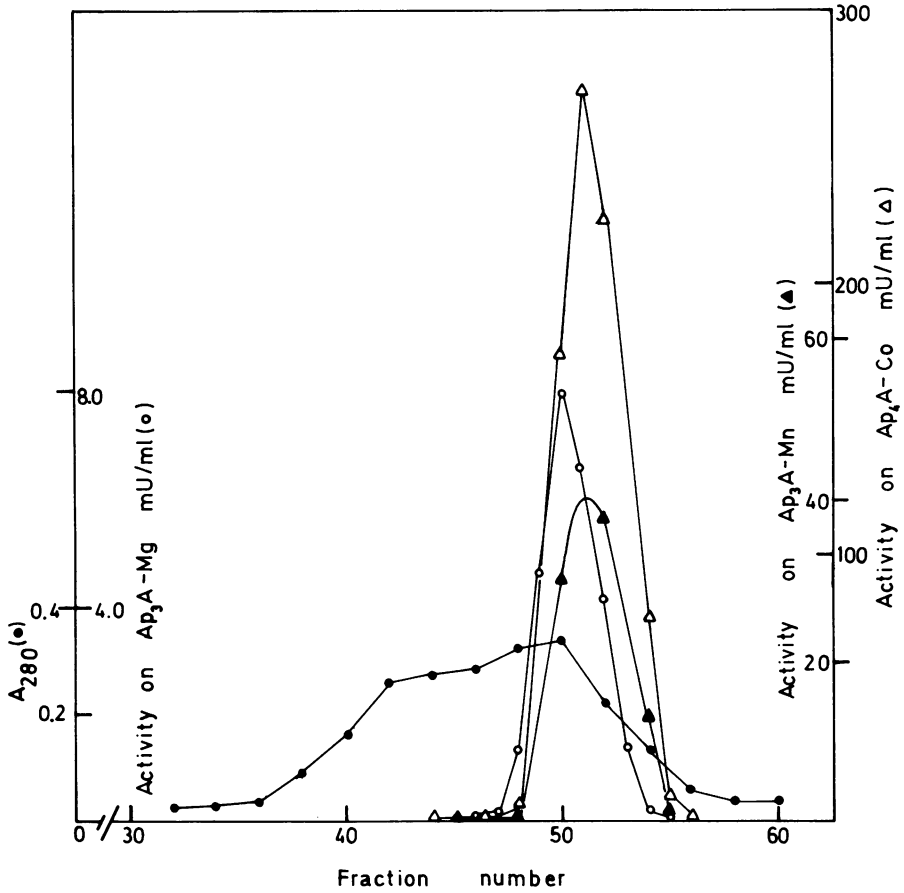

FIG. 3. Gel filtration on Sephadex G-75 (step 5 of purification). A 1.7-ml sample from the previous step and processed as described in the text was applied to a Sephadex G-75 column and eluted with buffer A.

