JOURNAL OF BACTERIOLOGY, Dec. 1989, p. 6703–6709
0021-9193/89/126703-07$02.00/0
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Vol. 171, No. 12

Hydrolysis of Bis(5'-Nucleosidyl) Polyphosphates by *Escherichia coli* 5'-Nucleotidase

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Received 21 February 1989/Accepted 27 June 1989

Two enzymatic activities that split diadenosine triphosphate have been reported in *Escherichia coli*: a specific Mg-dependent bis(5'-adenosyl) triphosphatase (EC 3.6.1.29) and the bis(5'-adenosyl) tetraphosphatase (EC 3.6.1.41). In addition to the activities of these two enzymes, a different enzyme activity that hydrolyzes dinucleoside polyphosphates is described. After purification and study of its molecular and kinetic properties, we concluded that it corresponded to the 5'-nucleotidase (EC 3.1.3.5) that has been described in *E. coli*. The enzyme was purified from sonic extracts and osmotic shock fluid. From sonic extracts, two isoforms were isolated by chromatography on ion-exchange Mono Q columns; they had a molecular mass of about 100 kilodaltons (kDa). From the osmotic shock fluid, a unique form of 52 kDa was recovered. Mild heating transformed the 100-kDa isoform to a 52-kDa form, with an increase in activity of about threefold. The existence of a 5'-nucleotidase inhibitor described previously, which associates with the enzyme and is not liberated in the osmotic shock fluid, may have been responsible for these results. The kinetic properties and substrate specificities of both forms (52 and 100 kDa) were almost identical. The enzyme, which is known to hydrolyze AMP and uridine-(5')-diphospho-(1)-α-D-glucose, but not adenosine-(5')-diphospho-(1)-α-D-glucose, was also able to split adenosine-(5')-diphospho-(5)-β-D-ribose, ribose-5-phosphate, and dinucleoside polyphosphates [diadenosine 5',5'''-P^1,P^2-diphosphate,diadenosine 5',5'''-P^1,P^3-triphosphate, diadenosine 5',5'''-P^1,P^4-tetraphosphate, and bis(5'-guanosyl) triphosphate]. The effects of divalent cations and pH on the rate of the reaction with different substrates were studied.

Diadenosine 5',5'''-P^1,P^3-triphosphate (Ap_3A) and diadenosine 5',5'''-P^1,P^4-tetraphosphate (Ap_4A) are present in eucaryotes and procaryotes. The metabolism and function of these compounds have been reviewed previously (14, 30, 35, 36). They have been implicated in the control of the stress response in procaryotes (3, 19, 20) and eucaryotes (1, 5, 9, 12). A number of hydrolases have been described for the catabolism of dinucleoside polyphosphates in eucaryotes (7, 17, 27, 29, 32) and procaryotes (15, 16, 28). In *Escherichia coli*, Ap_4A is split to ADP by a symmetrical dinucleoside pyrophosphohydrolase (Ap_4A hydrolase) (EC 3.6.1.41) (15, 28). This enzyme presents a rather broad substrate specificity, hydrolyzing dinucleotides with different numbers of inner phosphates. Its substrate specificity has been assayed in the presence of Co^{2+} or Mn^{2+} (15, 28). The comparison revealed that Co^{2+} favors the hydrolysis of the Ap_4A series (tetraphosphates), whereas Mn^{2+} favors that of the Ap_3A series (triphosphates). For a time it was thought that this enzyme was responsible for the hydrolysis of both Ap_4A and Ap_3A in vivo. Later, a different enzyme was described in *E. coli* extracts which specifically cleaved Ap_3A to AMP and ADP in the presence of Mg^{2+} (EC 3.6.1.29) (16).

While investigating the nature of the Ap_3A Mn-dependent hydrolytic activities present in *E. coli* extracts obtained by sonification, we noticed that after chromatography on Sephacryl-200, two peaks of activity appeared. One of them was in the same position as the Ap_4A hydrolase (15, 16, 28), and the other one, which chromatographed in a position with a higher molecular weight (Fig. 1 in reference 16; see Fig. 1A of this report), was of an unknown nature. Here we describe the characterization of that activity on Ap_3A-Mn, which was found to be caused by the 5'-nucleotidase or UDP-glucose hydrolase described previously in *E. coli* extracts (6, 11, 13, 24, 26, 33). As shown below, this enzyme also cleaves dinucleoside polyphosphates in the presence of certain metals.

MATERIALS AND METHODS

Materials. Molecular weight standards, alkaline phosphatase (EC 3.1.3.1; catalog no. 108146), AMP, ADP, ribose-5-phosphate, and bis-*p*-nitrophenylphosphate were from Boehringer Mannheim Biochemicals. Other substrates or nucleotides were obtained from Sigma Chemical Co., except for bis(5'-guanosyl) tetraphosphate (Gp_4G), which was purified from *Artemia* cysts as described previously (34). DEAE-SH cellulose (0.85 mEq/g) was from Serva. 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.

