The green alga *Scenedesmus obliquus* contains both diadenosine 5',5'''-P¹,P⁴-tetrphosphate (asymmetrical) pyrophosphohydrolase and phosphorylase activities

Alexander G. McLENNAN,* Elaine MAYERS, Steven HANKIN, N. Matthew H. THORNE, Mark PRESCOTT† and Roy POWLS

Cellular and Metabolic Regulation Group, Department of Biochemistry, University of Liverpool, P.O. Box 147, Liverpool L69 3BX, U.K.

Diadenosine 5',5'''-P¹,P⁴-tetrphosphate (Ap₄A) phosphorylase and Ap₄A pyrophosphohydrolase activities have been purified from extracts of the green alga *Scenedesmus obliquus*. Both activities were also detected in *Scenedesmus basiliensis*, *Scenedesmus quadricauda* and in *Chlorella vulgaris*. This is the first time that both types of enzyme have been detected in the same species. The Ap₄A phosphorylase has a molecular mass of 46–48 kDa, a broad pH optimum between 7.5 and 9.5, and requires a divalent ion for activity (Mg²⁺ > Co²⁺ > Ca²⁺ = Mn²⁺ = Cd²⁺ > Zn²⁺). It degrades substrates with at least four phosphate groups and always produces a nucleoside 5'-diphosphate product. The K_m values for Ap₄A and P_i are 5.3 μM and 160 μM, respectively, and k_{cat} = 1.8 s⁻¹. Arsenate, vanadate, molybdate, chromate and tungstate can substitute for phosphate.

The enzyme also catalyses Ap₄A synthesis (K_{eq} = [Ap₄A][P_i]/[ATP][ADP] = 9 × 10⁻⁴) and ADP arsenolysis. The Ap₄A hydrolase has a molecular mass of 26–28 kDa, an alkaline pH optimum of 8.8–9.8, and prefers Zn²⁺ as the stimulatory ion (Zn²⁺ > Mg²⁺ > Mn²⁺ > Co²⁺ > Cd²⁺). It degrades substrates with at least four phosphate groups, having a slight preference for Ap₅A, and always produces a nucleoside 5'-triphosphate product. The K_m value for Ap₄A is 6.6 μM and k_{cat} = 1.3 s⁻¹. It is inhibited competitively by adenosine 5'-tetrphosphate (K_i = 0.67 μM) and non-competitively by fluoride (K_i = 150 μM). A 50–54 kDa dinucleoside 5',5'''-P¹,P³-triphosphate (Ap₃A) pyrophosphohydrolase was also detected in *S. obliquus*, *S. quadricauda* and *C. vulgaris*. The corresponding enzyme in *S. basiliensis* (> 100 kDa) may be a dimer.

INTRODUCTION

The nucleotide diadenosine 5',5'''-P¹,P⁴-tetrphosphate (Ap₄A) is found in all cells in concentrations ranging from 0.05 to > 100 μM [1,2]. In many cases, the concentrations of Ap₄A and related dinucleoside polyphosphates, such as adenosine 5'-guanosine 5'-P¹,P⁴-tetrphosphate (Ap₄G) and adenosine 5'-guanosine 5'-P¹,P³-triphosphate (Ap₃G), increase after exposure of cells to various forms of metabolic stress (heat, oxidative, nutritional and DNA damage) and they have therefore been implicated in the regulation of the cellular response to such stresses [3–6]. Other evidence has supported a role for Ap₄A in DNA replication in eukaryotes [1,7]. However, definitive evidence for its role(s) in these situations is still lacking. Extracellular signalling and neurotransmitter functions for Ap₄A and its homologues diadenosine 5',5'''-P¹,P³-triphosphate (Ap₃A), diadenosine 5',5'''-P¹,P⁵-pentaphosphate (Ap₅A) and diadenosine 5',5'''-P¹,P⁶-hexaphosphate (Ap₆A) have also been suggested [8–10] and a specific cell-surface Ap₄A receptor has been characterized [11].

Any study of the function of a potential metabolic regulator must include an investigation of its synthesis and degradation. Ap₄A is synthesized in all cells by aminoacyl-tRNA synthetases [12,13]. Theoretically, any enzyme reaction involving an acyladenylate has the potential for Ap₄A synthesis; however, so far, firefly luciferase is the only other such enzyme that has been

found to synthesize this nucleotide at a significant rate at physiological pH [14].