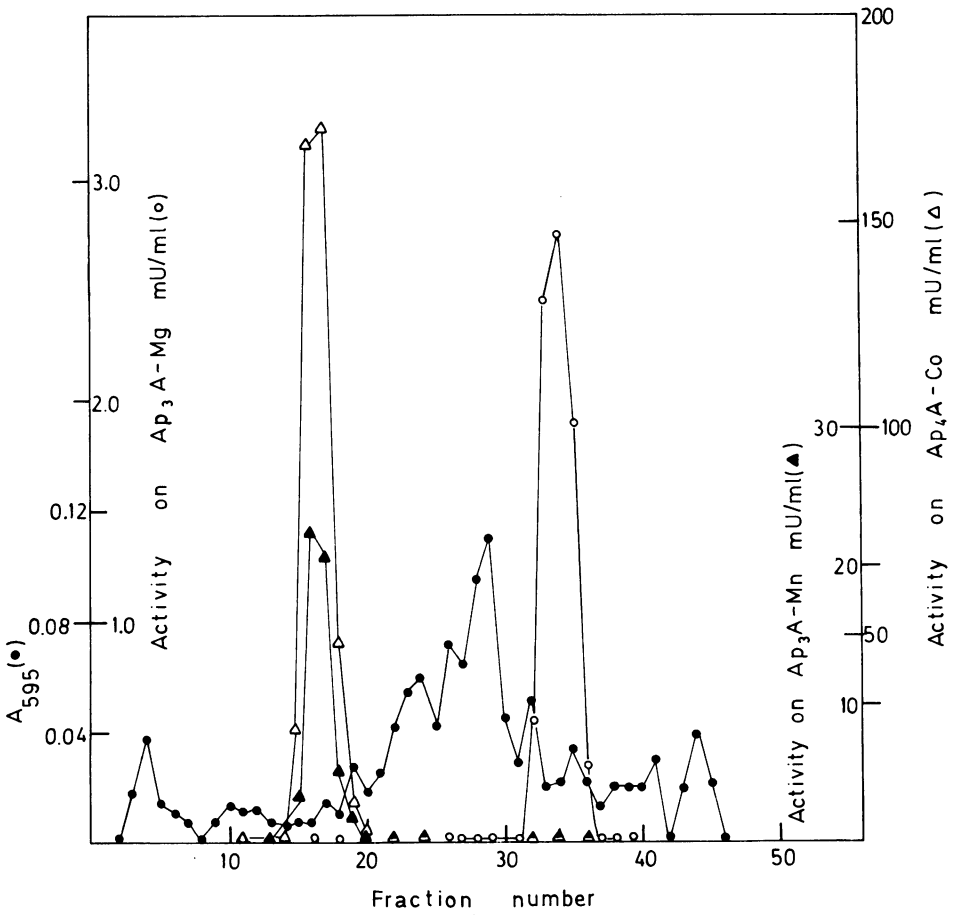

FIG. 4. High-pressure ion-exchange chromatography on a Mono Q HR 5/5 column. A 0.5-ml sample of concentrated material from step 5 of purification was injected into the column and eluted as described in the text with an HP 1090 chromatograph. Protein (●) was determined by reading the A_{595} of 25 μl of each fraction plus 1 ml of Bradford reagent (2).

ammonium sulfate at 0.8 fractional saturation. After centrifugation, the pellet was suspended in 1.8 ml of buffer A, applied to a Sephadex G-75 column (1.15 by 132 cm) equilibrated with buffer A, and eluted with the same buffer at a flow rate of 2 ml/h (Fig. 3). Fractions 48 to 53 were pooled and concentrated 10-fold with a Minicon type B15 membrane. A 0.5-ml sample (1.5 mg of protein) was injected into a Mono Q HR 5/5 column (coupled to an HP 1090 liquid chromatograph) and eluted at room temperature under a pressure of ca. 10 bar (ca. 1,000 kPa) and at a flow rate of 1 ml/min. The column, previously equilibrated with buffer A, was washed with this buffer containing 0.1 M KCl for 10 min, and the Ap_3A-Mg, Ap_3A-Mn, and Ap_4A-Co activities were eluted with 40 ml of a linear KCl gradient (0.1 to 0.4 M) in 20 mM Tris hydrochloride buffer (pH 7.5)–0.5 mM EDTA. Whereas the Ap_4A-Co and Ap_3A-Mn activities were coincident, the Ap_3A-Mg activity was clearly separable (Fig. 4). Overall, a 270-fold purification was obtained, with a yield of 4% (Table 1). Samples from purification steps 3 to 6 (Table 1) were analyzed by SDS-polyacrylamide gel electrophoresis. Two protein bands were present in the enzyme from the last step of purification (Fig. 5). As shown below, the enzyme was associated with the slower-moving band.