Growth. *E. coli* LP136 (kindly provided by F. Baquero, Hospital Santiago Ramón y Cajal, Madrid, Spain) was cultured in a medium containing the following, per liter: K_2HPO_4, 9 g; KH_2PO_4, 4.5 g; $(NH_4)_2SO_4 \cdot 7H_2O$, 0.31 g; and glucose, 2 g. Each 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 suspended at 0.33 g (wet weight) per ml in buffer A. Sonic and osmotic shock extracts were obtained as described previously (16, 24).

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Enzyme assays. The hydrolysis of Ap_3A, Ap_4A, bis(5'-adenosyl) diphosphate (Ap_2A), bis(5'-guanosyl) triphosphate (Gp_3G), Gp_4G, adenosine-(5')-diphospho-(5)-β-D-ribose (ADP-Rib), adenosine-(5')-diphospho-(1)-α-D-glucose (ADP-Glc), uridine-(5')-diphospho-(1)-α-D-glucose (UDP-Glc), adenosine 5'-(α,β-methylene)-triphosphate ($AdoPCH_2PP$), and adenosine 5'-(β,γ-methylene)-triphosphate ($AdoPPCH_2P$) was assayed by measuring the P_i liberated in an alkaline phosphatase coupled method (16). The reaction mixture contained the following, in 0.2 ml: 50 mM Tris hydrochloride buffer (pH 8.0) or 50 mM morpholinoethanesulfonic acid (MES)-KOH (pH 6.0), divalent cation (as indicated), 250 μM substrate (or as indicated), 1 μg of alkaline phosphatase, and extract.

The hydrolysis of ATP, ADP, 5'-AMP, 2'-AMP, 3'-AMP, adenosine 2',5'-bisphosphate (pA2'p), adenosine 3',5'-bisphosphate (pA3'p), and ribose-5-phosphate was measured by liberation of P_i as described above, but in the absence of alkaline phosphatase. Enzyme activity on bis-*p*-nitrophenylphosphate was assayed in 50 mM Tris hydrochloride (pH 8.0)–1 mM $MnCl_2$–3 mM substrate–enzyme following the liberation of *p*-nitrophenol at 405 nm. All the assays were run at 37°C. One unit of activity was defined as the amount of enzyme which transformed 1 μmol of substrate per min. The protein concentration was determined by the method of Bradford (4), with bovine serum albumin used as a standard.

Purification of the enzyme from *E. coli* extracts obtained by sonification. *E. coli* LP136 (5 g [wet weight]) was suspended in 15 ml of buffer A and disrupted by sonification. Cell debris was removed by centrifugation at 27,000 × *g* for 30 min, and the supernatant was further centrifuged at 290,000 × *g* for 100 min and fractionated with ammonium sulfate (0.35 to 0.60 saturation). The precipitate was suspended in buffer A and dialyzed against the same buffer. A portion of 5.3 ml (133 mg of protein) was applied to a Sephacryl S-200 column (2.5 by 90 cm) and eluted with buffer A. Two peaks of activity on Ap_3A-Mn were observed (Fig. 1A). The major peak (fractions 35 to 43) has been characterized previously and contained the activities on both Ap_4A-Co (15, 28) and Ap_3A-Mg (16). Fractions 27 to 32, corresponding to the small peak of activity on Ap_3A-Mn, 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 and with 120 ml of a linear KCl gradient (0.05 to 0.5 M) in buffer B (20 mM Tris hydrochloride, 0.5 mM EDTA [pH 7.5]; data not shown). Fractions 37 to 44 (eluting at about 0.2 M KCl) from the DEAE step were pooled, desalted by filtration through a Sephadex G-25 column (1.5 by 5 cm), and equilibrated in buffer B; and a 12-ml sample (1.1 mg of protein) was injected into a Mono Q column (0.5 by 5 cm) and coupled to a high-pressure liquid chromatograph (Fig. 1B). Elution was performed under a pressure of ca. 10 bar (ca. 1,000 kPa) and at a flow rate of 1 ml/min. The column was washed with 20 ml of equilibrating buffer (0.16 M KCl in buffer B) followed by a 40-ml linear KCl gradient (0.16 to 0.26 M) in the same buffer B. Two isoforms (I and II) with Ap_3A-Mn-splitting activity were then separated (see Fig. 1B).

Purification of the enzyme from *E. coli* fluid obtained by osmotic shock. *E. coli* LP136 (11 g [wet weight]) was suspended in 10 mM Tris hydrochloride–30 mM NaCl (pH 7.5), and the osmotic shock was performed as described previously (24). The osmotic shock fluid (440 ml) was concentrated by lyophilization and suspended in buffer A to a final volume of 11.2 ml. A 10-ml portion was applied to a column of Sephacryl S-200 (90 by 2.5 cm) and eluted with buffer A.