In contrast to this relatively simple and uniform picture, the specific enzymes responsible for Ap₄A degradation in different organisms vary considerably [15]. All prokaryotes studied so far possess an Ap₄A (symmetrical) pyrophosphohydrolase (Ap₄A → ADP + ADP; EC 3.6.1.41) of ~ 33 kDa [16,17] whereas higher eukaryotes (vertebrates, invertebrates and plants) have a small, 17–21 kDa (asymmetrical) Ap₄A pyrophosphohydrolase (Ap₄A → AMP + ATP; EC 3.6.1.17) [18–20]. Among the lower eukaryotes, however, the phylogenetic picture is very confusing: the acellular slime mould *Physarum polycephalum* has a symmetrical hydrolase [21], the fission yeast *Schizosaccharomyces pombe* has a dimeric (49 kDa) asymmetrical hydrolase [22], and the budding yeast *Saccharomyces cerevisiae* and the photosynthetic protozoan *Euglena gracilis* have a quite distinct enzyme, a reversible Ap₄A phosphorylase (Ap₄A + P_i ↔ ATP + ADP; EC 2.7.7.53) [23,24]. *S. cerevisiae* has in fact two phosphorylases (I and II, both 36–38 kDa), both of which have been cloned and shown to be distinct gene products, sharing 60 % sequence identity [25]. These enzymes are reversible and capable of a low rate of Ap₄A synthesis at physiological pH; however, it is likely that their primary role *in vivo* is catabolic [26]. The phylogenetic and functional significance of this cellular variation in the enzymes of Ap₄A catabolism is as yet unclear.

Despite this variation, all cells studied so far have been found

Abbreviations used: Ap₄A, diadenosine 5',5'''-P¹,P⁴-tetrphosphate; Ap₂A, diadenosine 5',5'''-P¹,P²-diphosphate; Ap₃A, diadenosine 5',5'''-P¹,P³-triphosphate; Ap₅A, diadenosine 5',5'''-P¹,P⁵-pentaphosphate; Ap₆A, diadenosine 5',5'''-P¹,P⁶-hexaphosphate; p₄A, adenosine 5'-tetrphosphate; p₅A, adenosine 5'-pentaphosphate; Gp₃G, diguanosine 5',5'''-P¹,P³-triphosphate; Gp₄G, diguanosine 5',5'''-P¹,P⁴-tetrphosphate; Gp₅G, diguanosine 5',5'''-P¹,P⁵-pentaphosphate; p₄G, guanosine 5'-tetrphosphate; BisTris Propane, (1,3-bis[tris(hydroxymethyl)-methylamino]propane; PMSF, phenylmethanesulphonyl fluoride; E-64, trans-epoxysuccinyl-L-leucylamido-(4-guanidino)butane.

* To whom correspondence should be addressed.

† Present address: Department of Biochemistry, Monash University, Clayton, Victoria, Australia.

to possess only a single type of enzyme species. Here, we describe for the first time both Ap_4A (asymmetrical) pyrophosphohydrolase and Ap_4A phosphorylase activities in the individual members of a group of organisms, the green algae of the genera *Scenedesmus* and *Chlorella*. Both enzymes have been purified to homogeneity from *Scenedesmus obliquus* and their properties determined. Ap_3A hydrolase activity was also detected in these algae.

EXPERIMENTAL

Materials

Phenylmethanesulphonyl fluoride (PMSF), benzamidine, *trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)butane (E64) and all nucleotides were obtained from Sigma. DEAE Sephacel was from Pharmacia and Ultrogel AcA44 from Réactifs IBF, Villeneuve la Garenne, France. ATP Monitoring Reagent, which contains firefly luciferase, luciferin and Mg^{2+} ions, was from BioOrbit, Turku, Finland. Procion Red P-3BN-Sepharose and homogeneous human placental Ap_4A (asymmetrical) pyrophosphohydrolase were prepared as described [27]. The breakage buffer was 30 mM Hepes/KOH, pH 8.0, 5 mM EDTA, 1 mM PMSF, 1 mM benzamidine, 5 μM E-64, and the running buffer was 20 mM Tris/HCl, pH 7.8, 1 mM 2-mercaptoethanol, 0.1 mM EDTA.

Cells and culture conditions

S. obliquus CCAP 276/6A and *Chlorella vulgaris* CCAP 211/11B from the Culture Collection of Algae and Protozoa (Ambleside, U.K.) were grown heterotrophically at 30 °C in 1 litre batches to late exponential phase as previously described [28]. *Scenedesmus basiliensis* CCAP 276/1A and *Scenedesmus quadricauda* CCAP 276/4B were grown in the light in EGM medium [0.1 % (w/v) sodium acetate trihydrate, 0.1 % (w/v) Lab Lemco (Oxoid L29), 0.2 % (w/v) tryptone (Difco), 0.2 % (w/v) yeast extract, 0.001 % (w/v) CaCl_2].

