Diadenosine 5′,5‴-P^1,P^4-Tetraphosphate Pyrophosphohydrolase from *Physarum polycephalum*. Substrate Specificity†

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ABSTRACT: The substrate specificity of diadenosine 5′,5‴-P^1,P^4-tetraphosphate pyrophosphohydrolase from *Physarum polycephalum* for dinucleoside polyphosphates has been determined by high-performance liquid chromatography (HPLC). Elution of a strong anion-exchange resin with a pH and ionic strength gradient of ammonium phosphate separates a series of monoadenosine and diadenosine polyphosphates. Most of the corresponding guanine nucleotides are also resolved on this HPLC system. One mole each of Ap_4A and Gp_4G is symmetrically hydrolyzed to 2 mol of ADP and GDP, respectively. Ap_3A, Ap_5A, Ap_6A, and Ap_4 are hydrolyzed, and in each case ADP is one of the products. Gp_3G, Gp_5G, Gp_6G, and Gp_4 are also substrates, and in each case GDP is one of the products. AMP, ADP, ATP, Ap_2A, ADPR, GMP, GDP, GTP, NAD^+, and $NADP^+$ are not substrates. No hydrolysis of the cap dinucleotides m^7Gp_3Am and m^7Gp_3Cm was detected by HPLC. Diadenosine tetraphosphate pyrophosphohydrolase preparations were also assayed for adenylate kinase, nucleotide diphosphate kinase, $NAD(P)^+$ pyrophosphohydrolase, phosphodiesterase, cyclic nucleotide phosphodiesterase, phosphatase, and ribonuclease activities. These enzymic activities were not detectable in diadenosine tetraphosphate pyrophosphohydrolase. The symmetrical hydrolysis of Ap_4A and Gp_4G is an unique catalytic property that distinguishes diadenosine tetraphosphate pyrophosphohydrolase from *P. polycephalum* from diadenosine tetraphosphate phosphohydrolases from other organisms.

We reported in the preceding paper (Barnes & Culver, 1982) the isolation and characterization of diadenosine 5′,5‴-P^1,P^4-tetraphosphate pyrophosphohydrolase from *Physarum polycephalum*. This enzyme symmetrically hydrolyzes diadenosine tetraphosphate (Ap_4A)[1] to ADP, is inhibited by Mg^{2+} and other divalent cations, and exhibits biphasic kinetics. These kinetic properties distinguish the enzyme from *Physarum* from Ap_4A-hydrolyzing enzymes isolated from other organisms. Enzymes from rat liver (Lobaton et al., 1975; Vallejo et al., 1976) and brine shrimp (Warner & Finamore, 1965a; Vallejo et al., 1974) asymmetrically hydrolyze Ap_4A, Gp_4G, and other dinucleoside tetraphosphates. Dinucleoside di- and triphosphates are apparently not substrates, while dinucleoside penta- and hexaphosphates have not been tested. Only Ap_4A has been assayed as a substrate with the enzyme from mouse ascites tumor cells (Ogilvie, 1981) and mouse liver (Höhn et al., 1982). The enzyme has been called diadenosine or diguanosine tetraphosphatase, dinucleoside tetraphosphatase, diadenosine tetraphosphate hydrolase, bis(5′-guanosyl)tetraphosphatase, and diadenosine or diguanosine tetraphosphate pyrophosphohydrolase by different investigators. This enzyme differs from a dinucleoside triphosphatase from rat liver that hydrolyzes only dinucleoside triphosphates (Sillero et al., 1977).

In this paper we determine the substrate specificity of diadenosine tetraphosphate pyrophosphohydrolase partially purified from *P. polycephalum* (Barnes & Culver, 1982). The enzyme was assayed for dinucleoside polyphosphate pyrophosphohydrolase, adenylate kinase, nucleoside diphosphate kinase, $NAD(P)^+$ pyrophosphohydrolase, phosphodiesterase, cyclic nucleotide phosphodiesterase, phosphatase, and ribonuclease activities.