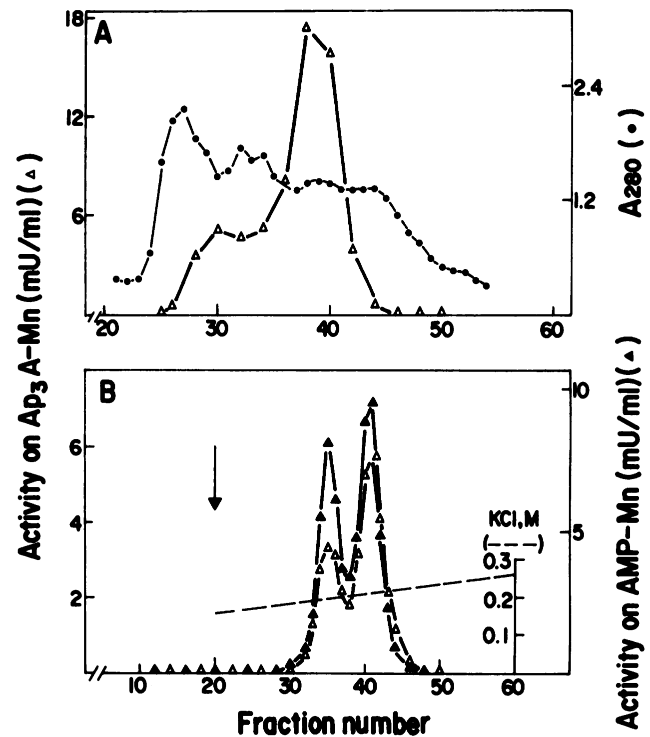

FIG. 1. Purification of Ap_3A-Mn hydrolytic activity from *E. coli* extracts obtained by sonification. An ammonium sulfate fraction (0.35 to 0.60 saturation) obtained from a 290,000 × *g* supernatant of *E. coli* LP136 (5 g [wet weight]) was successively chromatographed on columns of Sephacryl S-200 (A) DEAE-cellulose (data not shown), and Mono Q (B), as described in the text. The arrow indicates the start of the gradient.

Fractions of 6.2 ml were collected (Fig. 2A). The pooled fractions (41 to 43) from the previous step were concentrated with a Minicon type B 15 membrane and recovered in 25 mM imidazole hydrochloride (pH 7.4) by gel filtration through a Sephadex G-25 column. A sample of 2.5 ml containing 1.5 mg of protein was applied to a Polybuffer exchanger PBE 94 column (1 by 9 cm) that was equilibrated with 25 mM imidazole hydrochloride (pH 7.4). The chromatofocusing was performed by elution with 100 ml of Polybuffer 74 (diluted ninefold with water and adjusted to pH 4.0 with HCl). Fractions of 0.87 ml were collected at a flow rate of 18 ml/h (Fig. 2B). Fractions 55 to 60 were pooled, concentrated by $(NH_4)_2SO_4$ precipitation, suspended in 1.35 ml of buffer A (0.11 mg of protein), and applied to a Sephadex G-75 superfine column (1.15 by 132 cm). The enzyme was eluted with the same buffer (Fig. 2C). Fractions with activity (fractions 49 to 52) were pooled, dialyzed against buffer B, and injected into a Mono Q column (0.5 by 5 cm) that was equilibrated in the same buffer. After the column was washed with buffer B (for 15 min), the enzyme was eluted with 40 ml of a linear KCl gradient (0 to 0.1 M) in buffer B (Fig. 2D). Protein could not be detected under the peak fractions of the chromatography on the Mono Q column, even when the sensitive method of Bradford (4) was used. All operations were carried out at 4°C, except for the chromatographies on Mono Q columns, which were run at room temperature.

RESULTS

Detection and partial characterization of an Ap_3A-Mn splitting activity in sonic extracts. Initial data indicating the