Enzyme assays

Luminescence assay

Both hydrolase (asymmetrical) and phosphorylase activities were monitored during purification using a luminescence assay [19]. To measure hydrolase activity, the assay contained 40 mM Hepes/KOH, pH 8.0, 6 mM MgCl_2 , 50 μM Ap_4A , 10 μl of ATP-monitoring reagent and the enzyme fraction in a final volume of 50 μl . Phosphorylase assays in addition contained 5 mM potassium phosphate. Phosphorylase activity could be measured exclusively in the presence of hydrolase by the further inclusion of 0.5 mM NaF in the assay. The increase in luminescence was monitored over a 2 min period at 25 °C with an LKB 1250 luminometer. Ap_3A hydrolase activity was measured as previously described [29].

H.p.l.c. assay

Assays (50 μl) for hydrolase (asymmetrical) and phosphorylase activities contained 40 mM Hepes/KOH, pH 8.0, 6 mM MgCl_2 , 400 μM Ap_4A and 5 mM potassium phosphate (for phosphorylase only) and were incubated at 30 °C for 10 min. Reactions were terminated by the addition of 75 μl of 0.67 M ice-cold trichloroacetic acid. After 15 min at 4 °C, 125 μl of 0.6 M trioctylamine in Arcton (trichlorotrifluoroethane) was added and the mixtures were shaken for 2 min, then centrifuged at 5000 *g* for 2 min. Samples (90 μl) of the upper aqueous layers

were mixed with 20 μl of 0.1 M NH_4HCO_3 , pH 9.6, and 100 μl was injected on to a 50 mm $\times$ 7.8 mm Bio-Gel MA7Q column (BioRad) equilibrated in buffer A (20 mM NH_4HCO_3 , pH 9.6). The column was eluted at 2 ml/min with a 6 min linear gradient of 3–40 % (v/v) buffer B (0.7 M NH_4HCO_3 , pH 9.6). Areas of u.v.-absorbing peaks (254 nm) were integrated using a 1040 data capture unit and ChromMac software (Drew Scientific, London, U.K.).

Ap_4A synthesis

The synthetic capacity of the Ap_4A phosphorylase was assessed at 30 °C in a reaction mixture (50 μl) containing 40 mM 1,3-bis[tris(hydroxymethyl)-methylamino]propane (BisTris Propane) pH 5.3, 4 mM ATP, 4 mM ADP, 5 mM divalent cation and enzyme. Assays were incubated at 30 °C for up to 2 h then processed and analysed by h.p.l.c. as described above.

Other methods

Protein concentrations were measured by the Coomassie Blue binding method [30]. Molecular masses of the native enzymes were determined by gel filtration on a HiLoad 16/60 Superdex 75 column (16 mm $\times$ 600 mm, Pharmacia) calibrated with molecular-mass standards (Figure 1). SDS/PAGE was performed using a 100 mm $\times$ 100 mm $\times$ 0.75 mm 10 % (w/v) slab gel [31].

RESULTS

Demonstration of Ap_4A phosphorylase and Ap_4A hydrolase activities in *S. obliquus*

Cells (1 g wet weight) were suspended in 3 ml of breakage buffer and disrupted by shaking at 1600 rev./min for 4 min with glass

Figure 1 Demonstration of Ap_4A phosphorylase, Ap_4A hydrolase and Ap_3A hydrolase activities in *S. obliquus* by gel filtration on a Superdex 75 column

Crude extract (0.5 ml) was applied to a HiLoad 16/60 Superdex 75 column and the column run as described in the Results section. Samples (25 μl) of each fraction were assayed for Ap_4A phosphorylase (●), Ap_4A hydrolase (○) and Ap_3A hydrolase (□) activities using the luminescence assay (50 μl total volume). Phosphorylase assays contained 5 mM potassium phosphate and 0.5 mM NaF. The column was calibrated with the following molecular-mass standards (▲): 1, conalbumin (77 kDa); 2, BSA (67 kDa); 3, ovalbumin (45 kDa); 4, β -lactoglobulin (36.8 kDa); 5, carbonic anhydrase (29 kDa); 6, chymotrypsinogen A (25 kDa); 7, soybean trypsin inhibitor (20.1 kDa); 8, myoglobin (17 kDa); and 9, cytochrome c (12.4 kDa). The elution volumes of these standards are indicated by the appropriate numbers.

Table 1 Enzymes of diadenosine polyphosphate catabolism in various green algae

Enzyme activities were analysed by chromatography of crude cell extracts on a calibrated Superdex 75 column as described in the Experimental section. Specific activities were calculated from the summed activities of the appropriate column fractions obtained by chromatography of 0.5 ml of crude extract and the protein concentration of the crude extract. Results shown are means of duplicate determinations.

