Diadenosine Tetraphosphate Hydrolase from Mouse Liver

PURIFICATION TO HOMOGENEITY AND PARTIAL CHARACTERIZATION*

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An enzyme hydrolyzing diadenosine 5',5"-P1,P4'-tetraphosphate (Ap4A) to AMP and ATP has been purified to apparent homogeneity from mouse liver cell extracts. The isolation procedure comprised ammonium sulfate precipitation, chromatography on Sephadex G-75, DEAE-cellulose, blue Sepharose and AMP-Sepharose. The enzyme is a single polypeptide chain with a native Mr = 64,000 with a Kₘ of 1.66 μM and V_max of 1.25 μmol/min. AMP, ADP, Ap₃A, GTP, G₄P, Ap₃A, Ap₄A, Gp₄G, and Gp₃G are noncompetitive inhibitors of the Ap₄A hydrolase activity, whereas Gp₄G inhibits Ap₄A hydrolysis competitively with a Kᵢ of 8 μM. Theophylline, caffeine, and isobutylmethylxanthine do not or only slightly inhibit Ap₄A hydrolysis. Mitogenic factors have no effect on the enzymatic activity of Ap₄A hydrolase, excluding that a direct influence of internalized mitogens on Ap₄A degradation could be responsible for mitogen-dependent fluctuation of intracellular Ap₄A pool sizes.

Diadenosine 5',5"-P1,P4'-tetraphosphate is produced in the back reaction of the amino acid activation step of protein synthesis (1). This compound has been suggested, on the basis of its high metabolic lability and the correlation of its pool sizes with proliferative activities of eukaryotic cells, to act as a positive growth signal in these cells (2). We have recently observed that the Ap₄A pool of baby hamster kidney and mouse 3T3 fibroblasts is expanded 1000-fold after mitogenic stimulation of G₁-arrested quiescent cells. The Ap₄A concentration is elevated gradually during progression through the G₁-phase from less than 0.1 μM in early G₁ to 10 μM at the G₁/S phase boundary. Transformed and normal eukaryotic cells lacking a G₁-phase have a constitutively high Ap₄A basis level of about 0.3 μM which gradually is elevated during the S-phase to 1–10 μM.

The site of intracellular activity of Ap₄A has been shown to be the DNA replicative machinery. Addition of Ap₄A to permeabilized G₁-arrested baby hamster kidney cells yielded initiation of DNA replication in the resting cells (3, 4).

Similar stimulatory effects of Ap₄A on DNA synthesis have recently been demonstrated for adenovirus DNA replication (5, 6) and also in an in vitro replication system containing Escherichia coli DNA polymerase III holoenzyme and two additional initiation factors, F₁ and F₂ (7). Ap₄A has been demonstrated to bind as a specific ligand to calf thymus DNA polymerase α (deoxynucleosidetriphosphate:DNA deoxynucleotidyltransferase, EC 2.7.7.7) holoenzyme (M₀ = 404,000) and to affinity label a subunit of M₀ = 57,000 (8, 9). Also the high molecular weight (660,000) and low molecular weight (145,000) forms of DNA polymerase α of human HeLa cells were shown to possess a highly specific, noncovalent Ap₄A binding activity (10). These observations suggest that Ap₄A could play a role as a "second messenger" which transmits extracellular mitogenic signals into the intracellular response, i.e. onset of DNA replication. To get further insights into the metabolism of Ap₄A and to approach an understanding of the molecular mechanisms by which the Ap₄A pool is regulated, we are interested in the problem of intracellular degradation of this compound. Dinucleoside tetraphosphate-hydrolyzing enzymes have previously been described from Artemia salina and from rat liver but a purification to homogeneity was not achieved (11–13). We describe here the purification of a diadenosine tetraphosphate hydrolase to apparent homogeneity and report on some molecular and catalytic properties of this enzyme.

MATERIALS AND METHODS

Enzymes, Growth Factors, Resins, and Nucleotides—Snake venom phosphodiesterase was purchased from Boehringer Mannheim, and luciferin/luciferase (Lumit P.M.) was from Lumac. Epidermal growth factor, fibroblast growth factor, multiplication-stimulating activity, endothelial cell growth supplement, nerve growth factor, and thrombin were from Collaborative Research, Inc.; insulin and dexamethasone were from Sigma, Sephadex G-75 and Sepharose 4B were from Pharmacia, and DE52-cellulose was from Whatman. Blue Sepharose was kindly donated by Ulrich Huebscher (Stanford University School of Medicine). AMP-Sepharose was prepared according to the method of Caron et al. (14). AMP, ADP, ATP, Ap₃A, Ap₄A, Gp₄G, cGMP, and cAMP were purchased from Boehringer Mannheim; Ap₄A, Gp₄G, Gp₃G, and Gp₂G were from P-L Biochemicals.