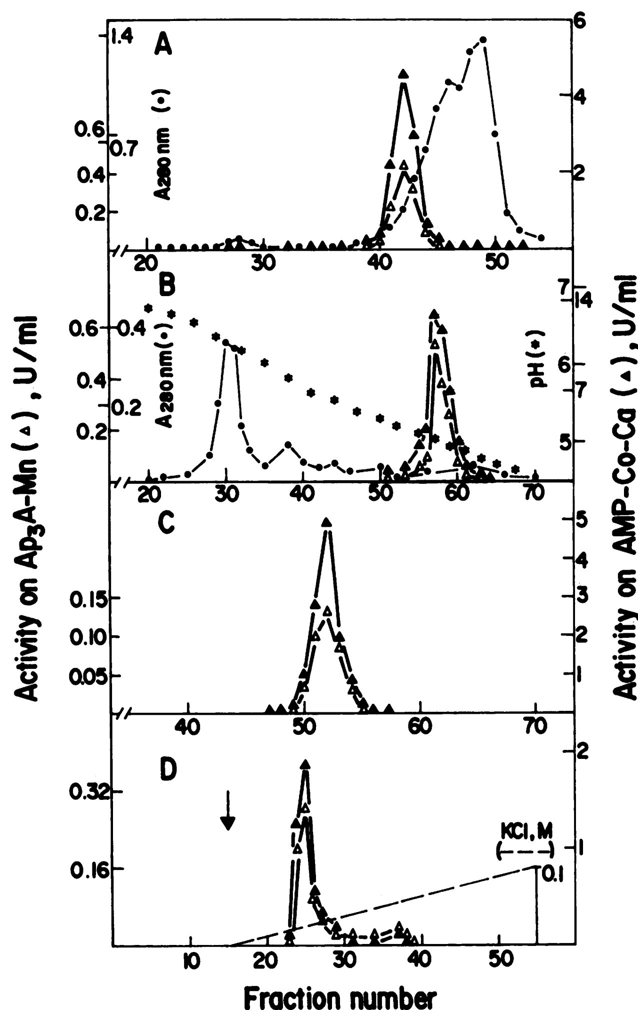

FIG. 2. Chromatographic steps in the purification of the activities on Ap_3A-Mn and AMP-Co-Ca present in the *E. coli* fluid obtained by osmotic shock. Osmotic shock fluid was obtained from *E. coli* LP136 (11 g [wet weight]) as described previously (24) and was concentrated and successively chromatographed on columns of Sephacryl S-200 (A), Polybuffer exchanger PBE 94 (B), Sephadex G-75 superfine (C), and Mono Q (D). Details are as described in the text.

presence of an Ap_3A-Mn hydrolytic activity (with a relatively high molecular weight and unrelated to both the Mg-dependent Ap_3A hydrolase [16] and to the Ap_4A hydrolase [15, 28] described previously) were gel filtration chromatographic profiles of an ammonium sulfate fraction from the 215,000 × *g* supernatants of *E. coli* sonic extracts (see Fig. 1 in reference 16 and the small peak in Fig. 1A of this report). The study for which the results are presented here was undertaken to characterize that activity.

Following standard purification procedures (see Materials and Methods and Fig. 1), two isoforms (I and II) with Ap_3A-Mn-splitting activity were separated after chromatography on a Mono Q column (Fig. 1B). A summary of a typical purification run is provided in Table 1. Both peaks were pooled separately and characterized as follows. Peaks I and II (Fig. 1B), which were assayed at pH 8.0 and in the presence of 1 mM $MnCl_2$ (see Materials and Methods), hydrolyzed not only Ap_3A (K_m, 10 μM) and other dinucleoside polyphosphates (Ap_2A, Gp_3G, Ap_4A) but also other compounds with inner phosphates (ADP-Rib, UDP-Glc, bis-*p*-nitrophenylphosphate) and even those with terminal phosphates (ATP, ADP, AMP, ribose-5-phosphate). In view of these results, the activity on AMP-Mn was also followed in the fractions obtained by Mono Q chromatography, and the elution profile was coincident with that obtained for the hydrolysis of Ap_3A-Mn (Fig. 1B).

Molecular properties. To characterize this activity further, samples from both the Sephacryl S-200 column (Fig. 1A) and from peaks I and II (Fig. 1B) were heated for 5 min at different temperatures. In all cases, the activities on both Ap_3A-Mn and AMP-Mn showed a parallel decay on heating. In those experiments it was also observed, with the material from the Sephacryl S-200 column, that the enzymatic activities on Ap_3A-Mn and AMP-Mn were elicited by heating at 50°C. This increase in activity was time dependent and reached an activation of 2.3-fold after 30 min of incubation. To try to correlate this activation with changes in the molecular properties of the enzyme, samples of Sephacryl S-200 material (Fig. 1A) that were unheated or heated at 50°C for different periods of time were analyzed by ion-exchange chromatography on a Mono Q column (Fig. 3) and by sucrose gradient centrifugation. The results showed that during heating there is a change in the molecular size of the protein from about 110 kilodaltons (kDa) to 52 kDa (data not shown) and, probably, a change in its charge, since peaks I and II were transformed to a form that was less negatively charged, as it was not retained by the Mono Q column under our working conditions. The molecular masses for peaks I and II and for the Mono Q-excluded peak were about 98 to 114, 94 to 102, and 52 kDa, respectively, as determined by sucrose gradient centrifugation, (data not shown). It seemed that mild heating could induce a change in the aggregation state of the protein or the dissociation of an inhibitory protein.