Organism	Ap ₄ A phosphorylase		Ap ₄ A hydrolase		Ap ₃ A hydrolase	
	Mass (kDa)	Specific activity (pkat/mg)	Mass (kDa)	Specific activity* (pkat/mg)	Mass (kDa)	Specific activity (pkat/mg)
<i>S. obliquus</i>	46	10.9	26	4.7	54	3.0
<i>S. basiliensis</i>	44	20.0	20	4.7	> 100	1.5
<i>S. quadricauda</i>	44	2.3	22	0.65	54	0.17
<i>C. vulgaris</i>	45	16.0	22	0.50	50	1.2

* For comparison, specific activities of 3.3–8.4 pkat/mg were measured for the Ap₄A hydrolases from various rat tissues.

Table 2 Purification of Ap₄A phosphorylase from *S. obliquus*

Ap₄A phosphorylase was purified from 175 g of exponential phase cells as described in the Results section. Specific activity was measured using the bioluminescence assay in the presence of 5 mM potassium phosphate and 0.5 mM NaF. Ap₄A phosphorylase specific activity in the crude extract was estimated by summing the activities of the fractions obtained by chromatography of 0.5 ml of extract on a Superdex 75 column, as described in Figure 1.

Fraction	Protein (mg)	Specific activity (nkat/mg)	Purification (-fold)	Activity yield (%)
Crude extract	3250	0.013	1	100
DEAE-Sephacel	296	0.20	16.3	148
Ultrogel AcA44	65	0.42	33.3	66
Procion Red-Sephrose	2.2	3.9	311	21
BioGel HPHT	0.1	32.6	2600	8

beads (0.45–0.5 mm) in a Braun Mikro-Dismembrator U. The cell homogenate was decanted and centrifuged at 100 000 *g* for 1 h and 0.5 ml of the filtered (0.2 μ m filter) supernatant was applied to a calibrated HiLoad 16/60 Superdex 75 column equilibrated in 30 mM Hepes/KOH, pH 8.0, 50 mM KCl, 1 mM 2-mercaptoethanol, 0.1 mM EDTA. The column was eluted with the same buffer at 1 ml/min and 1 ml fractions collected and assayed for Ap₄A hydrolase, Ap₄A phosphorylase and Ap₃A hydrolase activities. Figure 1 shows the presence of all three enzyme activities; their molecular masses and specific activities are shown in Table 1. This pattern was reproduced using cultures initiated from different stock slopes and also from a single colony. Furthermore, all culture supernatants were judged free of contamination by microscopic examination and by plating on Luria-Bertani agar and Sabouraud agar. Hence, both Ap₄A catabolic enzymes must have originated from *S. obliquus*.

A similar pattern for enzyme activities was observed in extracts of the related *S. basiliensis*, *S. quadricauda* and *C. vulgaris*, although their relative specific activities varied considerably (Table 1). In the case of the enzymes from *C. vulgaris*, their relative amounts did not differ greatly between cells grown in the light and in the dark or between different stages of the growth cycle (results not shown). The hydrolases from *S. obliquus* and *S. basiliensis* had specific activities similar to those from mammalian tissues, whereas those from *S. quadricauda* and *C. vulgaris* were an order of magnitude lower. Also, the Ap₃A hydrolase activity

of *S. basiliensis* eluted just behind the void volume of the column, suggesting a molecular mass of just over 100 kDa, twice that of the corresponding enzyme from the other algae (Table 1).

Purification of Ap₄A phosphorylase and Ap₄A hydrolase from *S. obliquus*

All operations were carried out at 4 °C unless otherwise stated. Cells (175 g wet weight) were harvested from 16 l of culture medium, washed, and then disrupted for 10 min with glass beads (0.25–0.30 mm) in breakage buffer in a Dynomill (type KDL; W. A. Bachofen, Basel, Switzerland). A high-speed supernatant (200 000 *g*, 2 h) was prepared as previously described [28]. The filtered supernatant was dialysed overnight against running buffer and was then applied at 130 ml/h to a 18 cm $\times$ 5 cm column of DEAE-Sephacel equilibrated in running buffer. The column was eluted with a 2.4 l linear gradient of 0–0.3 M NaCl in running buffer and 18 ml fractions were collected. Ap₄A hydrolase and Ap₄A phosphorylase activities eluted together at 0.12 M NaCl. Active fractions were pooled, precipitated with (NH₄)₂SO₄ (70 % saturation), redissolved in 30 ml running buffer and applied at 80 ml/h to a 95 cm $\times$ 5 cm column of Ultrogel AcA44 in running buffer. Fractions (18 ml) containing the separated Ap₄A phosphorylase (fractions 57–66) and Ap₄A hydrolase (fractions 69–80) activities were pooled separately, dialysed against 50 mM potassium phosphate buffer, pH 6.8, 0.1 mM EDTA, 10 % (v/v) glycerol and applied separately to two 60 mm $\times$ 8 mm columns of Procion Red P-3BN-Sephrose. After extensive washing of the columns with the same buffer, they were eluted as follows. Hydrolase activity was eluted biospecifically with 50 mM potassium phosphate buffer, pH 6.8, 10 % (v/v) glycerol containing 5 mM MgCl₂ and 25 μ M adenosine 5'-tetraphosphate (p₄A). After the first 5 ml of elution buffer had passed through the column, the flow was stopped for 1 h then restarted. Pooled active fractions (25 ml total) were dialysed for 48 h against two 1 l changes of 30 mM Hepes/KOH, pH 7.8, 50 % (v/v) glycerol, and stored at –20 °C. Phosphorylase activity was eluted with 50 mM potassium phosphate buffer, pH 6.8, 10 % (v/v) glycerol containing 1.5 M NaCl after first washing the column with the same buffer containing 0.7 M NaCl. Active fractions (2.5 ml each) were pooled, diluted 10-fold with water, then concentrated to 3 ml by ultrafiltration (Amicon stirred cell, 10 kDa cut-off membrane) to reduce the NaCl concentration and finally applied at 1 ml/min at room temperature to a 100 mm $\times$ 7.8 mm BioGel HPHT hydroxy-

