of the residues implicated in the crystallographic study. It is therefore possible that the assignments of the ATP site and the AMP site have been reversed in the crystallographic study illustrated in Figure 5A. While reversing the ATP and AMP sites in Figure 5A would satisfy the NMR data, it would not satisfy the observation (Hamada et al., 1979) that a peptide of residues 171–193 binds AMP. An alternative placement of ATP and AMP which would provide a better fit to the existing data is suggested in Figure 5B. The binding site for ATP partially overlaps with that proposed in the crystallographic study and is therefore consistent with the action of salicylate as a competitive inhibitor.

References

Andree, P. J. (1978) *J. Magn. Reson. 29*, 419–431.

Bothner-By, A. A., & Gassend, R. (1973) *Ann. N.Y. Acad. Sci. 222*, 668–676.

Cleland, W. W., & Mildvan, A. S. (1979) *Adv. Inorg. Biochem. 1*, 163–191.

Colowick, S. P., & Kalckar, H. M. (1943) *J. Biol. Chem. 148*, 37–45.

Dunaway-Mariano, D., & Cleland, W. W. (1980a) *Biochemistry 19*, 1496–1505.

Dunaway-Mariano, D., & Cleland, W. W. (1980b) *Biochemistry 19*, 1506–1515.

Hamada, M., & Kuby, S. A. (1978) *Arch. Biochem. Biophys. 190*, 772–792.

Hamada, M., Palmieri, R. H., Russell, G. A., & Kuby, S. A. (1979) *Arch. Biochem. Biophys. 195*, 155–177.

James, T. L. (1976) *Biochemistry 15*, 4724–4730.

Kalk, A., & Berendsen, H. J. C. (1976) *J. Magn. Reson. 24*, 343.

McDonald, G. G., Cohn, M., & Noda, L. (1975) *J. Biol. Chem. 250*, 6947–6954.

Meshitsuka, S., Smith, G. M., & Mildvan, A. S. (1981) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 40*, 1872.

Mildvan, A. S., & Gupta, R. K. (1978) *Methods Enzymol. 49G*, 322.

Mildvan, A. S., Granot, J., Smith, G. M., & Liebman, M. N. (1980) *Adv. Inorg. Biochem. 2*, 211–236.

Nageswara Rao, B. D., Cohn, M., & Noda, L. (1978) *J. Biol. Chem. 253*, 1149–1158.

Noda, L. (1973) *Enzymes, 3rd Ed. 8*, 279–305.

Pai, E. F., Sachsenheimer, W., & Schirmer, R. H. (1977) *J. Mol. Biol. 114*, 37–45.

Price, N. M., Reed, G. H., & Cohn, M. (1973) *Biochemistry 12*, 3322–3327.

Rhoads, D. G., & Lowenstein, J. M. (1968) *J. Biol. Chem. 243*, 3963–3972.

Rosevear, P. R., Smith, G. M., Meshitsuka, S., Mildvan, A. S., Desmeules, P., & Kenyon, G. L. (1981) *ACS Symp. Ser. No. 171*, 125–130.

Sachsenheimer, W., & Schulz, G. E. (1977) *J. Mol. Biol. 114*, 23–36.

Schulz, G. E., Elzinga, M., & Marx, F. (1974) *Nature (London) 250*, 120–123.

Vasak, M., Nagayama, K., & Wuthrich, K. (1979) *Biochemistry 18*, 5050–5055.

Wagner, G., & Wuthrich, K. (1979) *J. Magn. Reson. 33*, 675–680.

Isolation and Characterization of Diadenosine 5′,5‴-P^1,P^4-Tetraphosphate Pyrophosphohydrolase from *Physarum polycephalum*†

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ABSTRACT: A new enzyme that hydrolyzes diadenosine 5′,5‴-P^1,P^4-tetraphosphate has been purified by a factor of 250 from the acellular slime mold *Physarum polycephalum*. Activity was assayed radioisotopically with [^{3}H]Ap_4A. Isolation of the enzyme was facilitated by dye–ligand chromatography. The enzyme symmetrically hydrolyzes Ap_4A to ADP and exhibits biphasic kinetics for the substrate with values for the apparent K_m of 2.6 μM and 37 μM. The two values of V_{max} differ by a factor of 10. Mg^{2+}, Ca^{2+}, and other divalent cations inhibit the activity with 40–80% inhibition occurring at 0.5 mM. Mg^{2+}, at 0.5 mM, decreases both values of V_{max} by 50%, decreases the low K_m value by about 30%, and increases the high K_m value by about 100%. (Ethylenedinitrilo)tetraacetic acid (EDTA) and [ethylenebis(oxyethylenenitrilo)]tetraacetic acid (EGTA), at 10 mM, inhibit the activity by 50%. ADP, ATP, Ap_4, and Gp_4 are equipotent inhibitors with 50% inhibition occurring at 30 μM. AMP is a relatively weak inhibitor. The molecular weight of the enzyme is 26 000 on the basis of elution of activity from a calibrated Sephadex G-75 column.