A survey of previous reports showed that the characteristics of this enzyme fit well with those of the *E. coli* 5'-nucleotidase; the uridine-diphosphate sugar hydrolase

TABLE 1. Purification of Ap_3A-Mn-dependent hydrolytic activity present in *E. coli* extracts obtained by sonification

Purification step	Vol (ml)	Protein (mg)	Total activity (mU)[a]	Sp act (mU/mg)	% Recovery
1. 290,000 × *g* supernatant	16.5	198	550	2.8	100
2. $(NH_4)_2SO_4$ fraction	6.1	154	501	3.2	91
3. Sephacryl S-200 chromatography	49.9	63.8	160	2.5	29
4. DEAE-cellulose chromatography	53.1	6.9	474	68.7	86
5. Mono Q HR 5/5 chromatography					
Peak I	37.2	0.11	89	809	16.5
Peak II	37.2	0.022	134	6,090	24.4

[a] Activity was determined at pH 8.0, as indicated in the text, in the presence of 125 μM Ap_3A and 1.5 mM $MnCl_2$; mU refers to milliunits of enzyme.

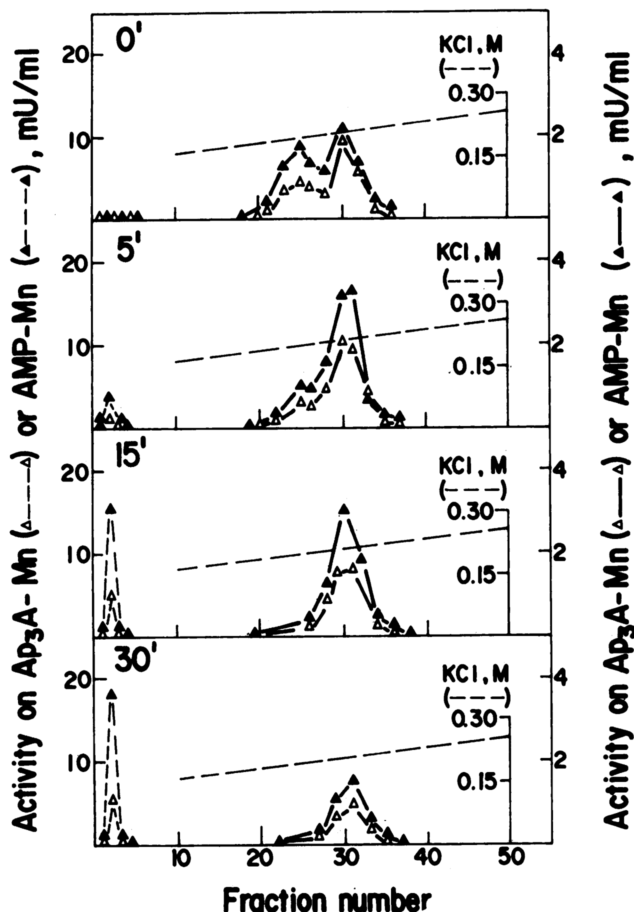

FIG. 3. Effect of heating on the molecular form of the Ap_3A-Mn hydrolytic activity eluted form the Sephacryl S-200 column. Analysis by chromatography on an ion-exchange Mono Q column. Four portions of 0.5 ml each from the pooled fractions (fractions 27 to 32) of the Sephacryl S-200 column (Fig. 1A) that were unheated or heated at 50°C for 5, 15, and 30 min were successively applied to a Mono Q column (5 by 0.5 cm) that was equilibrated in buffer B. After the column was washed with 10 ml of buffer B supplemented with 0.16 M KCl, a linear gradient of 40 ml of KCl (0.16 to 0.26 M) in buffer B was applied. Fractions of 1 ml were collected.

(EC 3.1.3.5), an enzyme that has been studied extensively by others (6, 11, 13, 24, 25); or both. This enzyme is known to have a cell surface (26) or a periplasmic (6) location and to possess a specific inhibitor of about 60 kDa in the cell cytoplasm (22, 25). When *E. coli* cells were subjected to osmotic shock, the 5'-nucleotidase was released whereas the inhibitor was not. So, while the enzyme obtained from sonic extracts had to be heated to destroy the inhibitor and to elicit activity (22, 25), the enzyme obtained from osmotic fluid was free from inhibitor and showed only slight activation by heating (24).