A HPLC system was developed to assay for the first four enzymic activities and to identify and quantitate reaction products of dinucleoside polyphosphate substrates. Two HPLC systems have been described for detection of Ap_4A in cells. Nierenberg et al. (1980) described an anion-exchange HPLC procedure performed at 50 °C that separated Ap_4A from UV-absorbing components in acid-soluble cell extracts. They also reported a reversed-phase HPLC system that separated Ap_4A from monoadenine nucleotides. Plesner & Ottesen (1980) detected Ap_4A in extracts from *Tetrahymena* by HPLC on an anion-exchange column isocratically eluted with a chloride-containing buffer. HPLC of diguanosine polyphosphates and diadenosine polyphosphates other than Ap_4A has not been reported. In our HPLC system cAMP, AMP, ADP, ATP, and Ap_nA (n = 2–6) were all separated by elution from an anion-exchange resin with a pH and ionic strength gradient of ammonium phosphate. Guanosine phosphates, diguanosine polyphosphates, NAD^+, $NADP^+$, and the cap dinucleotides m^7Gp_3Am and m^7Gp_3Cm were also assayed as substrates with this HPLC system.

Diadenosine tetraphosphate pyrophosphohydrolase from *P. polycephalum* has catalytic properties that have not been previously described for any enzyme. It catalyzes the symmetrical hydrolysis of Ap_4A and Gp_4G as well as the hydrolysis of Ap_3A, Ap_5A, Ap_6A, and the corresponding diguanosine polyphosphates. Preliminary accounts of part of this work have been reported (Garrison et al., 1981; Barnes et al., 1982).

Experimental Procedures

Materials. [2,8-^{3}H]Adenosine 3′,5′-phosphate (sp act. 36.4 Ci/mmol) and [8-^{3}H]guanosine 3′,5′-phosphate (sp act. 15 Ci/mmol) were obtained from Amersham. Unlabeled adenine and guanine nucleotides were purchased from Sigma Chemical Co. and P-L Biochemicals. All were at least 98% pure as analyzed by HPLC except for Ap_4 (Sigma Chemical Co.),

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[1] Abbreviations: Ap_nA, diadenosine 5′,5‴-P^1,P^n-polyphosphate, n = 2–6; Ap_4, adenosine tetraphosphate; ADPR, adenosine 5′-diphosphate ribose; cAMP, adenosine 3′,5′-phosphate; Gp_nG, diguanosine 5′,5‴-P^1,P^n-polyphosphate, n = 3–6; Gp_4, guanosine tetraphosphate; cGMP, guanosine 3′,5′-phosphate; HPLC, high-performance liquid chromatography; m^7Gp_3Cm, 7-methylguanosine 2′-*O*-methylcytidine 5′,5‴-P^1,P^3-triphosphate; m^7Gp_3Am, 7-methylguanosine 2′-*O*-methyladenosine 5′,5‴-P^1,P^3-triphosphate; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; NAD, nicotinamide adenine dinucleotide.

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which was about 10% ATP and 90% Ap_4. Two nanomoles of each nucleotide was analyzed, and a contaminant (detectable by absorbance at 254 nm and elution under the specified HPLC conditions, Figure 1) of 0.5% or greater would have been detected. The RNA terminal fragments (caps) m^7Gp_3Cm and m^7Gp_3Am were purchased from P-L Biochemicals. RNA, type III from bakers' yeast, ribonuclease A, *Escherichia coli* alkaline phosphatase, and beef heart cyclic nucleotide phosphodiesterase were from Sigma Chemical Co. Snake venom phosphodiesterase I was purchased from Worthington Biochemicals, and hog muscle adenylate kinase was from Boehringer/Mannheim. Additional compounds tested as potential substrates were at least reagent grade and were purchased from Sigma Chemical Co., P-L Biochemicals, or Boehringer/Mannheim.

Diadenosine Tetraphosphate Pyrophosphohydrolase Preparation. The enzyme was partially purified from microplasmodia of *P. polycephalum* (Barnes & Culver, 1982). Substrate specificity and possible enzymic activities were assayed with preparations of enzyme purified through the Sephadex G-75 step.