Diadenosine 5′,5‴-P^1,P^4-tetraphosphate (Ap_4A)[1] is synthesized from ATP and bound aminoacyl-AMP by some tRNA synthetases from *Escherichia coli*, yeast, and *Physarum polycephalum* (Zamecnik et al., 1966; Randerath et al., 1966; Plateau et al., 1981; Goerlich et al., 1982). Broad species distribution of this reaction and its occurrence in vivo have not been demonstrated, but Ap_4A has been detected in both procaryotic and eucaryotic species at concentrations of 10^{-8}–10^{-7} M (Zamecnik, 1969; Rapaport & Zamecnik, 1976). Zamecnik and co-workers proposed that Ap_4A may be a regulator by which the status of protein synthesis influences

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[1] Abbreviations: Ap_nA, diadenosine 5′,5‴-P^1,P^n-polyphosphate, n = 2–6; Ap_4, adenosine tetraphosphate; Gp_4, guanosine tetraphosphate; EDTA, (ethylenedinitrilo)tetraacetic acid; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; TLC, thin-layer chromatography; HPLC, high-performance liquid chromatography; DEAE, diethylaminoethyl; PEI, poly(ethylenimine); NADH, reduced nicotinamide adenine dinucleotide; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-ethanesulfonic acid.

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replication or transcription (Zamecnik & Stephenson, 1969; Rapaport & Zamecnik, 1976). This proposal was based on the correlation of increases in cellular levels of Ap_4A with short doubling times of cells, viral transformation, and tumorigenicity as well as the metabolic lability of Ap_4A (Rapaport & Zamecnik, 1976). This proposal was strengthened by Grummt's demonstration that Ap_4A induced DNA replication in permeabilized baby hamster kidney cells arrested in the G_1 phase of the cell cycle (Grummt, 1978). Ap_4A also may be involved in initiation of in vitro replication of adenovirus DNA (Reiter et al., 1980). Ap_4A binds to one of the subunits of calf thymus DNA polymerase α without a detectable effect on the activity of the enzyme (Grummt et al., 1979). Rapaport et al. (1981a) recently reported that tryptophanyl-tRNA synthetase and Ap_4A binding activities are tightly associated with DNA polymerase α from HeLa cells. The Ap_4A binding protein has been resolved from the high molecular weight form of DNA polymerase α (Rapaport et al., 1981b). Ap_4A can be used as a primer with poly(dT) as a template for DNA synthesis catalyzed by DNA polymerase α from HeLa cells (Rapaport et al., 1981b). Ap_4A also serves as a primer with a double-stranded, synthetic octadecamer template that represents a segment of the replication origin of the simian virus 40 genome (Zamecnik et al., 1982). Ap_4A neither activates nor inhibits DNA polymerase α, β, or γ from mouse myeloma, but it does inhibit terminal deoxynucleotidyltransferase activity (Ono et al., 1980). Ap_4A functions as an acceptor for ADP–ribose in a reaction catalyzed by poly(ADP–ribose) polymerase in vitro (Yoshihara & Tanaka, 1981).

Ap_4A phosphohydrolases have been previously isolated from rat liver (Vallejo et al., 1973; Lobaton et al., 1975), rat intestinal mucosa (Cameselle et al., 1982), brine shrimp (Vallejo et al., 1976), mouse ascites tumor cells (Ogilvie, 1981), and mouse liver (Höhn et al., 1982). These enzymes asymmetrically hydrolyze Ap_4A to AMP and ATP and require the presence of Mg^{2+}.

We have detected the presence of Ap_4A in the slime mold *P. polycephalum* by growing microplasmodial shake cultures in the presence of [^{3}H]adenosine (Garrison et al., 1981). We also have detected an enzyme in *P. polycephalum* that hydrolyzes Ap_4A as well as other dinucleoside polyphosphates. In this report we describe the isolation and some properties of this enzyme assayed radioisotopically with [^{3}H]Ap_4A as a substrate. The symmetrical hydrolysis of Ap_4A by the *Physarum* enzyme distinguishes it from previously reported enzymes that hydrolyze Ap_4A. Preliminary accounts of part of this work have been reported (Garrison et al., 1981; Barnes et al., 1982). In the following paper we describe the substrate specificity and identify the reaction products for this enzyme using HPLC (Garrison et al., 1982).

Experimental Procedures

Materials. Ap_4A and other nucleotides were purchased from Sigma Chemical Co. Ap_4A was custom labeled by Amersham by catalytic exchange in solution with tritium gas. [^{3}H]Ap_4A was purified every 3–4 months by chromatography on an 8 mm × 20 mm column of DEAE-cellulose eluted with 350 mM NH_4HCO_3, pH 8.2. The column eluate was lyophilized, dissolved in water, and relyophilized prior to storage in 50% (v/v) ethanol at −20 °C. [^{3}H]Ap_4A was 98–99% pure as analyzed by TLC and HPLC. [^{3}H]Ap_4A was subjected to chromatography on PEI–cellulose sheets developed in 1 M LiCl in the first dimension and 2.5 M CH_3COONH_4–4% (w/v) $Na_2B_4O_7 \cdot 10H_2O$, pH 9, in the second dimension. [^{3}H]Ap_4A also was analyzed on an HPLC anion-exchange resin isocratically eluted with 0.4 M ammonium phosphate, pH 5.2, as modified from the procedure of Garrison et al. (1982). The specific activity of [^{3}H]Ap_4A was 2.5–2.6 Ci/mmol. DEAE-cellulose (microgranular DE-52) was purchased from Whatman, Sephacryl S-200 superfine from Pharmacia, and Matrex Gel Green A dye–ligand resin from Amicon Corp. Proteins used as molecular weight standards, pyruvate kinase, and protamine sulfate were purchased from Sigma Chemical Co. NADH and lactate dehydrogenase were purchased from Boehringer/Mannheim.

