Regiospecificity of the Hydrolysis of Diadenosine Polyphosphates Catalyzed by Three Specific Pyrophosphohydrolases†

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ABSTRACT: The different patterns of enzymatic cleavage of diadenosine polyphosphates, Ap_nAs, where n = 3–5, have been established by fast atom bombardment mass spectrometry, FAB MS, of the nucleotide products formed in the presence of $H_2{}^{18}O$. The three specific pyrophosphohydrolases, Ap_3A hydrolase (EC 3.6.1.29) and (*asymmetrical*) Ap_4A hydrolase (EC 3.6.1.17) from lupin and the (*symmetrical*) Ap_4A hydrolase (EC 3.6.1.41) from *Escherichia coli*, manifest three different regiospecificities. The Ap_3A hydrolase cleaves all four substrates tested, Ap_3A, Ap_4A, $ApCH_2ppA$, and ApCHFppA, to give [^{18}O]AMP and the corresponding unlabeled adenosine nucleotide. In each case, the enzyme cleaves at the phosphate proximate to the bound adenosine moiety. The (*asymmetrical*) Ap_4A hydrolase cleaves both Ap_4A and Ap_5A to give unlabeled ATP plus [^{18}O]AMP and [^{18}O]ADP, respectively, and is thus seen to add water at the fourth phosphate from the bound adenosine moiety. Lastly, the (*symmetrical*) Ap_4A hydrolase from *E. coli* gives β-[^{18}O]ADP from Ap_3A, Ap_4A, and Ap_5A along with the unlabeled nucleotide coproducts. In addition, with $Ap_4A\alpha S$ (Ap_5pppA) as substrate for the bacterial enzyme, the products are β-[^{18}O]ADP and unlabeled $ADP\alpha S$. This *symmetrical* enzyme is thus characterized as cleaving the polyphosphate chain at the second phosphate from the bound adenosine moiety.

Dinucleoside polyphosphates (DNPPs) occur in both prokaryotic (Lee *et al.*, 1983; Coste *et al.*, 1987) and eukaryotic (Rapaport & Zamecnik, 1976; Plesner & Ottesen, 1980; Ogilvie, 1981; Flodgaard & Klenow, 1982; Ogilvie & Jacob, 1983; Garrison & Barnes, 1984; Brevet *et al.*, 1985; Miller & McLennan, 1986; Coste *et al.*, 1987) cells. Although diadenosine penta- and hexaphosphates (Ap_5A and Ap_6A) have been described very recently (Pintor *et al.*, 1992a,b, 1993), the predominate species are dinucleoside triphosphates (Np_3Ns) and dinucleoside tetraphosphates (Np_4Ns). Interestingly, the levels of DNPPs grow dramatically under conditions of stress (Lee *et al.*, 1983, Brevet *et al.*, 1985; Baker & Jacobson, 1986; Garrison *et al.*, 1986), but the biological role of those compounds is not actually clear. Based on studies *in vitro* (Zamecnik *et al.*, 1966; Goerlich *et al.*, 1982; Blanquet *et al.*, 1983; Guranowski *et al.*, 1990) and *in vivo* (Brevet *et al.*, 1989), it is believed that DNPPs are synthesized by certain ligases (Zamecnik *et al.*, 1966; Goerlich *et al.*, 1982; Blanquet *et al.*, 1983, Brevet *et al.*, 1989) and transferases (Warner *et al.*, 1974; Wang & Shatkin, 1984; Brevet *et al.*, 1987; Guranowski *et al.*, 1988a). Among the many factors which undoubtedly control the metabolism of DNPPs are various degrading enzymes, particularly ones that are highly specific. In higher eukaryotes, both animals (Warner & Finamore, 1965; Lobaton *et al.*, 1975; Sillero *et al.*, 1977; Ogilvie & Antl, 1983; Prescott *et al.*, 1989, 1992) and plants (Jakubowski & Guranowski, 1983; Guranowski, 1990), there exist a dinucleoside triphosphatase (EC 3.6.1.29), which cleaves preferentially Np_3Ns to NMP and N′DP (Sillero *et al.*, 1977; Jakubowski & Guranowski, 1983; Prescott *et al.*, 1992), and a distinct dinucleoside tetraphosphatase (EC 3.6.1.17), which *asymmetrically* cleaves Np_4Ns to NMP plus N′TP (Warner & Finamore, 1965; Lobaton *et al.*, 1975; Jakubowski & Guranowski, 1983; Ogilvie & Antl, 1983; Prescott *et al.*, 1989; Guranowski, 1990).