Table 3 Purification of Ap₄A pyrophosphohydrolase from *S. obliquus*

Ap₄A hydrolase was purified from 175 g of exponential cells as described in the Results section. Specific activity was measured using the bioluminescence assay in the absence of potassium phosphate and NaF. Ap₄A hydrolase specific activity in the crude extract was estimated as described in Table 1.

Fraction	Protein (mg)	Specific activity (nkat/mg)	Purification (-fold)	Activity yield (%)
Crude extract	3250	0.0057	1	100
DEAE-Sephacel	296	0.078	13.8	126
Ultrogel AcA44	32	0.44	77	76
Procion Red-Sepharose	0.08	48.4	8531	21

Figure 2 SDS/PAGE of *S. obliquus* Ap₄A phosphorylase and Ap₄A hydrolase

Electrophoresis was carried out in a 10% (w/v) polyacrylamide slab gel [31] and the gel stained with Coomassie Blue. Lane 1, standard proteins: BSA (67 kDa), ovalbumin (45 kDa), glyceraldehyde-3-phosphate dehydrogenase (36 kDa), carbonic anhydrase (29 kDa), trypsinogen (24 kDa), soybean trypsin inhibitor (20 kDa) and α -lactalbumin (14.2 kDa); lane 2, 1 μ g of Ap₄A phosphorylase final fraction; lane 3, 0.5 μ g of Ap₄A hydrolase final fraction.

apatite column (BioRad) equilibrated in 5 mM potassium phosphate buffer, pH 6.8, containing 10% (v/v) glycerol and 10 μ M CaCl₂. After elution of the unbound protein, the column was eluted with a 31.5 ml linear gradient of 5–300 mM potassium phosphate buffer, pH 6.8, containing 10% (v/v) glycerol and 10 μ M CaCl₂. Active fractions (which eluted at 80–100 mM potassium phosphate) were dialysed extensively against 30 mM Hepes/KOH, pH 7.8, 50% (v/v) glycerol, and stored at –20 °C. The purification procedures are summarized in Tables 2 and 3.

The purity of the final fractions was assessed by SDS/PAGE (Figure 2). The phosphorylase showed as a major band of 46 kDa (lane 2) and the hydrolase as a major band of 28 kDa (lane 3). These figures are in close agreement with those obtained by gel filtration and suggest that both enzymes consist of single polypeptides.

Effect of pH

Both enzymes exhibited maximum activity in the h.p.l.c. assay at alkaline pH in 40 mM BisTris Propane buffer (working pH range 6.0–9.8, $I = 0.15$). The phosphorylase had a very broad optimum between pH 7.5 and 9.5, with 20 and 80% activity remaining at pH 6.5 and 9.8, respectively. The hydrolase had a sharper

Figure 3 Effect of various divalent cations on the activities of (a) Ap₄A phosphorylase and (b) Ap₄A hydrolase from *S. obliquus*

Initial rates of Ap₄A degradation were determined using the h.p.l.c. assay in the presence of various concentrations of Zn²⁺ (●), Mg²⁺ (○), Mn²⁺ (×), Co²⁺ (■), Cd²⁺ (▲), or Ca²⁺ (□) ions.

optimum at pH 9.0, with 35 and 80% activity remaining at pH 6.5 and 9.8, respectively.

Divalent-ion requirements

Both enzymes had a strict requirement for a divalent cation in the h.p.l.c. assay. For the phosphorylase, the rank order of ability to activate the enzyme was Mg²⁺ > Co²⁺ > Ca²⁺ = Mn²⁺ = Cd²⁺ > Zn²⁺ whereas for the hydrolase the order was Zn²⁺ > Mg²⁺ > Mn²⁺ > Co²⁺ > Cd²⁺. Ca²⁺ ions did not support hydrolase activity at all. Maximum phosphorylase activity was obtained at 10 mM Mg²⁺ whereas maximum hydrolase activity was supported by 0.2 mM Zn²⁺ (Figure 3).