High-Performance Liquid Chromatography. Water was purified by reverse osmosis, deionization, distillation, and filtration through a Millipore filter (Millipore Corp.) with 0.45 μm diameter pores. Ammonium phosphate buffers were prepared by mixing $NH_4H_2PO_4$ and $(NH_4)_2HPO_4$ at equal concentrations to pH 5.2 or pH 5.7 at room temperature. Buffer A was 50 mM ammonium phosphate, pH 5.2, and buffer B was 1 M ammonium phosphate, pH 5.7.

Altex Model 110 pumps, Model 710 injector, Model 420 microprocessor controller, and Model 153 or Model 160 UV detector were used. The column effluent was monitored at 254 nm, and effluent absorbance was recorded on an Altex Model C-R1A integrator–recorder. Peaks were identified by retention time. Peak areas were integrated for quantitative analysis.

Nucleotides were separated by chromatography on a strong anion-exchange resin, Whatman Partisil PXS 10/25 SAX. The analytical column, 4.6 mm i.d. × 25 cm, was maintained at room temperature (23–24 °C). A Whatman Solvecon precolumn for solvent conditioning was in-line between the mixing chamber and injection valve. The buffer flow rate was 1.5 mL/min, and the pressure was about 1000 psi. Nucleotides were separated with a pH and ionic gradient of buffers A and B. Initially, the column was equilibrated with buffer A. At the time the sample was injected, the gradient concentration of buffer B was instantly increased to 5% and maintained constant for 10 min. After 10 min, the gradient concentration of buffer B was linearly increased from 5% to 45% over a 30-min period. The delay between the gradient mixing chamber and the detector was about 8.5 min, and the column dead time was 2.5 min.

Enzyme Assays. (A) Dinucleoside Polyphosphate Pyrophosphohydrolase. Previously, diadenosine tetraphosphate pyrophosphohydrolase was assayed radioisotopically with $[^3H]Ap_4A$ as the substrate (Barnes & Culver, 1982). We developed the HPLC system described above to assay this activity with unlabeled Ap_4A and to test for dinucleoside polyphosphate pyrophosphohydrolase activity. Standard solutions of nucleotides were prepared in 10 mM Hepes–NaOH, pH 7.5. Concentrations of adenine and guanine nucleotides were calculated from values of ϵ_M and absorbances at 260 and 252 nm, respectively. We assumed that the molar extinction coefficients for Ap_nA (n = 2, 3, 5, or 6) equaled the molar extinction coefficient, 2.54×10^4 M^{-1}, for Ap_4A (Randerath et al., 1966). Sillero et al. (1977) reported an ϵ_M value of 2.58×10^4 M^{-1} for Ap_3A. We used a molar extinction coefficient of 2.26×10^4 M^{-1} for Gp_nG (n = 3–6) assuming a 17.6% hypochromicity per guanosine residue in comparison to monoguanine nucleotides. This is analogous to the hypochromicity determined for Ap_4A (Randerath et al., 1966). Concentrations of the cap dinucleotides, m^7Gp_3Am and m^7Gp_3Cm, were based on A_{250nm} absorbance units (*P-L Biochemical Catalog 106*, 1982).

Ap_4A and other potential substrates examined by HPLC were incubated in the absence or presence of diadenosine tetraphosphate pyrophosphohydrolase (0.05–0.2 μg) in 10 mM Hepes–NaOH, pH 7.5, at 30 °C for 10 min in a volume of 200 μL. The reaction was stopped by quick freezing on dry ice or by immediately injecting the entire assay solution onto the HPLC column. Individual frozen assay solutions were thawed just before analyzing by HPLC. There was no detectable difference in results for fresh vs. frozen assay solutions. These are standard conditions for HPLC assays.

The percent hydrolysis of a substrate was calculated by dividing the peak areas of the substrate in the presence and absence of enzyme. When the percent hydrolysis of a particular substrate was less than 10%, the percent hydrolysis was determined by comparing the peak area of the product to the peak areas of known masses of product standard.