In the slime mold *Physarum polycephalum* (Barnes & Culver, 1982) and in various bacteria (Guranowski *et al.*, 1983), there occurs a (*symmetrical*) dinucleoside tetraphosphatase (EC 3.6.1.41). Although the latter enzyme also acts on Ap_3A, there occurs in *Escherichia coli* a distinct Mg^{2+}-dependent hydrolase which prefers Np_3Ns as substrates (Hurtado *et al.*, 1987). Finally, in lower eukaryotes, yeast (Guranowski & Blanquet, 1985) and protozoa (Guranowski *et al.*, 1988b), there has been found a dinucleoside oligophosphate phosphorylase (EC 2.7.7.53). A comparison of the substrate specificities exhibited by these different DNPP-degrading enzymes suggests that each of them performs the catalysis in a distinct way. The most comprehensive studies on the mechanism of hydrolysis of DNPPs by specific enzymes have been performed on the (*asymmetrical*) Ap_4A hydrolase from *Artemia* (McLennan *et al.*, 1989a) and yellow lupin seeds (Dixon & Lowe, 1989). McLennan and co-workers analyzed the products of Ap_4A enzyme in the presence of $H_2{}^{18}O$ and showed that ^{18}O was incorporated exclusively into AMP. Independently, Dixon and Lowe synthesized (R_p,R_p)-P^1,P^4-bis(5′-adenosyl) [1-^{17}O,1,1-$^{18}O_2$,4-^{17}O,4,4-$^{18}O_2$]tetraphosphate and demonstrated that the compound was hydrolyzed by the Ap_4A hydrolase from lupin with inversion of configuration at Pα yielding (R_p)-5′-[^{16}O,^{17}O,^{18}O]AMP.

We have extended our studies on the lupin enzyme by monitoring the hydrolysis of Ap_5A in $H_2{}^{18}O$ [for a preliminary report, see Blackburn *et al.* (1991)] and have performed

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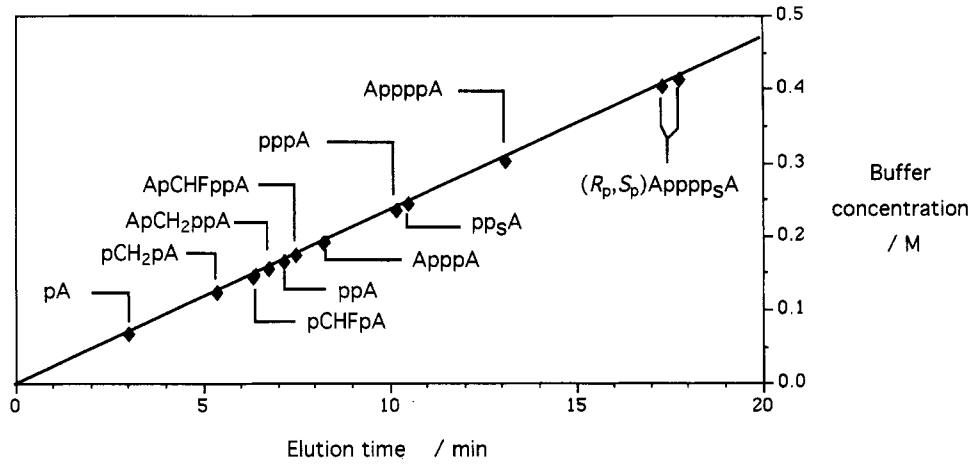

FIGURE 1: Elution time of nucleotides and diadenosine tri- and tetraphosphates and their analogues on a MonoQ column. The analyzed solution (0.02 mL) was injected into a 1-mL MonoQ column equilibriated with 50 mM ammonium bicarbonate (pH 8.5). The column was washed using an ammonium bicarbonate gradient (50–1000 mM) at a flow rate of 1.5 mL/min.

experiments directed at identification of the point of enzymatic cleavage of various diadenosine polyphosphates in two other systems: the Ap_3A hydrolase from yellow lupin seeds and the (*symmetrical*) Ap_4A hydrolase from *E. coli*.