Culture of Physarum. P. polycephalum strain M_3C VII was maintained in shake culture as previously described (Garrison & Barnes, 1980) except that the cultures were started with 0.5 mL of plasmodial sediment in 100 mL of media in 500-mL flasks every 2 days. Microplasmodia were harvested in log-phase growth when the cell density was 20–30 g wet wt/L of medium.

Enzyme Assay. Diadenosine tetraphosphate pyrophosphchydrolase activity was assayed radioisotopically with [^{3}H]Ap_4A as the substrate. The enzyme (0.02–10 μg of protein) was incubated in 50 mM Hepes–NaOH, pH 7.5, with [^{3}H]Ap_4A for 10 min at 30 °C in a final volume of 200 μL. The reaction was initiated by the addition of [^{3}H]Ap_4A (0.5–100 μM). The reaction was stopped by adding 10 μL of 2 N HCl and incubating for 5 min at 25 °C. Three milliliters of 20 mM NH_4HCO_3, pH 8.2, was added, and the entire assay solution was added to an 8 mm × 20 mm column of DEAE-cellulose equilibrated with 20 mM NH_4HCO_3, pH 8.2. The assay tube was rinsed with 4 mL of 20 mM NH_4HCO_3, pH 8.2, and the solution added to the column. The column was rinsed with an additional 4 mL of 20 mM NH_4HCO_3, pH 8.2. Sample and wash effluents were discarded. The [^{3}H]ADP formed in the reaction was eluted with successive washes of 4, 4, and 2 mL of 120 mM NH_4HCO_3, pH 8.2. The effluents were collected in scintillation vials containing 14 mL of cocktail. Unreacted [^{3}H]Ap_4A was retained on the DEAE-cellulose under these conditions. It could be eluted with 350 mM NH_4HCO_3, pH 8.2. The eluted [^{3}H]ADP was counted at 4 °C in a gel phase of the cocktail with 30–35% efficiency in a Beckman LS-230 or 7000 counter. Enzymic activity was assayed in duplicate, and each experiment on kinetic properties was performed at least twice on enzyme purified through the Green A resin or Sephadex G-75 steps. There were no observed differences in the properties of the enzyme from these columns except preparations purified through the Sephadex G-75 column had larger V_{max} values.

Enzymic activity also was measured spectrophotometrically at 340 nm by a coupled-enzyme assay. Partially purified enzyme was assayed at 30 °C in 50 mM Hepes–NaOH, pH 7.5, 0.1 mM $MgCl_2$, 0.5 mM phosphoenolpyruvate, 100 μM Ap_4A, and 0.25 mM NADH with 10 units of pyruvate kinase and 11 units of lactate dehydrogenase. Purification of the enzyme through Sephacryl S-200 gel filtration chromatography was necessary before the spectrophotometric assay could be used because of Ap_4A-independent oxidation of NADH in cruder preparations.

Gel Electrophoresis. Dissociated protein samples were analyzed by electrophoresis on 15% polyacrylamide gels containing 0.1% sodium dodecyl sulfate. Samples were subjected to electrophoresis with the discontinuous buffer system for slab gels as described by Laemmli (1970) and modified by Studier (1973). Protein samples were dialyzed against 10 mM sodium phosphate, pH 7.2, prior to dissociation in 1% sodium dodecyl sulfate and 1% 2-mercaptoethanol at 80–90 °C for 15 min. Gels were stained and destained with Coomassie blue R-250 (Fairbanks et al., 1971).

Nondissociated protein samples were analyzed by electrophoresis at 4 °C on slab gels composed of a 4.6–10% linear gradient of acrylamide. An alkaline pH discontinuous buffer system (Maizel, 1971) was used. After electrophoresis, the gels were stained with Coomassie blue R-250 or sliced and assayed for diadenosine tetraphosphate pyrophosphohydrolase activity. For the latter procedure, the gels were semifrozen by placing them on a glass plate over solid CO_2 and sliced into 2-mm segments with a razor blade. The segments were incubated at 4 °C for 16 h and at 30 °C for 10 min in 200 μL of 50 mM Hepes–NaOH, pH 7.5, containing 100 μM [^{3}H]-Ap_4A. The formation of [^{3}H]ADP was assayed as described previously. The relative intensities of protein bands stained with Coomassie blue were measured on a Helena Laboratories R & D densitometer fitted with an orange filter.