(B) Adenylate Kinase, Nucleoside Diphosphate Kinase, and NAD(P)$^+$ Pyrophosphohydrolase. The HPLC system was also used to assay for adenylate kinase, nucleoside diphosphate kinase, and NAD(P)$^+$ pyrophosphohydrolase activities. Concentrations of NAD^+ and $NADP^+$ were determined from A_{259nm} and ϵ_M values of 1.78×10^4 M^{-1} and 1.8×10^4 M^{-1}, respectively (Dawson et al., 1969). Assays for adenylate kinase and nucleoside diphosphate kinase were done in the absence or presence of 0.1 mM $MgCl_2$.

(C) Cyclic Nucleotide Phosphodiesterase. Diadenosine tetraphosphate pyrophosphohydrolase was assayed for cyclic nucleotide phosphodiesterase activity with [^{3}H]cAMP and [^{3}H]cGMP as substrates (Wells et al., 1975).

(D) Phosphodiesterase. Diadenosine tetraphosphate pyrophosphohydrolase was assayed for phosphodiesterase activity with *p*-nitrophenyl phenyl phosphonate (Kelly et al., 1975) and bis(*p*-nitrophenyl) phosphate (Razzell & Khorana, 1959) as potential substrates in the absence or presence of 0.5 mM $MgCl_2$.

(E) Phosphatase and Ribonuclease. Phosphatase activity of diadenosine tetraphosphate pyrophosphohydrolase was assayed by the formation of inorganic phosphate. Reactions were stopped by the addition of trichloroacetic acid, and the solutions were analyzed for inorganic phosphate by the malachite green procedure (Muszbek et al., 1977).

Ribonuclease and NAD^+ pyrophosphohydrolase activities were also assayed by measuring the formation of inorganic phosphate. RNA and NAD^+ were incubated with diadenosine tetraphosphate pyrophosphohydrolase and 0.3 unit of alkaline phosphate. Reactions were stopped and analyzed for inorganic phosphate as described above. Control assays contained only potential substrates and alkaline phosphatase in the buffer. NAD^+ phosphodiesterase activity would also be detectable with this assay.

Assays for cyclic nucleotide phosphodiesterase, phosphodiesterase, ribonuclease, and NAD^+ pyrophosphohydrolase activities were measured with 0.33–2 μg of diadenosine tetraphosphate pyrophosphohydrolase in 50 mM Hepes–NaOH, pH 7.5, in 200 μL for 10 min at 30 °C. The substrates tested, the concentrations used, and the limits of detection are

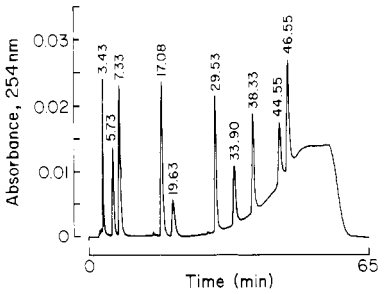

FIGURE 1: Chromatographic resolution of diadenine and monoadenine nucleotides by HPLC. Ap_2A, Ap_3A, Ap_4A, Ap_5A, Ap_6A, cAMP, AMP, ADP, ATP, and Ap_4, at 0.8–1.2 nmol each, were separated on a 4.6 mm × 25 cm column of Whatman Partisil PXS 10/25 SAX resin. Solutions: buffer A, 50 mM ammonium phosphate, pH 5.2; buffer B, 1 M ammonium phosphate, pH 5.7. Gradient elution with buffers A and B is described in detail under Experimental Procedures. Conditions: flow rate 1.5 mL/min; temperature 23–24 °C; AUFS 0.04 at 254 nm. Nucleotides and retention times: 3.43 min, cAMP; 5.73 min, AMP; 7.33 min, Ap_2A; 17.08 min, Ap_3A; 19.63 min, ADP; 29.53 min, Ap_4A; 33.9 min, ATP; 38.33 min, Ap_5A; 44.55 min, Ap_4; 46.55 min, Ap_6A.

