Diadenosine tetraphosphatase, an enzyme splitting diadenosine tetraphosphate to AMP and ATP, has been purified to apparent homogeneity from a permanent cell line derived from a leukemic child. The purification procedure consisted of fractionation by ammonium sulfate precipitation, followed by Sephacryl 200 and DEAE-cellulose chromatography, and finally a differential membrane filtration. The enzyme is a single polypeptide chain of $M_r = 17,500$ as determined by gel electrophoresis in the presence of sodium dodecyl sulfate. The apparent molecular weight of the native enzyme was calculated as 20,000 from gel filtration data. Two independent kinetic assays showed the following compounds were substrates of the enzyme: diadenosine triphosphate, NAD, nucleoside 5'-phosphates (AMP, ATP, GTP, GDP, and UTP). The enzyme had optimal activity in the presence of 1 mM Mg$^{2+}$, showing no activity in the presence of EDTA.

Several lines of evidence support the hypothesis of diadenosine 5',5''-P$^3$P$^4$-tetraphosphate to play an essential role in controlling growth and cell division. The intracellular concentration of the unusual dinucleotide fluctuates rapidly and is directly related to the proliferative activity of those cells (1). In resting permeabilized baby hamster kidney cells, the initiation of DNA replication could be stimulated by Ap4A$^i$ (2).

The possible molecular function of Ap4A was demonstrated with preparations of DNA polymerase-α from calf thymus showing specific noncovalent binding of labeled Ap4A (3) and with DNA polymerase-α from HeLa cells catalyzing DNA synthesis with utilizing Ap4A as a primer (4).

The synthesis of diadenosine tetraphosphate was discovered by Zamecnik et al. with a reaction mixture containing lysyl-tRNA synthetase, ATP, Mg$^{2+}$, and lysine, and seems to be a special property of some aminoacyl-tRNA synthetases (5-7).

A diadenosine tetraphosphate degrading enzyme (diadenosine tetraphosphatase) has been described and partially purified from rat liver (8), Artemia salina (9), Physarum polycephalum (10), and mouse ascites tumor cells (11). Here, we describe the homogeneous preparation of the enzyme from human cells and some of its characteristics.

**Experimental Procedures**

*Materials*

Tritium-labeled Ap4A (46 Ci/mmol) and diadenosine triphosphate (87 Ci/mmol) were from Amersham, mouse ascites tumor cells (11). Here, we describe tbe homogeneous preparation of the enzyme from human cells and some of its characteristics.

**Assay for Diadenosine Tetraphosphatase**

Coupled Luminescence Assay—The assay is based on luciferin-luciferase producing light with the ATP that is generated from Ap4A after diadenosine tetraphosphatase action. The assay has been described in detail (11). For determining enzyme activities in fractions of the purification steps, the assay (final volume 0.2 ml) contained 25-50 μl of luciferin-luciferase (Lumit PM; Lumac), MgCl$_2$ (5 mM), Hepes, NaOH (25 mM, pH 7.75), and 10 pmol of Ap4A (5 X 10$^{-4}$ M final concentration). Enzyme fractions (5-10 μl) were added, and the increase of luminescence was monitored with a photomultiplier (Lumacounter Model 2080; Lumac Systems, AG, Switzerland). For the determination of kinetic parameters, the concentration of Ap4A was varied as given in the legends to the figures.

Product Analysis by Thin Layer Chromatography—In a final volume of 0.03 ml, the mixture contained Hepes-NaOH (13 mM, pH 7.75), MgCl$_2$ (1 mM or otherwise indicated), $[^3H]$Ap4A (15 pmol), and diadenosine tetraphosphatase (75 ng). The reaction was started by the addition of enzyme. At different time intervals, aliquots (1 μl) were withdrawn and directly spotted on poly(ethyleneimine)-cellulose thin layer sheets. A mixture of marker nucleotides (ATP, Ap4A, ADP, and AMP) was added to each starting point. The thin layers were developed with 1 M LiCl containing acetic acid to give a pH of 4.25, dried, and washed for about 15 min in methanol under gentle shaking. After having dried the plates, a second run with 1 M LiCl was performed resulting in a clear separation of the nucleotides. The

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The abbreviations used are: Ap4A, diadenosine 5',5''-P$^3$P$^4$-tetraphosphate; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; SDS, sodium dodecyl sulfate.
nucleotides were marked under ultraviolet light and cut off. The thin layer pieces were overlaid with the scintillation mixture (toluene; 0.5% 2,5-diphenyloxazole, 0.03% 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene) and counted. Counting efficiency of tritium was 6%.