Determination of Molecular Weight and Stokes Radius. The molecular weight of diadenosine tetraphosphate pyrophosphohydrolase was determined by gel filtration chromatography on Sephadex G-75 (1.5 cm × 70 cm) equilibrated in 50 mM Hepes–NaOH, pH 7.5. One milligram of the following proteins was subjected to chromatography: hemoglobin (64 000), ovalbumin (43 000), β-lactoglobulin (36 800), and myoglobin (17 200). Blue dextran (1 mL of 0.2% solution) and 2-mercaptoethanol (1 mL of 5% solution) were markers for determination of the void volume and included volume, respectively, of the column. Absorbance of column eluates of standard proteins and markers was measured at 280 nm, and eluates of diadenosine tetraphosphate pyrophosphohydrolase were assayed radioisotopically. Column fractions of 0.67 mL were collected at a flow rate of 6 mL/h. The molecular weight and Stokes radius of the enzyme were calculated from standard curves of log molecular weight vs. K_{av} and R_S vs. $K_{av}^{1/3}$, respectively.

Miscellaneous. Phenylmethanesulfonyl fluoride was prepared as a 100 mM stock solution in 100% 2-propanol (James, 1978). It was diluted into buffers at 4 °C about 30–60 min before using. Conductivity of column eluates was measured at 4 °C with a Radiometer CDM2 conductivity meter and CDC 114 conductivity cell. Protein samples were diluted into 1% sodium dodecyl sulfate prior to analysis according to Lowry et al. (1951), and the absorbance values of samples were corrected for the absorbance of Hepes buffer. Bovine serum albumin was used as the standard protein.

Results

Purification of Diadenosine Tetraphosphate Pyrophosphohydrolase. All procedures were performed at 4 °C unless noted otherwise. NaCl concentrations in eluates from the DEAE-cellulose and Green A resin columns were determined by measurement of conductivity. Pooled fractions containing enzymic activity were concentrated under N_2 pressure in Amicon diaflo units containing a PM-10 membrane.

P. polycephalum microplasmodia, 250–300 g wet wt, were homogenized in 50 mM Hepes–NaOH, pH 7.5, 0.1 mM phenylmethanesulfonyl fluoride (2 mL of buffer/g wet wt) in a Waring blender at 17 000 rpm for 30 s. The homogenate was allowed to settle for 2 min; then it was rehomogenized. The homogenate was centrifuged at 43000*g* for 30 min to yield a clear, bright yellow crude supernatant.

Protamine sulfate (30 mg/mL) at pH 7.0 and at about 24 °C was added slowly to the crude supernatant fraction at 4 °C. About 0.15 mg of protamine sulfate was added per milligram of protein. After a 15-min stirring at 4 °C, the solution was centrifuged at 43000*g* for 20 min to yield a clear, yellow protamine sulfate supernatant.

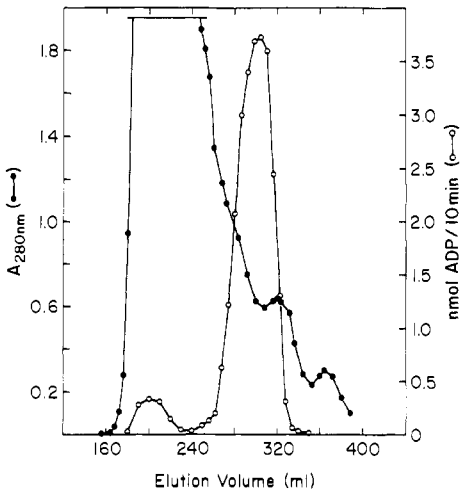

FIGURE 1: Purification of diadenosine tetraphosphate pyrophosphohydrolase on Sephacryl S-200. The concentrated DEAE-cellulose fraction was subjected to gel filtration chromatography on a column (2.5 cm × 101 cm) of Sephacryl S-200. The flow rate was 28 mL/h, and 2-mL fractions were collected. Activity was assayed with 20 μL of the fractions and 10 μM [^{3}H]Ap_4A as the substrate.

The protamine sulfate supernatant was applied to a 5.0 cm × 52 cm column of DEAE-cellulose (DE-52) equilibrated with 50 mM Hepes–NaOH, pH 7.5. The column was washed with this buffer at 100 mL/h and subsequently was eluted with a linear gradient (1800 mL) of 0–0.5 M NaCl in buffer. Diadenosine tetraphosphate pyrophosphohydrolase was eluted with 0.04–0.06 M NaCl in buffer. The pooled and concentrated DEAE-cellulose fraction was clear, pale yellow.

The concentrated DEAE-cellulose fraction was applied to a 2.5 cm × 101 cm column of Sephacryl S-200 equilibrated with 50 mM Hepes–NaOH, pH 7.5. The column was eluted with the same buffer at 28 mL/h, and 2-mL fractions were collected. The elution profile of the Sephacryl S-200 column is shown in Figure 1. Fractions between 260 and 330 mL containing about 94% of the eluted enzymic activity were pooled. The pooled fractions contained a small amount of yellow pigments while the majority of the pigments eluted between 180 and 230 mL, which contained about 6% of the enzymic activity.