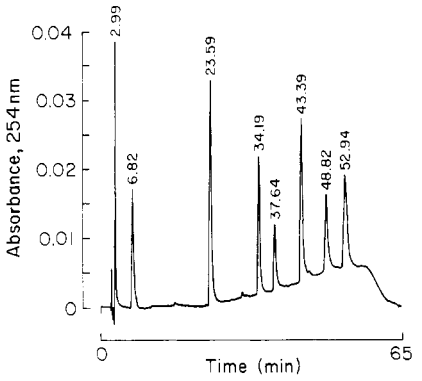

FIGURE 2: Chromatographic resolution of diguanine and monoguanine nucleotides by HPLC. Gp_3G, Gp_4G, Gp_5G, Gp_6G, cGMP, GMP, GTP, and Gp_4, at 0.8–1.2 nmol each, were separated on a 4.6 mm × 25 cm column of Whatman Partisil PXS 10/25 SAX resin. The buffer solutions and chromatographic elution conditions were the same as described in the legend of Figure 1. Nucleotides and retention times: 2.99 min, cGMP; 6.82 min, GMP; 23.59 min, Gp_3G; 34.19 min, Gp_4G; 37.64 min, GTP; 43.39 min, Gp_5G; 48.82 min, Gp_4; 52.94 min, Gp_6G.

specified in Table II. The validity of all assays was tested with commercial preparations of enzymes except for nucleoside diphosphate kinase and $NAD(P)^+$ pyrophosphohydrolase.

Results

HPLC System. Gradient elution with ammonium phosphate on a strong anion-exchange resin resolved each of the Ap_nA (n = 2–6), cAMP, AMP, ADP, ATP, and Ap_4 from one another (Figure 1). ADPR eluted at 7.4 min and coeluted with Ap_2A under these conditions. Reproducibility of all retention times was excellent for individual columns provided the resin was equilibrated with buffer A at the initiation of an analysis. However, we have found small differences in retention times with different columns from the same manufacturer. Resolution was still maintained on different columns except for Ap_6A and Ap_4, which coeluted. Gp_nG (n = 3–6), cGMP, GMP, GDP, GTP, and Gp_4 were resolved on the same HPLC system except for GDP and Gp_3G (Figure 2). GDP eluted 0.1–0.3 min after Gp_3G and was incompletely resolved. Retention times for other dinucleoside polyphosphates were as follows: NAD^+, 3.5 min; $NADP^+$, 15.4 min; m^7Gp_3Cm, 10.1 min; m^7Gp_3Am, 12.1 min.

Dinucleoside Polyphosphate Pyrophosphohydrolase Activity. One mole of Ap_4A was symmetrically hydrolyzed by diadenosine tetraphosphate pyrophosphohydrolase to 2 mol of ADP on the basis of a comparison to standards and integration of peak areas (Table I). Ap_5A was hydrolyzed to equimolar concentrations of ADP and ATP (Table I). Ap_3A, Ap_6A, and Ap_4 were substrates, and in each case ADP was a product (Table I). The percent hydrolysis of Ap_3A and Ap_4 was small with 0.05 μg of enzyme (Table I), but the percent hydrolysis was 4% and 52%, respectively, when the mass of enzyme was increased to 0.2 μg. Ap_4 was hydrolyzed to ADP and, presumably, PP_i (PP_i was not detectable by our HPLC methodology) since the enzyme did not hydrolyze PP_i (see phosphatase results). No hydrolysis of Ap_2A, AMP, ADP, ATP, or ADPR was detected by HPLC (Table I).

Table I: Substrate Specificity of *P. polycephalum* Diadenosine Tetraphosphate Pyrophosphohydrolase[a]

substrate (10 μM)	percent hydrolyzed[b]	products	relative percent hydrolyzed
AMP, ADP, ATP, ADPR	<0.5		
Ap_2A	<0.5		
Ap_3A	4[c]	ADP, AMP	
Ap_4A	40	ADP	100
Ap_5A	82	ADP, ATP	205
Ap_6A	50	ADP, Ap_4	125
Ap_4	11, 52[c]	ADP, PP_i (?)	28
Gp_4	16[c]	GDP, PP_i (?)	
GMP, GDP, GTP	<0.5		
Gp_3G	5[c]	GDP, GMP	
Gp_4G	35	GDP	88
Gp_5G	64	GDP, GTP	160
Gp_6G	85	GDP, Gp_4	212
m^7Gp_3Cm[d]	<0.5		
m^7Gp_3Am[d]	<0.5		