MATERIALS AND METHODS

Enzymes. Ap_3A hydrolase and (*asymmetrical*) Ap_4A hydrolase were isolated from yellow lupin (*Lupinus luteus* var. *topaz*) seeds as described previously (Jakubowski & Guranowski, 1983). The *E. coli* (*symmetrical*) Ap_4A hydrolase was purified according to Guranowski *et al.* (1983).

Chemicals. Water-^{18}O, normalized, 97 atom % ^{18}O, was purchased from Aldrich, U.K. AMP, ADP, and ATP as sodium salts and Ap_3A, Ap_4A, and Ap_5A as ammonium salts were obtained from Sigma. The monothio and dithio analogues of Ap_4A, Ap_sppA, and Ap_sppp_sA were synthesized according to Blackburn *et al.* (1987) and donated by Dr. G. E. Taylor (University of Sheffield). $ApCH_2ppA$ and ApCHFppA were synthesized as described earlier (Blackburn *et al.*, 1990) and provided by Drs. M.-J. Guo and S. P. Langston (University of Sheffield).

Chromatographic Systems. The time course of enzymatic reactions was analyzed by thin-layer chromatography (Guranowski *et al.*, 1987); aluminum plates precoated with silica gel containing fluorescent indicator (from E. Merck) were developed in dioxane/ammonia/water (6:1:4, v/v/v). The products of reactions to be analyzed by FAB MS (see below) were isolated on a 1-mL MonoQ column attached to a Pharmacia FPLC system. The column was equilibrated with 50 mM ammonium bicarbonate (pH 8.5) and the eluate monitored at 254 nm. The products were cleanly separated from traces of unconverted substrates by washing the column first for 2 min with the starting buffer and then for 20 min with the linear (50–500 mM) gradient of the same buffer. Chromatography of the mixtures containing $Ap_4A\alpha S$ was performed with a 50-1000 mM gradient run over 40 min. With this latter system, unchanged substrate, $Ap_4A\alpha S$, was eluted without any of the retardation which occurred when the former gradient was used. For the elution times of the substrates and corresponding products of the enzyme reactions, see Figure 1.

Enzymatic Hydrolysis of Diadenosine Polyphosphates in the Presence of $H_2{}^{18}O$ and Isolation of the Reaction Products. The incubation mixture for the lupin seed Ap_3A hydrolase contained the following in 0.1 mL total volume: 0.05 mL of $H_2{}^{18}O$, 50 mM Bicine/KOH (pH 8.1), 5 mM $MgCl_2$, 4 mM substrate (Ap_3A, Ap_4A, $ApCH_2ppA$, or ApCHFppA), and the enzyme solution.

The incubation mixture for the lupin seed (*asymmetrical*) Ap_4A hydrolase contained the following in 0.1 mL total volume: 0.05 mL of $H_2{}^{18}O$, 50 mM Bicine/KOH (pH 8.1), 5 mM $MgCl_2$, 4 mM substrate (Ap_4A or Ap_5A), and the enzyme solution.

The incubation mixture for *E. coli* (*symmetrical*) Ap_4A hydrolase contained the following in 0.1 mL total volume: 0.05 mL of $H_2{}^{18}O$, 50 mM Bicine/KOH (pH 8.1), 0.5 mM $MnCl_2$, 0.05 mM dithiothreitol, 4 mM substrate (Ap_4A, Ap_3A, Ap_5A, or $Ap_4A\alpha S$), and the enzyme solution.

Incubations were carried out at 30 °C, and the time course of hydrolysis was monitored by thin-layer chromatography. When the reactions had been brought to completion, the mixtures were applied onto a 1-mL MonoQ column connected to a Pharmacia FPLC system (see above). The product fractions, about 1 mL, were collected, freeze-dried, and analyzed by FAB MS.