Figure 4 Inhibition of the *S. obliquus* (●) and human placental (○) Ap₄A hydrolases by (a) sodium fluoride and (b) p₄A

Ap₄A hydrolase activity was determined using the luminescence assay in the presence of various concentrations of inhibitors and an Ap₄A concentration of 13 μM. The type of inhibition was shown by varying the substrate concentration in the presence of three different inhibitor concentrations (results not shown). Control experiments showed that neither NaF nor p₄A affected the activity of the firefly luciferase at the concentrations used (results not shown). K_i values were determined according to the Cheng–Prusoff relationship [34].

Kinetic parameters

Both enzymes exhibited Michaelis–Menten kinetics with Ap₄A as substrate up to a concentration of 400 μM using the luminescence assay. K_m values determined from Eadie–Hofstee plots were 5.3 and 6.6 μM for the Ap₄A phosphorylase and hydrolase, respectively. The rate constants were also very similar to each other: 1.8 and 1.3 s⁻¹ for the homogeneous Ap₄A phosphorylase and hydrolase, respectively. The K_m value for phosphate for the phosphorylase was 0.16 mM.

Inhibition by fluoride and adenosine 5′-tetraphosphate

Both of these agents are strong inhibitors of the mammalian Ap₄A hydrolase [32,33]. The *S. obliquus* hydrolase was also inhibited by these compounds, but less strongly than the human placental enzyme, to which it was compared directly. Fluoride inhibited the human and algal activities non-competitively with K_i values of 40 and 150 μM, respectively (Figure 4a) whereas p₄A was a competitive inhibitor with K_i values of 0.013 and 0.67 μM, respectively (Figure 4b). The *S. obliquus* phosphorylase was insensitive to both compounds over the concentration range tested.

Substrate specificity

Both enzymes were specific for dinucleoside polyphosphates containing at least four phosphate groups (Table 4). The phosphorylase exhibited strong specificity for dinucleoside tetraphosphates and always generated a nucleoside 5′-diphosphate product whereas the hydrolase displayed considerable activity with higher homologues and in fact preferred Ap₅A over Ap₄A. A nucleoside 5′-triphosphate was always one of the hydrolysis products of the hydrolase; hence it can be classified as an asymmetrically cleaving activity. AMP, ADP, ATP, NAD⁺, *p*-nitrophenylthymidine 5′-triphosphate and bis-*p*-nitrophenyl phosphate were inactive as substrates for either enzyme.

Table 4 Substrate specificity of Ap₄A phosphorylase and Ap₄A hydrolase

Reaction rates (V_{rel}) were determined using the h.p.l.c. assay (see the Experimental section). For substrates with more than four phosphate groups, the linear salt gradient was extended to 3–93% buffer B for 15 min. Products were identified from the retention times of authentic standards, except adenosine 5′-pentaphosphate (p₅A), whose identity was assumed from its increased retention time relative to p₄A. Activity is expressed as a percentage of that observed with Ap₄A. Ap₂A, diadenosine 5′,5′-*P*¹,*P*²-diphosphate; p₄G, guanosine 5′-tetraphosphate.

Compound	Ap ₄ A phosphorylase		Ap ₄ A hydrolase	
	V_{rel}	Products	V_{rel}	Products
Ap ₂ A	0	None	0	None
Ap ₃ A	0	None	0	None
Ap ₄ A	100	ADP + ATP	100	AMP + ATP
Ap ₅ A	19	ADP + p ₄ A	120	ADP + ATP
Ap ₆ A	2	ADP + p ₅ A	35	ATP + ATP
Gp ₃ G	0	None	0	None
Gp ₄ G	25	GDP + GTP	78	GMP + GTP
Gp ₅ G	1	GDP + p ₄ G	43	GDP + GTP

Degradation of Ap₄A phosphorylase by other anions

In common with the yeast Ap₄A phosphorylase [35], the *S. obliquus* enzyme was able to use arsenate, vanadate, molybdate, tungstate and chromate (but not sulphate) to promote Ap₄A breakdown. The corresponding values of K_m and V_{rel} (relative to phosphate) are given in Table 5 and show phosphate, arsenate and vanadate to be the preferred anions.