The pooled Sephacryl S-200 fractions were applied to a 2.5 cm × 16 cm column of Matrex Gel Green A resin equilibrated with 50 mM Hepes–NaOH, pH 7.5. The column was washed with the same buffer at 27 mL/h and subsequently was eluted with linear gradient (300 mL) of 0–0.5 M NaCl in the initial buffer. Enzymic activity eluted as a single peak with buffer containing 0.15–0.28 M NaCl (Figure 2). Fractions containing enzyme were pooled and concentrated. The concentrated Green A fraction was clear and colorless.

The concentrated Green A fraction was applied to a 1.5 cm × 72 cm column of Sephadex G-75 equilibrated with 50 mM Hepes–NaOH, pH 7.5. The column was eluted with the same buffer at 3.5 mL/h, and 0.67-mL fractions were collected. Enzymic activity eluted as a single peak (Figure 3). Fractions containing enzymic activity were pooled and concentrated.

A summary of a typical purification is presented in Table I. A purification of diadenosine tetraphosphate pyrophosphohydrolase of about 250 times with a recovery of 1% of total activity was obtained on the basis of an assay of the fractions with 100 μM [^{3}H]Ap_4A. The mass of the enzyme is maximally about 0.4% of the total soluble protein mass. During the isolation, column eluates were assayed with 10 μM [^{3}H]Ap_4A to decrease the probability of isolating a low-affinity, nonspecific pyrophosphohydrolase. The degree of pu-

Table I: Purification of Diadenosine Tetraphosphate Pyrophosphohydrolase from *P. polycephalum*[a]

fraction	vol (mL)	protein (mg)	total act.[b] (μmol of ADP min^{-1})	sp act. (nmol of ADP min^{-1} mg^{-1})	yield (%)
crude supernatant	650	4914	75	15.2	100
protamine supernatant	645	3225	24	7.4	32
DEAE-cellulose (concd)	17.6	524	5.3	10.2	7.2
Sephacryl S-200	71	71	2.3	32	3.1
Green A dye resin (concd)	1.7	3.1	1.0	312	1.3
Sephadex G-75	0.67	0.22	0.8	3774	1.1

[a] From 300 g wet wt of *P. polycephalum* microplasmodia. [b] Activity was assayed with 100 μM [^{3}H]Ap$_4$A.

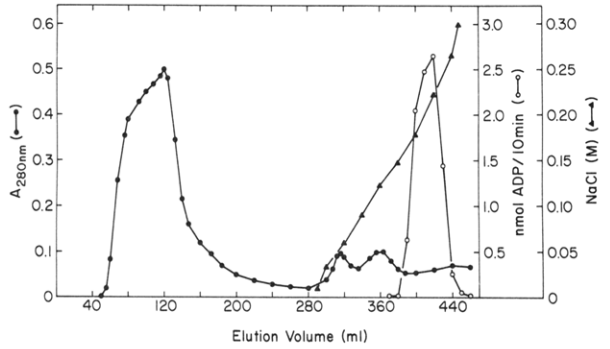

FIGURE 2: Purification of diadenosine tetraphosphate pyrophosphohydrolase on Matrex Gel Green A resin. The Sephacryl S-200 fraction was subjected to dye–ligand chromatography on a column (2.5 cm × 16 cm) of Green A resin. The flow rate was 27 mL/h, and 2-mL fractions were collected. Activity was assayed with 20 μL of the fractions and 10 μM [^{3}H]Ap$_4$A as the substrate. The enzyme was eluted with a linear gradient (300 mL) of 0–0.5 M NaCl in 50 mM Hepes–NaOH, pH 7.5. The concentration of NaCl in the fractions was determined by measuring the conductivity.

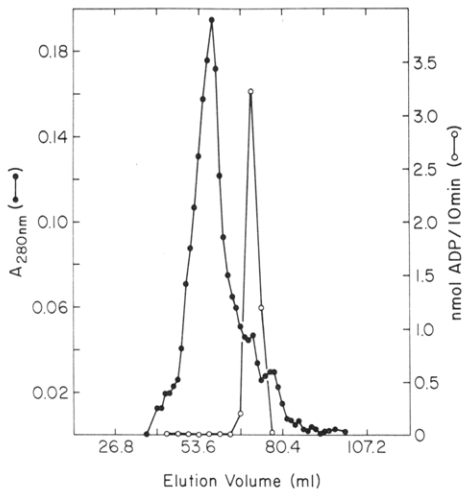

FIGURE 3: Purification of diadenosine tetraphosphate pyrophosphohydrolase on Sephadex G-75. The concentrated Green A fraction was subjected to gel filtration chromatography on a column (1.5 cm × 72 cm) of Sephadex G-75. The flow rate was 3.5 mL/h, and 0.67-mL fractions were collected. Activity was assayed with 10 μL of the fractions and 10 μM [^{3}H]Ap$_4$A as the substrate.

rification and percent recovery on the basis of an assay of activity with 10 μM [^{3}H]Ap$_4$A were 290-fold and 1%, respectively.