Assay for Ap₄A Hydrolase—The assay was based on the generation of ATP and AMP by splitting Ap₄A with snake venom phosphodiesterase. ATP was assayed by the luciferin/luciferase method using the Luminometer 1250 of LKB for measuring light emission. 1-ml assay samples contained 50 μl of enzyme fraction, 200 μl of luciferin/luciferase mixture (Lumit P.M.), 2.5 μg of snake venom phosphodiesterase (2 units/ml), 100 μl of 10⁻³ M Ap₄A in 50 mM Tris/acetate buffer, pH 7.5, 5 mM Mg²⁺.

RESULTS

Purification of Mouse Liver Ap₄A Hydrolase

All operations were performed at 0–4 °C. A summary of the purification is given in Table I. The procedure yielded an apparently homogeneous enzyme after one salt and four column separation steps (Table I). Enzymatic activity was followed by assaying ATP after incu-
bation of 1 nmol of ApA with respective fractions.

Step 1: Preparation of Postribosomal Supernatant Fraction—Mouse livers (50 g) were homogenized 1:2 (w/v) in 50 mm Tris-HCl, pH 7.5, 0.5 mm EDTA, 7 mm 2-mercaptoethanol, 0.25% sucrose by a motor-driven Potter Elvehjem homogenizer with a Teflon pestle. After centrifugation at 17,000 × g for 10 min, the postmitochondrial supernatant was centrifuged for 60 min at 50,000 rpm in a Spinco rotor Ti-60. The supernatant fluid was filtered through four layers of cheese-cloth to remove lipid-like material (Fraction I, 194 ml).

Step 2: Ammonium Sulfate Precipitation—Ammonium sulfate (175 g/liter) was added to Fraction I, with stirring that was continued for 15 min. The mixture was then centrifuged at 17,000 × g for 20 min. The precipitate was discarded and 216 g/liter of ammonium sulfate were added to the supernatant fluid was filtered through four layers of cheese-cloth (Fraction II, 1322 ml).

Step 3: Sephadex G-75 Chromatography—Fraction II was applied to a 1300-ml Sephadex G-75 column (40 × 9 cm). Filtration was carried out at 2.5 ml/min using buffer A. ApA hydrolyase activity appeared at Vr/V0 = 1.06 (Fig. 1A). 71% of the protein of the ammonium sulfate fraction is separated from the ApA hydrolyase-containing fractions at this sizing step. Active fractions were pooled (Fraction III, 305 ml).

Step 4: DEAE-Cellulose Chromatography—Fraction III was loaded at 0.4 ml/min onto a 250-ml DE52-cellulose column (20 × 4 cm) equilibrated with buffer A, 20% glycerol. The column was washed with 2 column volumes of buffer A and 20% glycerol, and it was eluted with 400 ml of a linear gradient from 150 to 300 mM KC1 in buffer A, 20% glycerol. The ion exchanger binds about 4% of the protein of Fraction III, including the ApA hydrolyase, which was then eluted at 0.235 mM KC1 (Fig. 1B). Active fractions were pooled (Fraction IV, 20 ml).

Step 5: Blue Dextran-Sepharose Chromatography—Fraction IV was dialyzed overnight against 1 liter of buffer A, 20% glycerol and then loaded onto a 7.5-ml blue dextran-Sepharose column (10 × 1 cm) equilibrated with buffer A, 20% glycerol. The column was washed with 2 column volumes of buffer A, 20% glycerol, and the ApA hydrolyase was eluted with a 200-ml gradient from 350 to 550 mM KC1 in buffer A, 20% glycerol. ApA hydrolyase activity eluted between 360 and 380 mM KC1. The active fractions were pooled (Fraction V, 12 ml).

Step 6: AMP-Sepharose Chromatography—Fraction V was loaded at 0.4 ml/min onto a 250-ml AMP-Sepharose column (20 × 10 cm) equilibrated with buffer A, 20% glycerol. Adsorption, washing, and elution were as described under "Results." 50 ml of the fractions were assayed with luciferin/luciferase and 1 nmol of ApA as substrate as described under "Materials and Methods." ApA hydrolyase given in units/ml.