[a] Each substrate was incubated in the absence and the presence of 0.05 μg of diadenosine tetraphosphate pyrophosphohydrolase under standard assay conditions. Assay solutions were analyzed by HPLC as described under Experimental Procedures. [b] The percent of substrate hydrolyzed was calculated from the peak areas of the substrate in the presence and absence of enzyme. When the percent hydrolysis was less than 10%, the peak area of the product was compared to the peak area of product standards to determine the percent hydrolysis. [c] Substrate was incubated with 0.2 μg of enzyme. [d] Concentration equivalent to 0.21 A_{250} unit.

Other Enzymic Activities. Results of assays for adenylate kinase, nucleoside diphosphate kinase, $NAD(P)^+$ pyrophosphohydrolase, cyclic nucleotide phosphodiesterase, phosphodiesterase, phosphatase, and ribonuclease activities are presented in Table II. None of these activities were detectable in partially purified diadenosine tetraphosphate pyrophosphohydrolase. Commercial preparations of the enzymes were active with designated substrates under the assay conditions.

Discussion

One mole of Ap_4A is symmetrically hydrolyzed to 2 mol of ADP by diadenosine tetraphosphate pyrophosphohydrolase from *Physarum* as detected by HPLC. This supports the

One mole of Gp_4G was symmetrically hydrolyzed to 2 mol of GDP, and Gp_5G was hydrolyzed to equimolar concentrations of GDP and GTP (Table I). Gp_3G, Gp_6G, and Gp_4 were substrates, and in each case GDP was a product (Table I). The percent hydrolysis of Gp_3G and Gp_4 was too small with 0.05 μg of enzyme to reliably measure. The percent hydrolysis was 5% and 16%, respectively, when the mass of enzyme was increased to 0.2 μg (Table I). No hydrolysis of the cap dinucleotides, m^7Gp_3Am and m^7Gp_3Cm, was detected by HPLC (Table I).

Table II: Enzyme Activities Assayed for in Preparations of Diadenosine Tetraphosphate Pyrophosphohydrolase from *P. polycephalum*[a]

activity assayed	substrates	concn (μM)	method of assay	% hydrolysis
adenylate kinase	ADP	10	HPLC	<0.5
	ATP + AMP	10 + 10		
$NAD(P)^+$ pyrophosphohydrolase	NAD^+	10	HPLC	<0.5
	NADP	10		
nucleoside diphosphate kinase	ATP + GDP	10 + 10	HPLC	<0.5
	GTP + ADP	10 + 10		
cyclic nucleotide phosphodiesterase	cAMP, cGMP	1, 5, 10	radioisotopic	<0.05
phosphodiesterase	*p*-nitrophenyl phenyl phosphonate	1000	spectrophotometric	<0.01
	bis(*p*-nitrophenyl) phosphate	1000		
ribonuclease	yeast RNA	0.1 mg/mL	alkaline phosphatase + P_i	<0.5
phosphatase	$NADP^+$, AMP, ADP, ATP, GMP, GDP, GTP, glucose 6-phosphate, fructose 6-phosphate, fructose 1,6-bisphosphate, ribose 5-phosphate, creatine phosphate, phosphoenolpyruvate, glyceraldehyde 3-phosphate, glycerol 3-phosphate, sodium pyrophosphate	300	P_i	<0.5

[a] Diadenosine tetraphosphate pyrophosphohydrolase was assayed for the presence of the indicated enzymic activities by the methodology described under Experimental Procedures.