Purity of Diadenosine Tetraphosphate Pyrophosphohydrolase. The Sephadex G-75 enzyme fraction exhibited two major and four minor protein-staining bands on nondissociating polyacrylamide gels. A single peak of enzymic activity was associated with the two major bands (Figure 4). Two major and several minor protein-staining bands were present on dissociating polyacrylamide gels (gels not shown).

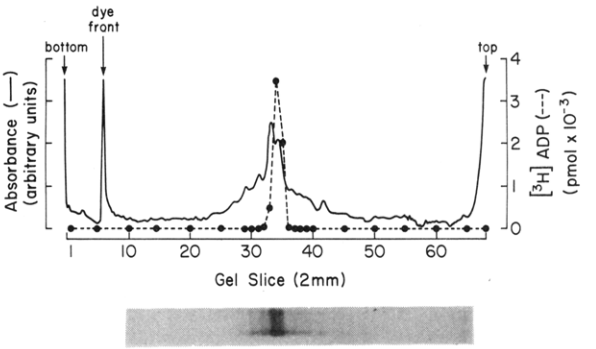

FIGURE 4: Electrophoresis of diadenosine tetraphosphate pyrophosphohydrolase on a nondissociating polyacrylamide gel. The Sephadex G-75 enzyme fraction was subjected to electrophoresis at 4 °C on a slab gel composed of a 4.6–10% linear gradient of acrylamide. One track of the gel was stained for protein (15 μg applied) with Coomassie blue R-250, and the relative intensities of the bands were measured with a densitometer. Another track of the gel with 2 μg of protein applied was sliced into 2-mm segments and assayed for enzymic activity with 100 μM [^{3}M]Ap$_4$A (20 nmol) as described under Experimental Procedures. All gel slices were assayed, but only the data for every fifth slice are illustrated except in the region of activity. The slice with peak activity represents about 2 × 10^4 dpm of [^{3}H]ADP.

Molecular Weight. The molecular weight of diadenosine tetraphosphate pyrophosphohydrolase was 26 200 ± 1000 (mean and SD for four different preparations) on the basis of elution of activity from a calibrated Sephadex G-75 column. The value of the Stokes radius was 23 ± 1 Å (mean and SD for four different preparations).

Product of the Hydrolysis of Ap$_4$A. The reaction product was identified as ADP by coelution with standard nucleotide from DEAE-cellulose and by a coupled-enzyme assay. The tritiated reaction product coeluted with ADP, which partially overlapped with AMP, upon washing the DEAE-cellulose assay columns with 120 mM NH_4HCO_3, pH 8.2. Hydrolysis of Ap$_4$A to ADP also was detected by an assay system of pyruvate kinase and lactate dehydrogenase in the presence of phosphoenolpyruvate, NADH, and diadenosine tetraphosphate pyrophosphohydrolase. With 0.3–1.3 μg of the latter enzyme and 100 μM Ap$_4$A (100 nmol), 4 nmol of NADH was oxidized per minute per milligram. Diadenosine tetraphosphate pyrophosphohydrolase preparations did not contain detectable adenylate kinase activity. When 1 mM ATP and 1 mM AMP or 10 μM ATP and 10 μM AMP instead of 100 μM Ap$_4$A were incubated with diadenosine tetraphosphate pyrophosphohydrolase in the coupled-enzyme assay system, no oxidation of NADH was observed. Conversion of 0.1% of ATP and AMP to ADP by contamination of diadenosine tetraphosphate pyrophosphohydrolase with adenylate kinase would have been detected. The stoichiometry of the reaction and identification of ADP as the product as well as the absence of adenylate kinase activity also were determined by HPLC (Garrison et al., 1982).

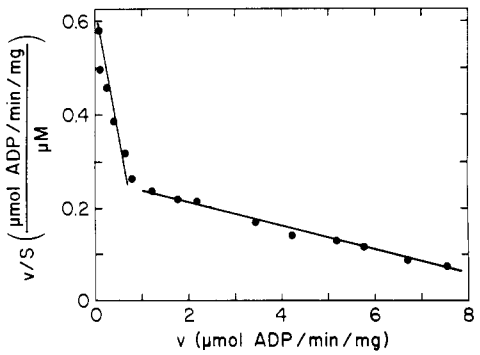

FIGURE 5: Eadie–Scatchard plot of the hydrolysis of Ap_4A by diadenosine tetraphosphate pyrophosphohydrolase. [^{3}H]Ap_4A, 0.5–100 μM, was incubated with 0.06 μg of protein in 50 mM Hepes–NaOH, pH 7.5, for 10 min at 30 °C. The mass of ADP formed was measured as described under Experimental Procedures.