Ap₄A synthesis by Ap₄A phosphorylase

Ap₄A synthesis by the yeast Ap₄A phosphorylase is promoted at low pH. At pH 5.5 and 4 mM Ca²⁺, a K_{eq} of 0.03 (i.e. $[Ap_4A] \cdot [P_i] / [ATP][ADP]$) was measured [26]. At pH 5.5 and 5 mM Mg²⁺ (the preferred cation), the *S. obliquus* enzyme was also able to

Table 5 Kinetic parameters for Ap₄A degradation by anions

Kinetic parameters were determined using the luminescence assay and derived from Eadie-Hofstee plots. The rate of phosphorolysis (V_{rel}) was 1.9 $\mu\text{mol Ap}_4\text{A degraded/min per mg protein}$. Degradation rates are expressed relative to the rate of phosphorolysis (= 1.00).

Anion	K_m (mM)	V_{rel}	V_{rel}/K_m (mM ⁻¹)
Phosphate	0.16	1.00	6.25
Arsenate	0.13	1.04	8.00
Vanadate	0.07	1.14	16.30
Molybdate	0.45	0.32	0.70
Chromate	0.52	0.18	0.35
Tungstate	1.75	0.36	0.20

synthesize Ap₄A, as determined by h.p.l.c. However, the K_{eq} value calculated from the integrated substrate and product peak areas after 2 h incubation was 9×10^{-4} . Thus, the *S. obliquus* enzyme appears less able to synthesize Ap₄A than its yeast counterpart.

Arsenolysis of ADP by Ap₄A phosphorylase

ADP/P_i exchange and ADP arsenolysis are also catalysed by the yeast Ap₄A phosphorylase [36]. Although optimal conditions for this reaction were not determined for the *Scenedesmus* phosphorylase, the algal enzyme was found to catalyse the arsenolysis of ADP at pH 7.4 in the absence of divalent cations (results not shown).

DISCUSSION

The data presented in this paper provide conclusive evidence for the presence of both Ap₄A phosphorylase and Ap₄A (asymmetric) hydrolase activities in *S. obliquus* and related green algae. Both enzymes are slightly larger than those found in most other organisms: the *Scenedesmus* phosphorylase has a molecular mass of 46–48 kDa whereas the enzymes from *S. cerevisiae* and *E. gracilis* have masses of 36–38 kDa [25] and 30 kDa [24], respectively. No evidence was found for more than one species of phosphorylase. The *Scenedesmus* hydrolase has a mass of 26–28 kDa whereas most other hydrolases have a mass of 17–21 kDa, although the enzyme from *Drosophila* has a molecular mass of 26 kDa [37] whereas the unusual hydrolase from *S. pombe* is a dimer of a 22 kDa species [22]. Interestingly, the 54 kDa *S. obliquus* Ap₃A hydrolase appears to be roughly half the size of the corresponding enzyme from *S. basiliensis*. On the basis of the sizes of the yeast (55 kDa) and *Artemia* (115 kDa) Ap₃A hydrolases, we have already suggested that this class of enzyme may be found in monomeric and dimeric forms [29]. The present results support this suggestion.

Purification of the Ap₄A hydrolase activity took advantage of biospecific elution from an immobilized triazine dye by the competitive inhibitor p₄A, a method first reported for the rat liver enzyme [38]. Even though the *Scenedesmus* enzyme has a higher K_i for p₄A than the mammalian enzymes, it is still eluted with high purity from the column. The phosphorylase also binds to the dye column but requires salt for elution because it is not inhibited by p₄A.

In terms of their general properties, both the Ap₄A-catabolizing enzymes are similar to those that have been separately isolated from other cells and tissues, although there are some interesting differences. The preference of a hydrolase for Zn²⁺ as a divalent ion has not been observed before. Generally, these enzymes are either weakly activated or inhibited to varying degrees by this ion [15,19,20]. The rate constant for the hydrolase of 1.3 s⁻¹ is very close to values previously measured for the lupin hydrolase (1.2 s⁻¹ [20]) and the low activity form of the *Artemia* hydrolase (1.5 s⁻¹ [19]). The K_m of 6.6 μM is at the upper end of the range of typical values (0.5–5 μM [15]).

The sensitivity of the hydrolase to inhibition by fluoride and p₄A clearly establishes it as a typical asymmetrically cleaving Ap₄A hydrolase [15]. However, with a K_i of 150 μM , it is less sensitive to fluoride than plant (K_i typically 2–3 μM) and animal (K_i typically 20–30 μM) hydrolases [15,32], and the K_i for p₄A is 50-fold higher than that of the human placental and other mammalian hydrolases [33]. Nevertheless, the K_i for p₄A (0.67 μM) is still 10-fold lower than the K_m for Ap₄A (6.6 μM), making p₄A a potent competitive inhibitor and a useful ligand for the elution of the enzyme from immobilized triazine dyes. Also, the insensitivity of the phosphorylase to fluoride allows this enzyme to be specifically assayed in the presence of contaminating hydrolase by the addition of 0.5–1.0 mM fluoride ions.