Isolation of the Ap_3A Mn-dependent hydrolase from *E. coli* osmotic shock fluid. In order to assess whether the activity on Ap_3A-Mn corresponded to the 5'-nucleotidase described above, the splitting activity on Ap_3A was also purified from an *E. coli* osmotic shock fluid (Fig. 2). In this case, the activities on both Ap_3A-Mn and on AMP-Co-Ca were followed in parallel. The hydrolysis of Ap_3A was assayed in the presence of 0.125 mM substrate–1.5 mM $MnCl_2$ (pH 8.0), and the activity on AMP was assessed in the presence of 0.5 mM substrate and 5 mM $CoCl_2$–20 mM $CaCl_2$ at pH 6.0 (optimal conditions that have been described previously [24] for the assay of 5'-nucleotidase). An overall purification of about 1,000-fold, with a yield of 10 and 17%, was obtained for the activities on AMP-Co-Ca and Ap_3A-Mn, respectively (see Materials and Methods, Fig. 2, and Table 2). Samples from purification steps 1 to 5 (Table 2) were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Enzyme from the last step of purification (Fig. 4, lane E) presented an intense band (58 kDa) that was enriched with respect to the starting material. We assumed that both activities (on Ap_3A-Mn and AMP-Co-Ca) were associated with the main band.

Substrate specificity and kinetic properties. To assess substrate specificity for the enzyme isolated form the osmotic shock fluid, material from step 4 (Table 2) was assayed against several substrates. The results (Table 3) were essentially the same as those obtained with the partially purified material prepared from the sonic extracts. In view of these results it can be concluded that *E. coli* 5'-nucleotidase does not only hydrolyze AMP, UDP-Glc, ADP, ATP, and bis-*p*-nitrophenylphosphate, as previously described by others (13, 24), but it is also able to hydrolyze dinucleoside polyphosphates (Ap_2A, Ap_3A, Gp_3G, and Ap_4A). The purified enzyme did not hydrolyze ADP-Glc, as has been reported previously (13).

The hydrolysis of Ap_3A-Mn (pH 8.0), UDP-Glc-Mn (pH 8.0), and AMP-Co-Ca (pH 6.0) followed typical Michaelis-Menten kinetics in the substrate concentration range from 25 to 500 μM. Apparent K_m values of 35, 45, and 45 μM, respectively were obtained for the substrates listed above under the pH conditions given in parentheses. The products of the hydrolysis of Ap_3A were investigated by high-pressure liquid chromatography (16). As expected, the final product was adenosine, but AMP and ADP were detected as intermediary products of the reaction (data not shown).

TABLE 2. Purification of the activity on Ap_3A-Mn and AMP-Co-Ca present in *E. coli* osmotic shock fluid

Purification step	Vol (ml)	Protein (mg)[a]	Total activity (U)		Sp act (U/mg)		% Recovery	
			AMP-Co-Ca	Ap_3A-Mn	AMP-Co-Ca	Ap_3A-Mn	AMP-Co-Ca	Ap_3A-Mn
1. Lyophilized osmotic shock fluid	11.2	38.3	119.6	10.4	3.1	0.27	100	100
2. Sephacryl S-200 chromatography	20.8	4.8	66.5	5.8	13.8	1.2	56	56
3. PBE 94 chromatography	11	0.21	45.1	2.5	215	11.9	38	24
4. Sephadex G-75 chromatography	12.8	33[b]	37.1	3.1	1,124	93.9	31	29
5. Mono Q HR 5/5 chromatography	12.2	~5[b]	12.2	1.8	2,440	360	10	17

[a] Protein was determined by the method of Bradford (4) (steps 1 to 4). In step 5 an estimation was made based on the intensity of the staining of the corresponding band in the gel (Fig. 4).
[b] Units of the amount of these proteins are in micrograms.

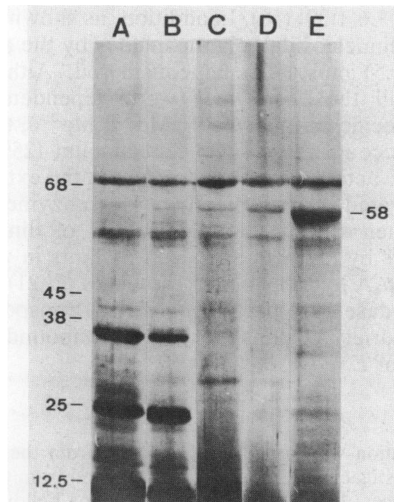

FIG. 4. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of the Ap_3A-Mn and AMP-Co-Ca hydrolytic activities at various stages of purification. Sample preparation and electrophoresis were performed by the method of Laemmli (18) in slabs containing 10% acrylamide and were stained by the method of Morrissey (23). Lanes A to E, Osmotic fluid, Sephacryl S-200, chromatofocusing, Sephadex G-75, and Mono Q steps of purification (Table 2), respectively. In the case of step 5, fractions 24 to 26 (2.9 ml) from the Mono Q column were pooled, lyophilized to dryness, and prepared for electrophoresis as described previously (18). The standard marker proteins (masses indicated on the left [from top to bottom, respectively] in kilodaltons) were bovine serum albumin, ovalbumin, calf intestine adenosine deaminase, α-chymotrypsinogen, and cytochrome *c*. The mass of the main band of the Mono Q step is indicated on the right.