**Gel Electrophoresis**

SDS-polyacrylamide gel electrophoresis was performed in slab gels with Laemmli's buffer system (15) using a Bio-Rad apparatus (Model 220; 1.5 mm spacers). The gradient gels were polymerized (from acrylamide (7-20%; 7-30%), N,N'-diallyltartardiamide (0.27-0.8%; 0.27-1.2%), ammonium persulfate (0.05-0.016%), and N,N,N',N'-tetramethyl ethylenediamine (0.16-0.2%) and glycerol (0-10%). The separation gel (12-cm length) was overlayed by a spacer gel of about 1 cm (5% acrylamide; 0.2% N,N'-diallyltartardiamide). The electrophoresis was performed at room temperature with a constant current of 15 mA/gel for approximately 4 h. The gel was stained for protein by means of 0.1% (w/v) Coomassie blue in methanol:water:acetic acid (50:50:10) for several hours and destained in the same solution without Coomassie blue.

**RESULTS**

**Purification of Diadenosine Tetraphosphatase**—All steps were carried out at 2-4 °C unless otherwise indicated.

**Preparation of Crude Extract**—About 5 ml of packed, washed cells from the human leukemic cell line RU-3 (13) were suspended in 25 ml of phosphate-buffered saline. Disruption of cells was performed by homogenizing with a Potter-Elvehjem homogenizer equipped with a Teflon pestle for 10 min. The homogenate was first centrifuged for 15 min at 800 X g. The supernatant was again centrifuged for 1 h at 48,000 X g. The homogenization of the pellet was repeated two times. To the combined supernatants, magnesium chloride (2 mM), Na2EDTA (0.2 mM), and 2-mercaptoethanol (10 mM) were added. The enzyme was kept frozen at -25 °C.

**Ammonium Sulfate Fractionation**—Ammonium sulfate was added to the supernatant to 30% saturation. After being stirred for 1 h, the suspension was centrifuged for 15 min at 11,000 X g. To give 80% saturation, the supernatant of the previous step was again stirred with crystalline ammonium sulfate. After centrifugation, the precipitate was dissolved in 5 ml of buffer (50 mM Tris-HCl, pH 7.7, 2 mM MgCl2, 10 mM 2-mercaptoethanol, 0.2 mM Na2EDTA).

**Sephacryl 200 Chromatography**—The enzyme obtained from the previous step (22.5 mg) was applied to a Sephacryl 200 column (2.5 X 85 cm) equilibrated with buffer A. The enzyme was eluted from the column with a constant flux (15 ml/h) of buffer A. Fractions of 2.5 ml were collected and tested for enzyme activity with the coupled luminescence assay (Fig. 1). The peak of the enzyme approximately coincided with the peak of myoglobin as measured by a separate chromatography, suggesting a low molecular weight of the enzyme.

**DEAE-cellulose Chromatography**—The fractions of the Sephacryl chromatography with enzyme activity were pooled and directly applied to a DEAE-cellulose column (2.5 X 50 cm) equilibrated with buffer A. The enzyme was eluted (12 ml/h) from the column with a linear gradient of 0-0.2 M NaCl (in buffer A) for 16 h, and the fractions (3 ml) were again tested for enzyme activity and pooled (Fig. 2). This purification step seemed to be rather ineffective, yielding only a small increase in specific activity (Table I). No explanation for the lability of the enzyme during this procedure could be found. On the other hand, no further efforts have been made to stabilize the enzyme by additives, for instance albumin, etc.

**Ultrafiltration**—Using an Amicon concentrating system (8

**FIG. 1. Sephacryl 200 column chromatography of human diadenosine tetraphosphatase.** The 30-80% (NH4)2SO4 fraction of the crude enzyme (22.5 mg) was applied to a Sephacryl 200 column and chromatographed as described in the text. An aliquot (10 ml) from each fraction was tested for diadenosine tetraphosphatase activity with the coupled luminescence assay. Enzyme activity is expressed in arbitrary units (1 unit corresponds to an increase of luminescence of 10^4 cpm).