previous evidence that ADP is the product (Barnes & Culver, 1982). The formation of ADP is not the result of hydrolysis of Ap_4A to AMP and ATP by the *Physarum* enzyme and subsequent conversion to ADP by adenylate kinase. First, the equilibrium constant for the reaction catalyzed by adenylate kinase is 0.4–1.2 (Noda, 1962), so AMP, ADP, and ATP should all be present and detectable by HPLC if adenylate kinase is present. Secondly, no adenylate kinase activitiy was detectable in diadenosine tetraphosphate pyrophosphohydrolase preparations by HPLC or by a coupled-enzyme assay system (Barnes & Culver, 1982). Additional adenine nucleotide substrates for this enzyme are Ap_3A, Ap_5A, Ap_6A, and Ap_4, and ADP is always one of the products. Similarly, Gp_4G is symmetrically hydrolyzed to 2 mol of GDP. Gp_3G, Gp_5G, Gp_6G, and Gp_4 are also substrates, and GDP is always one of the products.

A minimum of three phosphates is necessary, but insufficient, for a nucleotide to be a substrate. Ap_3A and Gp_3G are substrates, but ATP, GTP, m^7Gp_3Cm, and m^7Gp_3Am are not. Among the Ap_nA (n = 3–6) substrates, Ap_5A is the optimal one, while Gp_6G is the optimal substrate among the Gp_nG (n = 3–6) series. Ap_4A, Ap_5A, and Ap_4 are better substrates than Gp_4G, Gp_5G, and Gp_4, while Gp_6G is a better substrate than Ap_6A. Both Ap_3A and Gp_3G are relatively weak substrates. These relative percents of hydrolysis were determined under one set of conditions. Measurement of values of K_m and V_{max} may change the consideration of the optimal substrate.

The capacity of diadenosine tetraphosphate pyrophosphohydrolase from *Physarum* to hydrolyze Ap_3A, Gp_3G, Ap_4, and Gp_4 distinguishes it from the rat liver diadenosine tetraphosphate phosphohydrolase (Lobaton et al., 1975). These nucleotides have not been directly tested as substrates for the enzyme from other tissues. Ap_5A, Ap_6A, Gp_5G, and Gp_6G have not been directly examined as substrates for any of the enzymes from other organisms, so we do not know if the hydrolysis of these nucleotides is a unique property of the *Physarum* enzyme. Only the enzyme from *Physarum* hydrolyzes Ap_4 and Gp_4, although both of these mononucleotides are potent inhibitors of Ap_4A hydrolysis by enzymes from *Physarum* (Barnes & Culver, 1982), rat liver (Lobaton et al., 1975), brine shrimp (Vallejo et al., 1976), and mouse liver (Höhn et al., 1982). Ap_4 and Gp_4 are better substrates than Ap_3A and Gp_3G for the enzyme from *Physarum* and are the only mononucleotides determined to be substrates. Diadenosine tetraphosphate pyrophosphohydrolase from *Physarum* is distinct from nucleoside tetraphosphate hydrolase from rabbit muscle since it hydrolyzes Ap_4 to ATP and P_i (Small & Cooper, 1966a).

We do not know if only a single enzyme is responsible for the substrate specificity exhibited by the *Physarum* preparation. Although the preparation is heterogeneous, only a single peak of activity for Ap_4A hydrolysis was detected on polyacrylamide gels (Barnes & Culver, 1982). Several enzyme activities that could account for hydrolysis of Ap_nA (n = 3–6), Ap_4, Gp_nG (n = 3–6), and Gp_4 were not detected in diadenosine tetraphosphate pyrophosphohydrolase from *Physarum*. Previously, only the enzyme from rat liver had been assayed for any contaminating activities, and no phosphodiesterase or phosphatase activities were detected. (Lobaton et al., 1975).

Ap_4A (Rapaport & Zamecnik, 1976), Ap_4 (Small & Cooper, 1966b), Gp_3G (Warner & Finamore, 1965b), and Gp_4G (Finamore & Warner, 1963) have been detected in eucaryotic organisms. The presence of an enzyme with specificity toward these nucleotides supports the idea that these compounds have a physiologic role.