Substrate specificity, Michaelis constant, and products of the reaction. The following nucleotides were tested as substrates of the reaction in the presence of the indicated cofactors: Ap_3A-Mg, Ap_3A-Mn, Ap_4A-Co, Ap_4A-Mg, bis(5′-adenosyl) pentaphosphate-Mg, NAD^+-Mg, ATP-Mg, ADP-Mg, AMP-Mg, glucose 6-phosphate, *p*-nitrophenylphosphate, bis-*p*-nitrophenylphosphate, and deoxyribosylthymine-5′-(4-nitrophenylphosphate). The nucleotides with inner phosphates were assayed in the presence of alkaline phosphatase, and the metal concentration was as indicated

TABLE 1. Purification of *E. coli* Ap_3A-Mg hydrolase

Purification step	Vol (ml)	Protein (mg)[a]	Total activity (mU)	Sp act (mU/mg)	% Recovery
1. 215,000 × *g* Supernatant	58	663	315	0.47	100
2. $(NH_4)_2SO_4$ fraction	29.5	588	242	0.41	76.8
3. Sephacryl S-200 chromatography	135	115	63.4	0.55	20.1
4. DEAE-cellulose chromatography	45.3	13.6	56.6	4.16	17.9
5. Sephadex G-75 chromatography	11.8	2.4	41.9	17.4	13.3
6. Mono Q HR 5/5 chromatography	8.5	0.1	12.7	127	4.0

[a] Protein was determined by the method of Lowry et al. (13) (steps 1 to 4) or Bradford (2) (steps 5 and 6).

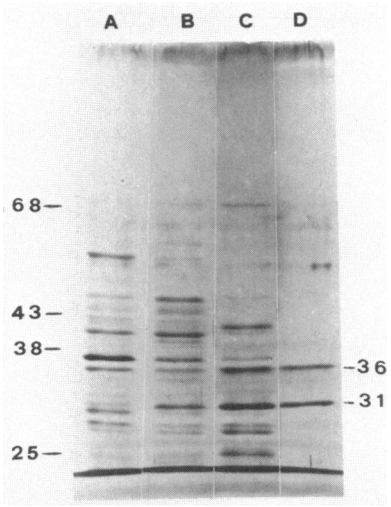

FIG. 5. SDS-polyacrylamide gel electrophoretic analysis of Ap_3A-Mg hydrolase at various stages of purification. Sample preparation and electrophoresis were performed by the method of Laemmli (10) in slabs containing 10% acrylamide and stained by the method of Morrissey (17). Lanes A to D correspond to Sephacryl S-200, DEAE-cellulose, Sephadex G-75, and Mono Q HR 5/5 steps of purification (Table 1), respectively. The standard marker proteins (masses indicated on the left [from top to bottom] in kilodaltons) were bovine serum albumin, ovalbumin, calf intestine adenosine deaminase, and α-chymotrypsinogen.

in Materials and Methods. None of the compounds tested, except for Ap_3A-Mg, were substrates of the reaction. The enzyme preparation from step 6 also hydrolyzed bis(5′-adenosyl)diphosphate-Mg at a rate of around 20% of that measured for Ap_3A-Mg. However, the activity on these two substrates corresponded to different proteins, as was apparent upon chromatofocusing on Polybuffer exchanger PBE 94 (results not shown).

The hydrolysis of Ap_3A-Mg followed typical Michaelis-Menten kinetics in the substrate concentration range 10 to 125 μM. An apparent K_m value of 12 ± 5 μM (average ± standard deviation of three determinations) was calculated. Higher K_m values (up to 100 μM) were obtained if the final KCl concentration in the assay (when enzyme solution from step 4 or 6 [Table 1] was used) was more than 50 mM. The hydrolysis of Ap_3A-Mg yielded stoichiometric amounts of ADP and AMP, as determined by high-pressure liquid chromatography on a Nova Pak C_{18} column (see Materials and Methods).