Kinetic Properties. The activity was linear with time to 10 min and was linear with 0.01–3 μg of protein for partially purified enzyme. The reaction velocity was a biphasic function of the Ap_4A concentration from 0.5–100 μM as illustrated by the Eadie–Scatchard plot (Figure 5). Calculation of kinetic parameters from this plot yielded apparent K_m values of 2.6 μM and 37 μM for the high substrate affinity and low substrate affinity activities, respectively. The corresponding values of V_{max} were 0.83 and 9.15 μmol of ADP formed per minute per milligram. Values of K_m and V_{max} were determined after resolution of the curves by the graphical–mathematical method described by Feldman (1972). A one-ligand, two-binding site model was assumed for the calculations. (This is strictly a mathematical assumption without implying multisites on one protein or single sites on different proteins.) A Hill plot of these data with a V_{max} value of 9.98 μmol of ADP min^{-1} mg^{-1} had a slope of 0.89.

The divalent cations Mg^{2+}, Mn^{2+}, Ca^{2+}, Co^{2+}, Fe^{2+}, Zn^{2+}, and Cu^{2+} at 0.5 mM inhibited the activity by 40–80%. $MgCl_2$, $MgSO_4$, and $CaCl_2$ inhibited diadenosine tetraphosphate pyrophosphohydrolase in the same concentration-dependent manner. Mg^{2+}, at 0.5 mM, decreased both values of V_{max} by 50%, decreased the low K_m value by 30%, and increased the high K_m value by 100%. The chelator EDTA inhibited the activity in a concentration-dependent manner with inhibition of about 50% at 10 mM. Inhibition by EGTA paralleled the inhibition by EDTA.

The product of the reaction, ADP, inhibited the enzyme by about 50% and 75% at 30 and 100 μM, respectively. ATP and Ap_4 inhibited the activity as strongly as ADP while 100 μM AMP only inhibited the enzyme by 20%. Guanosine tetraphosphate inhibited the enzyme in the same concentration-dependent manner as Ap_4.

None of the inhibitory compounds significantly decreased the activity if the maximal mass of inhibitor tested was added to the assay after the reaction was stopped. The sulfhydryl reducing agents dithiothreitol and 2-mercaptoethanol, at 0.5–10 mM, did not significantly affect the activity.

Discussion

Diadenosine tetraphosphate pyrophosphohydrolase partially purified from *P. polycephalum* has catalytic properties that are previously unreported for the enzyme from any organism. This enzyme symmetrically hydrolyzes Ap_4A to ADP, is inhibited by Mg^{2+} and other divalent cations, and exhibits biphasic kinetics for Ap_4A. These properties distinguish the *Physarum* enzyme from diadenosine tetraphosphate phosphohydrolases of rat tissues (Lobaton et al., 1975; Vallejo et al., 1976; Cameselle et al., 1982), brine shrimp (Vallejo et al., 1976), mouse ascites tumor cells (Ogilvie, 1981), and mouse liver (Höhn et al., 1982). Enzymes from these mammalian and crustaceal organisms all asymmetrically hydrolyze Ap_4A to AMP and ADP and require Mg^{2+} or Mn^{2+} for activity. Ap_4 and Gp_4 are potent inhibitors of the enzyme from rat liver (Lobaton et al., 1975) and mouse liver (Höhn et al., 1982) with values of K_i less than 0.5 μM. The rat liver enzyme does not hydrolyze Ap_4 and Gp_4 (Lobaton et al., 1975). Ap_4 and Gp_4 inhibit the enzyme from *Physarum* less strongly with about 30 μM each nucleoside tetraphosphate required for 50% inhibition of Ap_4A. However, both Ap_4 and Gp_4 are hydrolyzed by diadenosine tetraphosphate pyrophosphohydrolase from *Physarum* (Garrison et al., 1982).

Presently, we do not know the basis for the biphasic kinetics exhibited by diadenosine tetraphosphate pyrophosphohydrolase. Such kinetics could be explained by the existence of two different states of the same enzyme or by two different enzymes. Although only one peak of activity was detected on native gels, the resolution of protein-staining bands and activity were inadequate to correlate the activity with one specific protein. Negative cooperativity, as analyzed by Hill plots, is not the basis for the biphasic kinetics.

Plateau et al. (1981) demonstrated that homogeneous *E. coli* phenylalanyl-tRNA synthetase catalyzed the synthesis of Ap_4A. This enzyme also hydrolyzed 0.5–2 mM Ap_4A to ADP provided Zn^{2+} and phenylalanine were present. Ap_4A pyrophosphohydrolase from *Physarum* may be related to or derived from a tRNA synthetase, but the inhibition by divalent cations and absence of an amino acid requirement make this possibility unlikely.