$AdoPCH_2PP$ and $AdoPPCH_2P$ (which were not hydrolyzed by the enzyme [Table 3]) were tested as inhibitors of the 5'-nucleotidase by using AMP or Ap_3A as substrates and in the presence in each case of Mn^{2+} or Ca^{2+}-Ca^{2+} as cations. Both compounds were strong inhibitors of the reaction, with K_i values ranging from 0.2 to 4 μM.

TABLE 3. Substrate specificity of *E. coli* Ap_3A-Mn hydrolytic activity (5'-nucleotidase) against different substrates

Substrate	Relative activity[a]
Ap_3A	100
Gp_3G	82
Ap_4A	24
Gp_4G	0
Ap_2A	128
ADP-Rib	108
ADP-Glc	0
UDP-Glc	140
pA2'p	0
pA3'p	0
2'-AMP	0
3'-AMP	0
5'-AMP	381 (100)[b]
ADP	(93)
ATP	(53)
$AdoPCH_2PP$	0
$AdoPPCH_2P$	0
Rib-5-P	140
Bis(*p*-nitrophenylphosphate)	572

[a] Relative activities were determined at pH 8.0, as indicated in the text, in the presence of 1 mM $MnCl_2$. Activity on Ap_3A was arbitrarily set at 100.

[b] As shown in parentheses, when ADP and ATP were assayed as substrates, the P_i liberated was related to AMP, which was set at 100.

Effect of divalent cations and pH on the rate of reaction with different substrates. Experiments performed in the early phases of this investigation showed that the hydrolysis of Ap_3A in the presence of the cation Mn^{2+} occurs at an optimum pH of 8.0. The same was true when AMP was used as the substrate. This last result was in disagreement with the pH optimun of 6.0 determined by Neu (24) for the 5'-nucleotidase under the working conditions of that study, in which the reaction mixture included Co^{2+} and Ca^{2+} as divalent cations. Therefore, we reinvestigated the pH dependence of the enzymatic reactions in the presence of Mn^{2+} or Co^{2+}-Ca^{2+}. The results obtained (data not shown) were as follows. In the presence of Mn^{2+}, the dinucleotides tested (Ap_3A, Ap_4A, Ap_2A) and AMP presented an optimum pH value of about 8.0. For all those substrates, the optimum Mn^{2+} concentration in the assay mixture was about 1 mM. With UDP-Glc as the substrate, the results were different. The optimum Mn^{2+} concentration was about 0.1 mM, and in the presence of Mn^{2+} the activity increased with an increase in the pH. In the presence of Co^{2+}-Ca^{2+}, the conditions optimized by Neu (24) for AMP, a clear peak of activity occurred at pH 6.0. This was not the case with Ap_3A, Ap_2A, and UDP-Glc. With Ap_3A and Ap_2A the optimum pH range was broad (from pH 5.5 to 8.5), and with UDP-Glc the optimum pH range was between 7 and 8, as reported previously (22).

DISCUSSION

The aim of this investigation was to elucidate the nature of the enzymatic activity on the Ap_3A-Mn that was present in *E. coli* extracts that were obtained by sonification and that was unrelated to both the Ap_4A hydrolase and the Ap_3A-Mg-dependent hydrolase described previously (15, 16, 28). Studying its substrate specificity, we observed that the enzyme was able to cleave both internal phosphoanhydride and terminal phosphoester bonds. This fact, together with the characteristics of the molecular properties described above, led us to the presumption that this Ap_3A-Mn hydrolase activity could correspond to the 5'-nucleotidase (EC 3.1.3.5) described by others (6, 11, 13, 24). As this enzyme was released by osmotic shock, we undertook another purification procedure by starting with osmotic shock fluid. Under those conditions, the activities on both AMP-Co-Ca and Ap_3A-Mn were followed in parallel. In all the chromatographic steps, both activities eluted in the same position and had coincident profiles and similar yields. The molecular properties of the enzyme isolated from *E. coli* extracts obtained by sonification or by osmotic shock were different. In the first case, heating of partially purified preparations for 30 min at 50°C increased the enzymatic activity about threefold. This property had been described previously for the 5'-nucleotidase of *E. coli* and is assumed to be due to the presence of an inhibitor associated with the enzyme when *E. coli* extracts are obtained by sonification (10, 25). Here, we also showed that two isoforms (peaks I and II) are resolved by chromatography of the partially purified extracts on Mono Q columns (Fig. 1B). Both peaks had a molecular mass of about 100 kDa, as determined by sucrose gradient centrifugation. Mild heating of the material applied to the Mono Q column caused a change in the chromatographic behavior of the enzyme. In parallel with the increase in total activity, there was a gradual transformation of isoforms I and II to a different form with less affinity for the resin (Fig.