**FIG. 2. DEAE-cellulose chromatography of diadenosine tetraphosphatase from the Sephacryl 200 step.** The enzyme (9 mg) was applied to a DEAE-cellulose column and eluted by a NaCl gradient as described in the text. Aliquots (10 ml) of each fraction were assayed for diadenosine tetraphosphatase activity. Enzyme activity is expressed in arbitrary units (1 unit corresponds to an increase of luminescence of 10^4 cpm).

| Purification step | Total protein* (mg) | Total activity* (nmol/min/mg) | Specific activity* (nmol/min/mg) | Purification fold | Recovery % |
|-------------------|---------------------|------------------------------|---------------------------------|------------------|-----------|
| Cytoplasm (48,000 X g supernatant) | 33 | 49.5 | 1.5 | 0.15 | 1a | 100b |
| Ammonium sulfate (30-80%) | 22.5 | 82a | 3.6 | 0.2 | 2.5 | 16b |
| Sephacryl 200 | 9 | 45.2 | 5 | 3.3 | 91 |
| DEAE-cellulose | 1.3 | 8 | 6.2 | 4.1 | 16 |
| Amicon filtration (PM-10/UM-2) | 0.065 | 6.8 | 108 | 11.4 | 73 |

*The protein concentration of all enzyme fractions was determined by the method of Bio-Rad.

*Activity is the increase in luminescence in the standard assay at 25 °C expressed in counts/min X 10^-4, specific activity is expressed in cpm/mg X 10^-4.

*Activity is measured by following the degradation of [3H]Ap4A at 25 °C.

The purity of the S-48 fraction is taken as 1.

The activity of the S-48 fraction is taken as 100%.

The increase in total activity upon differential precipitation with ammonium sulfate is thought to be due to the removal of inhibitors of the coupled enzymatic assay, i.e. other ATP-consuming proteins.
tein staining with Coomassie blue (Fig. 3).

The residual concentrate was diluted with an equal volume of buffer and again filtered. The combined eluates were concentrated in the same apparatus equipped with a Diaflo membrane PM-10. About 50% of enzyme activity passed through the membrane and was obtained from the eluate. The eluate was free of enzymatic activity. Part of the concentrated enzyme was frozen at -25 °C, another part was diluted with an equal volume of pure glycerol and also kept at -25 °C. This preparation step was very effective, achieving about a 20-fold purification. When kept frozen, the enzyme was stable for several weeks. To characterize the enzyme, all of the following experiments were performed with this material. The complete purification procedure is summarized in Table I.

**Determination of Molecular Weight and Purity**—When examined with SDS-polyacrylamide gel electrophoresis on gradient gels as outlined under "Experimental Procedures," diadenosine tetraphosphatase showed a single band after protein staining with Coomassie blue (Fig. 3) and was judged essentially homogeneous. By comparison of this band with the migration of proteins of known molecular weight, that of denatured diadenosine tetraphosphatase (Fig. 4) appeared to be 17,500 which is the mean of several determinations.

The enzyme was also chromatographed together with molecular weight standards on Sephadex G-75 in the absence of SDS. Under these conditions, diadenosine tetraphosphatase was found to have a molecular weight of about 20,000 (Fig. 5).

**Products of the Reaction of Diadenosine Tetraphosphatase**—The hydrolysis of [³²P]Ap₄A by diadenosine tetraphosphatase was followed by product analysis on polyethyleneimine-cellulose thin layers with authentic markers as described under "Experimental Procedures." A complete degradation of Ap₄A to ATP plus AMP was obtained (Fig. 6).

Under these conditions, no indication for a symmetric cleavage or a further degradation of ATP to AMP was obtained. The difference in the counting efficiency of the two products (Fig. 6) could be explained by the more expanded spot of AMP on the thin layer plate relative to that of ATP which is caused by the much longer migration distance of AMP.

**Enzyme Kinetics**—The initial velocity of enzymatic hydrolysis of Ap₄A was measured by the coupled luminescence assay as well as by thin layer analysis of the products as described under "Experimental Procedures." The double reciprocal plots of the initial velocity versus Ap₄A concentration also shows lysozyme (1 μg; slot 1), myokinase (1.5 μg, slot 3), chymotrypsinogen (2 μg; slot 4, partially degraded), and ovalbumin (1.5 μg, slot 5).

**Fig. 3.** SDS-polyacrylamide gel electrophoresis. Purified human diadenosine tetraphosphatase (1.2 μg) was electrophoresed on a 7-20% polyacrylamide gradient gel (slot 2) as described under "Experimental Procedures." As molecular weight markers, the figure also shows lysozyme (1 μg, slot 1), myokinase (1.5 μg, slot 3), chymotrypsinogen (2 μg; slot 4, partially degraded), and ovalbumin (1.5 μg, slot 5).

**Fig. 4** (left). Molecular weight determination by SDS-polyacrylamide gel electrophoresis. Human diadenosine tetraphosphatase (Ap₄Aase) was electrophoresed together with molecular weight standards on a 7-30% polyacrylamide gradient gel as described under "Experimental Procedures." Ov, ovalbumin; Ch, chymotrypsinogen; Myok, myokinase; Pa, papain; Myoglobin, myoglobin; Ly, lysozyme; Cyt, cytochrome c. The dashed line indicates the location of diadenosine tetraphosphatase.