Fast Atom Bombardment Mass Spectrometry. FAB MS was performed on a Kratos MS 80 instrument using a DS90 data system. Ionization of samples was effected by fast atom bombardment using an adapted saddle field source (Ion Tech Ltd.) operating at 8 keV and 2 mA tube current. Xenon was used to provide a primary beam of atoms, and samples were run from a matrix of glycerol:4-toluenesulfonic acid which had previously been coated onto a stainless steel tip probe. Spectra were recorded in the positive ion mode at a scan speed of 30 s/decade. The system was calibrated using AMP [m/z 348 ($M + H^+$, 100), 370 ($M + Na^+$, 22)]; ADP [m/z 428 ($M + H^+$, 100), 450 ($M + Na^+$, 33), 472 ($M + 2Na^+ - H^+$, 9)]; and ATP [m/z 508 ($M + H^+$, 100), 530 ($M + Na^+$, 75), and 552 ($M + 2Na^+ - H^+$, 32)].

RESULTS AND DISCUSSION

Comments on the Methods Used. As shown in Figure 1, the concentration gradient of the ammonium bicarbonate buffer applied onto the MonoQ column gave a fast, "baseline" separation of the products from the appropriate DNPP substrate.

In our hands, the glycerol:4-toluenesulfonic acid matrix gave MS data with a superior signal-to-noise ratio than any other system examined for adenine nucleotides. We were thus able to employ incubation mixtures containing $H_2{}^{18}O$

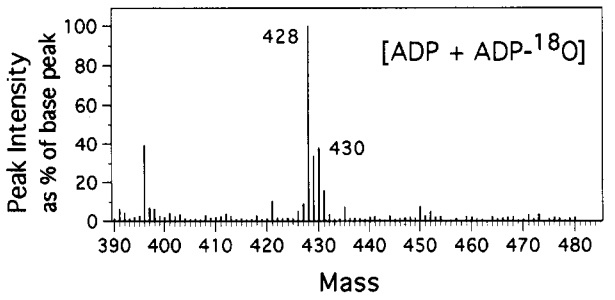

FIGURE 2: Fast atom bombardment mass spectrometry analysis of the ADP degradation product of Ap_4A hydrolyzed in 50% $H_2{}^{18}O$ by the *symmetrical* Ap_4A hydrolase from *E. coli*. A sample of ADP purified on the MonoQ column (3–5 nmol) was analyzed by positive ion FAB MS as described in Materials and Methods.

adjusted to 50% isotopic enrichment with two clear advantages over the previous use of mixtures constituted in *ca.* 100% $H_2{}^{18}O$ (McLennan *et al.*, 1989a). Firstly, they are much simpler to prepare. Secondary, the FAB MS data show M and (M + 2) doublets of equal intensity, thus providing an internal calibration of the isotope-labeled peaks. This is especially valuable in the case of the *symmetrical* cleavage of Ap_4A by the *E. coli* Ap_4A hydrolase, where the ratio obtained for [^{16}O]ADP:[^{18}O]ADP (m/z 428:430) is close to 3:1 after correction for the intensity of the (M + 2) peak in the control ADP spectrum (Figure 3C) and reflects the incorporation of water into half of the released nucleotide product (Figure 2).

Action of Ap_3A Hydrolase from Lupin. Ap_3A hydrolases which are typical in higher eukaryotes cleave dinucleoside polyphosphates having a chain of at least three phosphates (Sillero *et al.*, 1977; Jakubowski & Guranowski, 1983; Prescott *et al.*, 1992). Prescott *et al.* first reported that the dinucleoside triphosphatase from *Artemia* hydrolyzes Gp_4G asymmetrically to GMP plus GTP but not symmetrically to 2GDPs. In correction of the results of earlier studies (Jakubowski & Guranowski, 1983), we have shown in the present work that the lupin enzyme Ap_3Ase also hydrolyzes Ap_4A *asymmetrically*. In contrast to the (*asymmetrical*) Ap_4A hydrolases, details of the reactions catalyzed by the eukaryotic Ap_3A hydrolases were not known hitherto. We have carried out hydrolyses of Ap_3A, Ap_4A, and two new Ap_3A analogues, namely $ApCH_2ppA$ and ApCHFppA, with the lupin Ap_3A hydrolase in the presence of 50% $H_2{}^{18}O$. In every instance we observed the appearance of labeled [^{18}O]AMP and unlabeled ADP, ATP, pCH_2pA, and pCHFpA, respectively (Figure 3). This pattern of O^{18}-labeling/nonlabeling is also reflected in the peaks at 22 mass units higher corresponding to the protonated monosodium salt of the nucleotides.