TABLE I

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Total protein</th>
<th>Volume</th>
<th>Total activity</th>
<th>Specific activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>I Postribosomal supernatant</td>
<td>11,058</td>
<td>194</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>II Ammonium sulfate precipitation</td>
<td>2,202</td>
<td>43</td>
<td>53.75</td>
<td>24</td>
</tr>
<tr>
<td>III Sephadex G-75</td>
<td>638</td>
<td>305</td>
<td>734</td>
<td>1,149</td>
</tr>
<tr>
<td>IV DE52-cellulose</td>
<td>24</td>
<td>20</td>
<td>317</td>
<td>1,322</td>
</tr>
<tr>
<td>V Blue dextran-Sepharose</td>
<td>5</td>
<td>12</td>
<td>120</td>
<td>25,000</td>
</tr>
<tr>
<td>VI AMP-Sepharose (immeasurable)</td>
<td>6</td>
<td>111</td>
<td>N.D.</td>
<td></td>
</tr>
</tbody>
</table>

*1 unit = amount of enzyme able to transform 1 nmol of substrate/min at room temperature.
*2 N.D., not determined.

**Fig. 1. Purification of ApA hydrolase. A, gel filtration on Sephadex G-75. B, ion-exchange chromatography with DE52-cellulose. C, affinity chromatography on blue dextran-Sepharose. D, affinity chromatography on AMP-Sepharose. Adsorption, washing, and elution were as described under "Results." 50 ml of the fractions were assayed with luciferin/luciferase and 1 nmol of ApA as substrate as described under "Materials and Methods." ApA hydrolysis given in units/ml.
dialyzed against 1 liter of buffer A, 20% glycerol and loaded onto a 2-ml AMP-Sepharose column (0.5 x 10 cm). Washing the column with 2 volumes of buffer A, 20% glycerol was followed by a linear 40-ml gradient from 5 to 1000 mM KCl in the same buffer. ApA hydrolase activity eluted at about 80 mM KCl. Active fractions were pooled (Fraction VI, 6 ml). ApA hydrolase also binds to hydroxyapatite and can be eluted at 0.11 M phosphate buffer (not shown here).

Stability and Homogeneity of ApA Hydrolase

Purified ApA hydrolase (0.5 µg/ml, in 50 mM Tris-HCl, pH 7.5, 7 mM 2-mercaptoethanol, 0.5 mM EDTA, 20% glycerol) was stable for several months at -20 °C. The enzyme lost no activity after 60 min at 30 °C but lost 30% of its activity after 60 min at 56.5 °C. When the purified enzyme (Fraction VI) was analyzed by electrophoresis in a 10% sodium dodecyl sulfate gel under denaturing and reducing conditions, a single protein band was observed after radioiodination of the protein fraction (inset in Fig. 2).

Physical Properties

A native molecular weight of about 62,000 was estimated by Sephadex G-75 gel filtration. On a 10% polyacrylamide gel, the ApA hydrolase migrated as a polypeptide of 64,000 daltons under denaturing and reducing conditions (Fig. 2). Thus, ApA hydrolase is judged to be a single polypeptide chain.

Influence of pH, Salts, and Sulfhydryl Reagents

A broad pH rate profile (maximal at pH 7.5 and half-maximal at pH values of 6 and 8.5) was observed. Experiments on the influence of mono- and divalent cations on ApA hydrolase activity revealed that ApA is best hydrolyzed in the presence of 1 mM MgSO4 and in the absence of monovalent cations. The influence of the thiol reagent N-ethylmaleimide on ApA was studied. 10 mM N-ethylmaleimide inhibited ApA hydrolysis by 93%. This result suggests that sulfhydryl groups are essential for the enzyme activity.

Km and V of ApA Hydrolase

To determine the Km and V of ApA hydrolase, kinetic studies were carried out and the data plotted according to Lineweaver and Burk (Fig. 3). A Km of 1.86 µM and V of 1.25 nmol/min were obtained for ApA hydrolysis.

Inhibition Studies

Kinetic studies with ApA hydrolase at three different substrate concentrations and in the presence of increasing amounts of AMP, ADP, adenosine tetraphosphate (ApA), GTP, guanosine tetraphosphate (GpA), ApA, ApA, GpA, GpA, respectively, have shown that these nucleotides are noncompetitive inhibitors of ApA hydrolysis (Table II). GpA, however, is a competitive inhibitor of ApA hydrolysis. Cyclic nucleotides inhibit ApA hydrolysis only partially at concentrations above 10 mM (maximum inhibition by 56% with cAMP and by 49% with cGMP).