**Fig. 5** (center). Molecular weight determination of native diadenosine tetraphosphatase by Sephadex G-75 column chromatography. Human diadenosine tetraphosphatase or standard proteins were successively applied to a Sephadex G-75 column and chromatographed as described under "Experimental Procedures." The abbreviations used for the molecular weight standards are given in Fig. 4. The dashed line indicates the position of diadenosine tetraphosphatase.

**Fig. 6** (right). Kinetics of Ap₄A hydrolysis by human diadenosine tetraphosphatase and product analysis. The degradation of [³²P]Ap₄A by diadenosine tetraphosphatase was followed by withdrawal of aliquots of the assay at different times and chromatography on polyethyleneimine-cellulose thin layers as described under "Experimental Procedures." The sum of the radioactivity found in Ap₄A ( ), ATP ( ), ADP (Δ), and AMP ( ) was set at 100%.
Human Diadenosine Tetraphosphatase

Fig. 7. Double reciprocal plot of initial velocity studies with variable Ap4A and [3H]Ap4A concentrations. Ap4A diadenosine tetraphosphatase action was measured with the coupled luminescence assay as described under "Experimental Procedures." The concentration of Ap4A ranged from 0.14-0.9 μM. Enzyme activity is expressed in arbitrary units (1 unit corresponds to an increase of luminescence of 104 cpm). The Lineweaver-Burk plot gives a Michaelis constant of 0.5 μM. B, the degradation of [3H]Ap4A by human diadenosine tetraphosphatase was followed by thin layer chromatography as described under "Experimental Procedures." The concentration of [3H]Ap4A ranged from 0.17-1.65 μM. Enzyme activity is expressed in arbitrary units (1 unit corresponds to 0.5 pmol of Ap4A hydrolyzed/minute). The Lineweaver-Burk plot gives a Michaelis constant of 0.5 μM.

Fig. 8. Effect of magnesium concentration on the rate of Ap4A degradation by human diadenosine tetraphosphatase. The activity of the enzyme was measured following the degradation of [3H]Ap4A on thin layer plates as described under "Experimental Procedures." Activity in the absence of magnesium was measured with Na2EDTA at 0.3 mM in excess over Mg2+, which was added with the stored enzyme.

(MgCl2) m M

| M g C I2 | Activity |
|----------|----------|
| 2        | 80       |
| 1        | 60       |
| 2        | 50       |
| 1        | 40       |
| 2        | 30       |
| 1        | 20       |

The intracellular concentration of diadenosine tetraphosphate (Ap4A) is different from the diadenosine tetraphosphatase isolated from the same source (8). Other dinucleotides as NAD or GTP are also not substrates of the enzyme.

The human enzyme revealed a Michaelis constant for Ap4A of 0.5 μM as determined by two independent kinetic assays. The corresponding values reported for the partially purified enzymes from rat liver (21,000) or from A. salina (17,500) as estimated by Sephadex G-75 filtration (9). From mouse liver a diadenosine tetraphosphatase hydrolyase has been described with a molecular weight of 64,000 (16).

The discrepancy in the molecular weights of the human enzyme obtained from SDS-gel electrophoresis and from gel filtration as well as the unexpected feature of the enzyme to pass an ultrafiltration membrane with a nominal exclusion limit of 10,000 suggests a molecular size of the enzyme that is nonspherical.

In accordance with the rat liver and A. salina enzyme, diadenosine tetraphosphatase from human cells splits Ap4A asymmetrically to ATP and AMP. The enzyme shows a high specificity for diadenosine tetraphosphate. Diadenosine triphosphate is not degraded, in accordance with reports on analogous enzymes from other sources (9, 16). For diadenosine triphosphate, a specific hydrolysis has been isolated from rat liver (17). This enzyme has a molecular weight of 29,800 and is different from the diadenosine tetraphosphatase isolated from the same source (8). Other dinucleotides as NAD or nucleoside 5'-phosphates (AMP, ATP, GDP, GTP, and UTP) are also not substrates of the enzyme.

DISCUSSION

This report represents the first homogeneous purification of a human diadenosine tetraphosphatase. The apparent molecular weight as determined by SDS-polyacrylamide gel electrophoresis was 17,500. This value is in good agreement with those for the partially purified enzymes from rat liver (21,000) or from A. salina (17,500) as estimated by Sephadex G-75 filtration (9). From mouse liver a diadenosine tetraphosphate hydrolyase has been described with a molecular weight of 64,000 (16).