Resolution in this HPLC system was obtained with a pH and ionic strength gradient of ammonium phosphate. The initial isocratic phase of the gradient was necessary to separate nucleotides with less than four negative groups. The linear portion of the gradient permitted separation of the more negatively charged nucleotides. A completely linear gradient was unsuccessful even though several different values of pH and ionic strengths of ammonium phosphate were tested. Relative elutions of the monoadenine and diadenine nucleotides indicate that the separation is not a simple function of actual charge. Ap_4A, Ap_5A, and probably Ap_6A have a stacked conformation of adenine rings at pH 7 (Kolodny et al., 1979). The conformation of Ap_2A has been proposed to differ from the conformation of Ap_3A and Ap_4A at pH 7 (Scott & Zamecnik, 1969). At pH 4–5, Ap_4A and Ap_5A have a folded, unstacked conformation in which the phosphate chain is shielded by the adenine rings. Such a conformation is not possible for Ap_2A and Ap_3A (Kolodny et al., 1979). Different conformations and the resulting effective charge probably affect the elution of the diadenosine polyphosphates.

In conclusion, diadenosine tetraphosphate pyrophosphohydrolase from *P. polycephalum* has catalytic properties that distinguish it from Ap_4A-hydrolyzing enzymes from other organisms. Symmetrical hydrolysis of Ap_4A and Gp_4G, hy-

drolysis of Ap_3A, Ap_5A, Ap_6A, Ap_4, and the corresponding guanine nucleotides, biphasic kinetics for Ap_4A, and inhibition by Mg^{2+} (Barnes & Culver, 1982) are specific characteristics of the *Physarum* enzyme.

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Molecular Aspects of Bovine Erythrocyte Bis[(heme *b*)copper] Protein†

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ABSTRACT: An erythrocyte Cu_2(heme $b)_2$ protein of M_r 400 000 was successfully isolated. Incubating the protein in sodium dodecyl sulfate prior to polyacrylamide gel electrophoresis caused the splitting into M_r 70 000, 120 000, and 200 000 units. The copper was fully electron paramagnetic resonance detectable of the type II ($g_\perp = 2.0309$, $g_\parallel = 2.2122$, $A_\parallel = 175$ G). The high-spin ($d^5_{5/2}$) iron(III) showed a g value of 6.05. No magnetic interaction between copper and heme iron was detected. In a comparison of more than ten different enzymatic oxidase activities, not a single one could be assigned to the Cu_2(heme $b)_2$ protein. Azide, CO, cyanide, fluoride, and imidazole were bound to the heme iron. The binding of imidazolate suggests the accessibility of the sixth coordination site of the heme *b* group to fairly large ligands. Removal of the copper by ethylenediaminetetraacetic acid or cyanide resulted in an irreversible precipitation of the protein. This supports the structural contribution of the copper.

The copper concentration in both serum and red blood cells is near 10 μM (Underwood, 1971). The origin of the serum copper is fairly well understood; it is almost entirely bound in ceruloplasmin (Carrico & Deutsch, 1969a; Malkin & Malmström, 1970; Frieden, 1980). Specific copper binding sites have been characterized in this protein (Malkin & Malmström, 1970; Fee, 1975). All three copper chromophores as defined by Malkin & Malmström (1970) are detectable.

At present, only 38% of the copper content in red blood cells has been assigned to a copper and zinc containing protein called erythrocuprein (Mann & Keilin, 1938; Carrico & Deutsch, 1969b, 1970; Weser, 1973). The latter protein has had many different names and is presently called superoxide dismutase (McCord & Fridovich, 1969). The occurrence of another erythrocyte copper protein called "pink copper protein" has been reported by Reed et al. (1970). Its existence could not be confirmed (H. Deutsch et al., unpublished results; G. Rotilio et al., unpublished results; U. Weser et al., unpublished results).

In a preliminary study a Cu_2(heme $b)_2$ protein of M_r 400 000 was reported (Sellinger & Weser, 1981). While the functional side of erythrocuprein or superoxide dismutase copper is debated, at least, the biochemical role of the copper heme protein is completely unknown. It was of interest to characterize this copper heme protein in more detail. How many subunits

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