![Fig. 2. Sodium dodecyl sulfate-polyacrylamide slab gel electrophoresis of purified ApA hydrolase.](image)

**Fig. 2.** Sodium dodecyl sulfate-polyacrylamide slab gel electrophoresis of purified ApA hydrolase. Purified ApA hydrolase (Fraction VI, 20 µl) and a set of reference proteins were reduced and denatured by heating for 2 min at 100 °C in 1% sodium dodecyl sulfate and 5% 2-mercaptoethanol prior to electrophoresis. The ApA fraction was iodinated with 125I by lactoperoxidase and glucose oxidase treatment before denaturation. Slab gel electrophoresis was conducted in 10% polyacrylamide gels (5% concentration of bisacrylamide) with 0.1% sodium dodecyl sulfate in a Tris-glycine buffer system (15). Gels were stained with Coomassie brilliant blue, dried, and autoradiographed with a Kodak X-Omat X-R5 film. Mobilities are measured relative to a bromphenol blue marker. The inset shows the molecular weight determination by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Protein standards were: phosphorylase b (94,000), bovine serum albumin (67,000), ovalbumin (42,000), carbonic anhydrase (30,000), trypsin inhibitor (20,100), and α-lactalbumin (14,400). ApA, ApA hydrolase.

**Table II**

Noncompetitive inhibition of ApA hydrolase activity by various nucleotides

Standard assays were as described under “Material and Methods.” 50 µl of ApA hydrolase (Fraction IV/V) were assayed with 5, 10, and 20 µM ApA, respectively, as substrate and various amounts of the respective inhibitors. The Ki values were derived from Dixon plots (not shown).

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Ki (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AMP</td>
<td>100</td>
</tr>
<tr>
<td>ADP</td>
<td>40</td>
</tr>
<tr>
<td>ApA</td>
<td>0.4</td>
</tr>
<tr>
<td>GTP</td>
<td>5</td>
</tr>
<tr>
<td>GpA</td>
<td>0.075</td>
</tr>
<tr>
<td>ApA</td>
<td>65</td>
</tr>
<tr>
<td>ApA</td>
<td>34</td>
</tr>
<tr>
<td>GpA</td>
<td>7.0</td>
</tr>
<tr>
<td>GpA</td>
<td>1.5</td>
</tr>
</tbody>
</table>
from mouse liver cells. Here we report on experiments carried out to purify this enzyme to apparent homogeneity and to characterize some of its physical and enzymatic properties.

In a different series of experiments, we studied whether mitogens have a direct effect on the Ap4A hydrolase activity. At concentrations of 10 and 100 ng/ml, however, none of the growth-promoting agents tested (fibroblast growth factor, epidermal growth factor, nerve growth factor, multiplication-stimulating activity, endothelial cell growth complement, insulin, thrombin, dexamethasone) has any effect on the enzyme activity.

**DISCUSSION**

The Ap4A hydrolase of mammalian cells is of special interest since the intracellular concentration of its substrate, Ap4A, was shown to fluctuate in correlation with the growth rate and the cell cycle state (2). The dramatic changes observed in the Ap4A concentration could be brought about at various regulatory levels, e.g. by control of the biosynthetic pathway, by structural modification of this compound, or by affecting Ap4A degradation. To decide whether the latter possibility is actually underlying the control mechanism determining the pool sizes of Ap4A, we started to study the Ap4A hydrolase from mouse liver cells. Here we report on experiments carried out to purify this enzyme to apparent homogeneity and to characterize some of its physical and enzymatic properties.

Our results suggest that mouse liver Ap4A hydrolase has a high degree of substrate specificity for dinucleoside tetrophosphates since only Ap4A and GpG from all the nucleotides tested are substrates of the enzyme. In contrast, all nucleotides with free phosphate groups are noncompetitive inhibitors of the Ap4A splitting reaction. The mouse enzyme described here shares this property with dinucleoside tetraphosphate-hydrolyzing enzymes found in A. salina and also from rat liver (11–13). These previously described enzymes were reported to be proteins with relatively low molecular weight (17,000–21,000). The purified mouse enzyme, however, has a molecular weight about four times higher than the enzyme isolated from rat and Artemia enzymes have not yet been purified to homogeneity, it is difficult to assess the validity of the molecular weights determined for these enzymes by gel filtration. The Ap4A-degrading enzyme hydrolizes the anhydride bond between an α and β phosphate residue in the Ap4A yielding 5‘AMP and 5‘ATP. This activity is not or only very slightly affected by inhibitors of cyclic nucleotide-specific phosphodiesterase. We also studied whether mitogens could affect the Ap4A pool size of cells by directly inhibiting Ap4A degradation on the enzyme level. However, all the mitogens and hormones tested so far did not affect the Ap4A hydrolase activity at all. This seems to rule out the possibility of direct effect on Ap4A degradation of mitogens internalized into living cells. Experiments are now in progress to study whether changes of the Ap4A hydrolase activity correlate with fluctuations of intracellular Ap4A pools at different growth and cell cycle states.

**REFERENCES**

Diadenosine tetrphosphate hydrolase from mouse liver. Purification to homogeneity and partial characterization.
M Höhn, W Albert and F Grummt


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