The magnesium requirement of diadenosine tetraphosphatase—Dialysis of the enzyme against buffer containing Na2EDTA resulted in a completely irreversible loss of enzyme activity. When Na2EDTA was present at 2-fold excess over Mg2+, no activity could be measured in the enzymatic assay, suggesting an absolute requirement for Mg2+. Enzyme activity was measured at various concentrations of MgCl2 (Fig. 8). At 0.2 mM MgCl2, the activity was about 80% of its maximal value, which is reached at 1 mM.

Substrate Specificity—The following radioactively labeled compounds were tested as substrates: diadenosine triphosphate, NAD, ATP, AMP, GTP, GDP, and UTP. In every case the hydrolysis of the potential substrates was followed for 3 h under standard conditions. Aliquots of the assays were chromatographed on polyethyleneimine-cellulose thin layers with suitable developing systems (Ap4A: H2O, 0.8 M LiCl; NAD: 0.5 M LiCl; ATP: H2O, 0.8 M LiCl; AMP: H2O, 0.8 M LiCl; GTP: 1 M LiCl; GDP: 1 M LiCl; and UTP: 0.8 M LiCl). All nucleotides were tested at two final concentrations in the assay (2 μM; 100 μM). No hydrolysis of the tested compounds could be measured during the incubation time.

The intracellular concentration of diadenosine tetraphosphate of proliferating cells was found to be in the range of 0.1-1 μM (1). Changes in metabolic conditions or inhibition of protein synthesis triggers an immediate degradation of Ap4A that is more rapid and drastic than the drop of ATP, suggesting an essential role of an Ap4A consuming activity in regul-
lating the intracellular concentration of the nucleotide. Considering the high cytoplasmic activity of diadenosine tetraphosphatase as well as its apparent Michaelis constant of 0.5 μM, the enzyme probably plays that essential role in human cells. Whether the steady state level of Ap4A could be controlled by the rate of synthesis or by modulating the activity of diadenosine tetraphosphatase is an important question to be investigated.

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REFERENCES
1. Rapaport, E., and Zamecnik, P. C. (1976) Proc. Natl. Acad. Sci. U. S. A. 73, 3984–3988
2. Grummt, F. (1978) Proc. Natl. Acad. Sci. U. S. A. 75, 371–375
3. Grummt, F., Waltl, G., Jantzen, H. M., Hamprecht, K., Huebcher, U., and Kuenzle, C. G. (1979) Proc. Natl. Acad. Sci. U. S. A. 76, 6081–6085
4. Rapaport, E., Zamecnik, P. C., and Baril, E. F. (1981) J. Biol. Chem. 256, 12148–12151
5. Zamecnik, P. C., Stephenson, M. L., Janeway, C. L., and Randeth, K. (1966) Biochem. Biophys. Res. Commun. 24, 91–97
6. Plateau, F., Mayaux, J.-F., and Blanquet, S. (1981) Biochemistry 20, 4654–4662
7. Mayaux, J.-F., and Blanquet, S. (1981) Biochemistry 20, 4647–4654
8. Lobaton, D., Vallejo, C. G., Sillero, A., and Sillero, M. A. G. (1975) Eur. J. Biochem. 50, 495–501
9. Vallejo, C. G., Lobaton, C. D., Quintanilla, M., Sillero, A., and Sillero, M. A. G. (1976) Biochim. Biophys. Acta 438, 304–309
10. Garrison, P. N., Culver, C. A., and Barnes, L. D. (1981) Fed. Proc. 40, 1905
11. Ogilvie, A. (1981) Anal. Biochem. 116, 302–307
12. Reiss, J. R., and Moffat, J. G. (1965) J. Org. Chem. 30, 3381–3387
13. Schneider, U., Schwenk, H. U., and Herzog, K. H. (1979) Klin. Pediatr. 191, 189–196
14. Bradford, M. (1977) Anal. Biochem. 72, 248–254
15. Laemmli, U. K. (1970) Nature (Lond.) 227, 680–685
16. Hohn, M., Albert, W., and Grummt, F. (1982) J. Biol. Chem. 257, 3003–3006
17. Sillero, M. A. G., Villalba, R., Moreno, A., Quintanilla, M., Lobaton, C. D., and Sillero, A. (1977) Eur. J. Biochem. 78, 331–337
Diadenosine tetraphosphatase from human leukemia cells. Purification to homogeneity and partial characterization.
A Ogilvie and W Antl

J. Biol. Chem. 1983, 258:4105-4109.

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