Like the two yeast enzymes, the *Scenedesmus* phosphorylase is unable to utilize Ap₃A as a substrate. In contrast, the phosphorylase from *Euglena* can degrade Ap₃A [24]. Yeast Ap₄A phosphorylase I, but not phosphorylase II, can slowly degrade Gp₃G [25]. The algal enzyme is inactive with this substrate as well; it behaves therefore most like the yeast phosphorylase II. The slight preference of the hydrolase for Ap₅A as a substrate is probably not significant and the enzyme may still be classified as an Ap₄A hydrolase on the basis of its requirement for a minimum of four phosphate groups; also, Gp₃G is less effective than Gp₄G as a substrate.

Comparison of the *Scenedesmus* hydrolase with the only other asymmetrically cleaving hydrolase isolated from a lower eukaryote (*S. pombe*) reveals several differences. The fission yeast enzyme is a dimer, is totally resistant to fluoride, has significant activity with Ap₃A (but not Ap₅A) as a substrate and poor activity with guanine dinucleotides [22]. In these respects, the algal enzyme is more like those from higher eukaryotes.

The ability of the yeast Ap₄A phosphorylases to synthesize Ap₄A has highlighted the possibility that they might act as synthetases *in vivo*. However, measurements of K_{eq} at physiological pH, and the fact that disruption of both yeast Ap₄A phosphorylase genes leads to an increase in the concentration of intracellular Ap₄A, suggests that *in vivo* they act as catabolic enzymes [25,26,39]. The data reported here indicate that the *Scenedesmus* phosphorylase is also primarily a catabolic enzyme. This then raises the question of why this organism has two enzymes, with almost identical K_m values and rate constants, whose apparent purpose is the specific breakdown of dinucleoside polyphosphates with at least four phosphate groups (the principal substrate probably being Ap₄A) whereas all other cells studied so far appear to have either one type or the other.

The similarities in the kinetic parameters of these two enzymes argue against specific roles at the different concentrations of Ap₄A that may arise under conditions of stress. Because satisfactory subcellular fractionation is not possible with *Scenedesmus* sp., we could not determine the intracellular location of these enzymes directly. However, both *Scenedesmus* enzymes were readily isolated from the soluble fraction of the cellular homogenate at low ionic strength in the absence of detergents. It seems unlikely that one of the enzymes is cytosolic while the

other is located and functions in the algal chloroplasts. In general, neither phosphorylases nor hydrolases can be specific chloroplast enzymes because both are found in non-photosynthetic organisms. Ap₄A hydrolases are widespread [15] and we have detected only the typical low-molecular-mass hydrolase activity in green leaf tissues of lettuce and cabbage, as well as in potato tubers, wheat germ and cultured celery cells (E. Mayers, K. Boyle, S. Jose and A. G. McLennan, unpublished work). Furthermore, Ap₄A phosphorylase is present in many non-photosynthetic fungi, e.g. *Aspergillus nidulans*, *Candida albicans*, *Mucor mucedo* and *Coprinus cereus* (E. Mayers, K. Boyle, S. Jose and A. G. McLennan, unpublished work) in addition to *S. cerevisiae* [23] and *Acanthamoeba castellanii* [24]. Even among green algae of the division Chlorophyta, the existence of both enzymes may not be widespread. *Scenedesmus* and *Chlorella* belong to the class Chlorophyceae, order Chlorococcales, whereas *Chlamydomonas reinhardtii* belongs to the class Chlorophyceae, order Volvocales. So far, we have been able to detect only a 50 kDa Ap₄A phosphorylase in *Chlamydomonas* (E. Mayers, K. Boyle, S. Jose and A. G. McLennan, unpublished work). Thus, the occurrence of both enzymes appears to be unrelated simply to the presence of chloroplasts. It is also unlikely that one of the enzymes is nuclear; if such a location were of any significance then one would expect the observed phenomenon to be much more widespread.

Although a catabolic role for Ap₄A phosphorylases has been highlighted, there is no doubt that they can synthesize Ap₄A *in vivo*. Indeed, enhanced expression of yeast Ap₄A phosphorylase I by insertion of a multicopy plasmid carrying the *APAI* gene leads, paradoxically, to an increase in intracellular Ap₄A [40]. Thus, the synthetic capacity of Ap₄A phosphorylases clearly depends on the environment of the enzymes, and possibly on some reversible post-translational modification. An anabolic role for the *Scenedesmus* enzyme cannot, therefore, be entirely excluded.

At present, there is no obvious explanation for the presence of both an Ap₄A hydrolase and an Ap₄A phosphorylase within a single species. However, this is clearly a discovery of some significance for our eventual understanding of the metabolism and function of Ap₄A and related nucleotides.

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