Molecular properties. The apparent molecular mass was investigated with enzyme from step 4 (Table 1) in a Sephadex G-75 column (1.15 by 132 cm) equilibrated with buffer A. The markers were bovine serum albumin (68 kilodaltons [kDa]), calf intestine adenosine deaminase (38 kDa), and cytochrome *c* (12.5 kDa). The elution volume of the Ap_3A-Mg hydrolase corresponded to a molecular mass of 36 kDa. In the same experiment, the Ap_4A hydrolase showed a molecular mass of 33 kDa, as reported previously (20). SDS-polyacrylamide gel electrophoresis of the 270-fold-purified enzyme was performed as described by Laemmli (10), and the gel was stained with the sensitive method of Morrissey (17). Two bands (36 and 31 kDa) were apparent (Fig. 5D). To ascertain which of the two bands corresponded to the Ap_3A-Mg hydrolase, we rechromatographed a pool of the three fractions with maximum enzymatic activity (Fig. 4) on the Mono Q HR 5/5 column under the same experimental conditions as in step 6 of purification, except that the KCl gradient was between 0.2 and 0.4 M. Two protein peaks were resolved, and one of them contained the Ap_3A-Mg hydrolase activity. SDS-polyacrylamide gel electrophoresis of this peak showed the presence of a unique band of 36 kDa (results not shown).

An isoelectric point of 4.84 ± 0.05 (average and ± standard deviation of six experiments) was determined by chromatofocusing on a column of Polybuffer exchanger PBE 94 (0.9 by 23 cm). The column was first equilibrated with degassed 25 mM imidazole hydrochloride buffer (pH 7.4) and then washed with 5 ml of Polybuffer 74 (diluted ninefold with glass-distilled water, adjusted to pH 4 with HCl, and degassed). Enzyme from step 4 of purification (Table 1), concentrated by ammonium sulfate precipitation and suspended in 25 mM imidazole hydrochloride (pH 7.4), was applied to the column and eluted with 210 ml of the Polybuffer 74 used in the previous wash at a flow rate of 24 ml/min. Other conditions were as previously described (5).

Other properties. The effect of varying the pH on the activity of the enzyme was monitored with Tris hydrochloride and glycine-NaOH buffers at pH 6.6 to 9.5. Maximum velocities were found at pH 8.2 to 8.5.

The reaction had a strict requirement for Mg^{2+}. A typical Michaelian curve was obtained when the enzymatic activity was assayed in the presence of various Mg^{2+} concentrations, attaining maximum velocity at around 1.5 mM. Other divalent cations, such as Mn^{2+}, Ca^{2+}, and Co^{2+}, were not effective. Zn^{2+}, a known potent inhibitor of bis(5′-adenosyl)-triphosphatase (EC 3.6.1.29) (4, 5, 22) and bis(5′-guanosyl)-tetraphosphatase (3.6.1.17) (3) from brain and rat liver, did not affect the activity of the *E. coli* Ap_3A-Mg hydrolase when present in the reaction mixture at a concentration of up to 0.1 mM.

DISCUSSION

Two publications have already reported the purification and properties of a symmetrical Ap_4A hydrolase in *E. coli* extracts (9, 20). The molecular masses of the purified enzyme were 27 kDa (9) and 33 kDa (20). In both cases, the pattern of cleavage of bis(5′-nucleosidyl) polyphosphates and its stimulation by Co^{2+} were rather similar. When the substrate specificities were studied, the hydrolysis of Ap_4A and Ap_3A was preferentially stimulated by Co^{2+} and Mn^{2+}, respectively (20). Both studies probably concerned the same enzyme, which can hydrolyze Ap_4A and Ap_3A in *E. coli* (9, 20).

Our own preliminary results suggested the occurrence of an Mg^{2+}-dependent enzyme that hydrolyzes Ap_3A and is different from the symmetrical Ap_4A hydrolase previously described (9, 20). Here we report a purification procedure which very clearly separates the Ap_3A-Mg activity from the Ap_4A-Co and Ap_3A-Mn activities. The present work both confirms previous findings related to the specificity of the Ap_4A hydrolase (9, 20) and shows the occurrence in *E. coli* extracts of a specific enzyme that hydrolyzes Ap_3A-Mg.

In summary, we have purified a bis(5′-adenosyl) triphosphatase which differs markedly from a related enzyme(s) which hydrolyses Ap_4A and was previously described (9, 20). Although both can hydrolyze Ap_3A in vitro, we are unable at present to assess the relative contributions of these enzymes to its metabolism in vivo.

ACKNOWLEDGMENTS

This investigation was supported by the Comisión Asesora de Investigación Científica y Técnica (grant 1159/84).

We thank Pablo Lozano for typing the manuscript.

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