3) and a lower molecular mass (52 kDa). This behavior was compatible with the loss, on heating, of an inhibitory protein of about 60 kDa (25) that was associated with the enzyme. The occurrence of two enzyme-inhibitor complexes (Fig. 1B and 3) could have been due either to the probable existence of two isoforms for the enzyme (6, 11) or to differences in both the inhibitory protein itself or in the type of enzyme-inhibitor association.

The native enzyme isolated from osmotic shock fluid had a molecular mass of 52 kDa and was not activated by mild heating, since it was free of the inhibitory protein, which is located in the cytoplasm, and is not liberated when the 5′-nucleotidase is isolated by the osmotic shock procedure (24).

The pI of the Ap_3A Mn-dependent hydrolase was determined by chromatography on PBE 94 columns (Fig. 2B). The pI value for the enzyme purified from extracts obtained by sonification or osmotic shock were similar: 4.87 and 5.04, respectively. The molecular mass of the enzyme obtained by both procedures and after elution from the cromatofocusing PBE 94 column was about 52 kDa, indicating that during this step, the complex enzyme-inhibitor dissociated, hence, the similarity of the pI values indicated above. Recently, we have developed methods (8, 31) to calculate theoretical pI values of proteins, provided that their amino acid compositions are known. The experimental pI value (about 5.0) differed from the theoretical one (9.7) deduced from the amino acid composition described for the 5′-nucleotidase (24) and calculated as described previously (8, 31). This discrepancy can be interpreted as either an indication of the presence of impurities in the enzyme preparation, from which the amino acid composition was determined, or as being due to a particular folding of the protein that hinders part of the positively charged amino acids of the protein. In this sense, it can be calculated that a good fit between the experimental and theoretical pI values could be met if about 22% (lysines and arginines) was sequestered in the interior of the native protein.

Although it was not our aim to analyze the reaction mechanism of this enzyme, some of its more relevant properties, as deduced from the data in Table 3 and from previous publications (13, 24), are worth mentioning. The nucleotidase hydrolyzed AMP and Ap_3A, showing that the enzyme cleaves phosphoester and phosphoanhydride bonds (Table 3); this enzyme also split UDP-Glc and other uridine (5′) diphospho(1)-sugars (UDP-Gal, UDP-*N*-acetylglucosamine, and UDP-*N*-acetylgalactosamine), but not ADP-Glc (13, 24) (Table 3), thus discriminating between two similar compounds: UDP-Glc and ADP-Glc. Interestingly, it was also able to split ADP-Rib. The nucleotidase may distinguish both the nucleotide base and either the sugar joined to the terminal phosphate (glucose or ribose) or the type of union (hemiacetalic or not). Related to the importance of the residues joined to the PP_i chain, neither $AdoPCH_2PP$ nor $AdoPPCH_2P$ were substrates of the reaction. The presence of a nucleotide base did not seem to be essential for the catalysis as, in our hands, the enzyme hydrolyzed ribose-5-phosphate (Table 3), in disagreement with the results of a previous report (24). Nucleoside 3′- or 2-monophosphates were not substrates of the reaction. Neither were the nucleoside 5′-phosphate derivative substrates, such as pA2′p or pA3′p with their phosphorylated 2′ or 3′ hydroxyl group (Table 3) (24).

In sonic extracts of *E. coli*, dinucleoside polyphosphates are cleaved by at least two specific enzymes: an Ap_4A hydrolase (EC 3.6.1.41) (15, 28) and an Ap_3A Mg-dependent hydrolase (EC 3.6.1.29) (16). In addition, as shown here, the hydrolysis of dinucleoside polyphosphates by the nucleotidase (EC 3.1.3.5) must also be considered. Although the activities of all those enzymes were dependent on the presence of specific cations (Co^{2+}, Mn^{2+}, Mg^{2+}), the nucleotidase may account for a significant amount (25%) of the total hydrolytic activity on Ap_3A present in the extracts that were not activated by mild heating. This enzyme must be considered when studying the hydrolysis of dinucleoside polyphosphates by extracts of *E. coli* mutants in which the gene for the Ap_4A hydrolase is manipulated (2, 21). In vivo, the 5′-nucleotidase may participate in the transport or hydrolysis of a variety of phosphorylated compounds present in the habitat of *E. coli*.

ACKNOWLEDGMENTS

This investigation was supported by grants from the Dirección General de Investigación Científica y Técnica and from the Fundación Ramón Areces. J.M.R. holds a Severo Ochoa Fellowship from Fundación Ferrer.

We are indebted to Isabel de Diego for technical assistance.

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