The physiologic significance of this enzyme for Ap_4A catabolism in *P. polycephalum* is presently unknown. If the previous results and proposals that Ap_4A is a regulator of DNA replication (Rapaport & Zamecnik, 1976; Grummt, 1978; Rapaport et al., 1981b; Zamecnik et al., 1982) are applicable to *Physarum*, then diadenosine tetraphosphate pyrophosphohydrolase could alter the cellular level of Ap_4A and thereby affect DNA replication. From published data (Plateau et al., 1981; Goerlich et al., 1982) rates of synthesis of Ap_4A by phenylalanyl-tRNA synthetases can be estimated as 0.25, 2.5, and 1.5 s^{-1} for the enzyme from *E. coli*, yeast, and *Physarum*, respectively, in the presence of 2 mM ATP and 60–80 μM Zn^{2+}. Rates in the absence of Zn^{2+} are 20–50 times less. Estimation of the intracellular concentration of tRNA synthetases yields values from about 0.2 to 1.6 μM [calculated from the data of Schmidt et al. (1971), Fayat et al. (1974), and Kalousek & Konigsberg (1974)]. If a mean value of 1 μM is assumed and only a few of the tRNA synthetases synthesize Ap_4A at significant rates (Goerlich et al., 1982), the stimulated rate of Ap_4A synthesis would be about 1–10 μM/s. The activity of diadenosine tetraphosphate pyrophosphohydrolase in the crude supernatant fraction from *Physarum* can be used to estimate a degradation rate of about 2.5 μM/s at 100 μM Ap_4A. Thus, stimulated rates of Ap_4A synthesis may be sufficient to rapidly produce the large increases in Ap_4A observed by Rapaport & Zamecnik (1976). Under these conditions, small changes in either the rate of synthesis or degradation could alter markedly the cellular level of Ap_4A. For example, elevation of free Zn^{2+} intracellularly could increase the Ap_4A concentration by stimulating phenylalanyl-tRNA synthetase and inhibiting diadenosine tetraphosphate pyrophosphohydrolase.

The total intracellular levels of Mg^{2+} and Ca^{2+} in *P. polycephalum* have been estimated as about 12 and 23 mM, respectively (Kuroda & Kuroda, 1980). If 1% of the total

Mg^{2+} and Ca^{2+} concentrations is free and accessible to the enzyme, the activity would be inhibited strongly. Similarly, the concentration of the monoadenine nucleotides in *Physarum* (Sachenmaier et al., 1969) is sufficient to almost completely inhibit diadenosine tetraphosphate pyrophosphohydrolase. However, the activity measured in the crude supernatant fraction indicates that additional regulatory factors may offset the effect of these inhibitors. Additional unique properties of diadenosine tetraphosphate pyrophosphohydrolase from *Physarum* concerning substrate specificity and product formation are reported in the following paper (Garrison et al., 1982).

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References

Barnes, L. D., Culver, C. A., & Garrison, P. N. (1982) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 41*, 650.

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

Fairbanks, G., Steck, T. L., & Wallach, D. F. H. (1971) *Biochemistry 10*, 2606–2617.

Fayat, G., Blanquet, S., Dessen, P., Batelier, G., & Weller, J. P. (1974) *Biochimie 56*, 35–41.

Feldman, H. A. (1972) *Anal. Biochem. 48*, 317–338.

Garrison, P. N., & Barnes, L. D. (1980) *Biochim. Biophys. Acta 633*, 114–121.

Garrison, P. N., Culver, C. A., & Barnes, L. D. (1981) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 40*, 1905.

Garrison, P. N., Roberson, G. M., Culver, C., & Barnes, L. D. (1982) *Biochemistry* (following paper in this issue).

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

Grummt, F. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 371–375.

Grummt, F., Waltl, G., Jantzen, H.-M., Hamprecht, K., Huebscher, U., & Kuenzle, C. C. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 6081–6085.

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

James, G. T. (1978) *Anal. Biochem. 86*, 574–579.

Kalousek, F., & Konigsberg, W. H. (1974) *Biochemistry 13*, 999–1006.

Kuroda, R., & Kuroda, H. (1980) *J. Cell Sci. 44*, 75–85.

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

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

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

Maizel, J. V., Jr. (1971) *Methods Virol. 5*, 179–246.

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

Ono, K., Iwata, Y., Nakamura, H., & Mutsukage, A. (1980) *Nucleic Acids Res. 8*, S187–S190.

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

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., Zamecnik, P. C., & Baril, E. F. (1981a) *Proc. Natl. Acad. Sci. U.S.A. 78*, 838–842.

Rapaport, E., Zamecnik, P. C., & Baril, E. F. (1981b) *J. Biol. Chem. 256*, 12148–12151.

Reiter, T., Futterer, J., Temple, M., Antoine, G., & Winnacker, E. L. (1980) in *Cell Compartmentation and Metabolic Channeling* (Nover, L., Lynen, F., & Mothes, K., Eds.) pp 169–175, Gustav Fischer Verlag, Jena, West Germany.

Sachenmaier, W., Immich, H., Grunst, J., Scholz, R., & Bücher, T. (1969) *Eur. J. Biochem. 8*, 557–561.

Schmidt, J., Wang, R., Stanfield, S., & Reid, B. (1971) *Biochemistry 10*, 3264–3268.

Studier, F. W. (1973) *J. Mol. Biol. 79*, 237–248.

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.

Yoshihara, K., & Tanaka, Y. (1981) *J. Biol. Chem. 256*, 6756–6761.

Zamecnik, P. C. (1969) *Cold Spring Harbor Symp. Quant. Biol. 34*, 1–16.

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-Patterson, A., Ottersen, M., & Thaysen, J. H., Eds.) pp 276–291, Academic Press, New York.

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

Zamecnik, P. C., Rapaport, E., & Baril, E. F. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 1791–1794.