The conclusions to drawn from these results are threefold. (i) The enzyme has somewhat broader substrate specificity than have (*asymmetrical*) Ap_4A hydrolases, although the *Artemia* hydrolase degrades Ap_3A rather slowly (Prescott *et al.*, 1989). (ii) It recognizes various dinucleoside polyphosphates bearing a P^1–O–P^2 scissile bond, with a preference for dinucleoside triphosphates. (iii) It adds the nucleophilic oxygen always to the substrate at P^1.[1] Thus, the enzyme directs attack of water at the phosphate proximate to the 5′-oxygen of the nucleoside moiety that is more strongly recognized. A scheme illustrating the mode of action of the lupin Ap_3A hydrolase is shown (Figure 4).

Action of (Asymmetrical) Ap_4A Hydrolase from Lupin. It was demonstrated in earlier studies on various substrates specificities that both the lupin enzyme (Jakubowski & Guranowski, 1983) and the (*asymmetrical*) Ap_4A hydrolases from other sources (Lobaton *et al.*, 1975; Ogilvie & Antl, 1983; Prescott *et al.*, 1989) hydrolyze dinucleoside polyphosphates with a requirement for at least four phosphate residues in the polyphosphate chain. Thus, Ap_4A, Ap_5A, and Ap_6A give AMP + ATP, ADP + ATP, and 2ATP, respectively. Recently, it was demonstrated for the enzymes from *Artemia* (McLennan *et al.*, 1989) and yellow lupin (Dixon & Lowe, 1989) that Ap_4A is cleaved at either P^1 or P^4 and ^{18}O is incorporated always into AMP.[1]

In our studies, we have confirmed these observations and, moreover, shown that the Ap_4A homologue, Ap_5A, is cleaved with incorporation of ^{18}O only into ADP [for the FAB MS profile, see Blackburn *et al.* (1991)]. These data show that the enzyme accepts the nucleoside tetraphosphate moiety in the substrate and directs water, as second substrate, at P^4. The target for hydrolytic attack is thus identified as the fourth phosphate remote from that adenosine (nucleoside) which better fits the enzyme binding site. Finally, it can be added that in this mode of hydrolysis the water molecule is directed only at an unmodified phosphate residue in the substrate molecule. Neither a $-CH_2-P^4$ bond as in $ApCH_2ppCH_2pA$ (Guranowski *et al.*, 1987; McLennan *et al.*, 1989b) nor an O–P_S bond in (R_p,S_p)-$Ap_4A\alpha S$ (Lazewska & Guranowski, 1990) is cleaved. Other phosphonate analogues, $AppCH_2ppA$ and $ApCH_2pppA$, are good enzyme substrates (Guranowski *et al.*, 1987), while the two diastereomers of $Ap_4A\alpha S$ were found to be cleaved at P^4, yielding AMP and corresponding diastereomers of $ATP\alpha S$ (Lazewska & Guranowski, 1990). In Figure 5, we show a scheme for the mode of action of the (*asymmetrical*) Ap_4A hydrolase that is consistent with these observations.

It is worth adding here that, while the lupin enzyme shows a resemblance to the enzyme from animal sources, it also degrades adenosine 5′-tetraphosphate (p_4A) to ATP and inorganic phosphate (Jakubowski & Guranowski, 1983). It follows that a nucleoside bearing at C-5′ a chain of four phosphates appears to be the most important feature of substrate recognition for the lupin enzyme, and this particular enzyme might thus also be considered as an adenosine 5′-tetraphosphatase (EC 3.6.1.14), as first described for this activity from animal tissues (Small & Cooper, 1966).

Action of (Symmetrical) Ap_4A Hydrolase from E. coli. The (*symmetrical*) Ap_4A hydrolase from *E. coli* was shown to cleave DNPPs having at least three phosphate chains. It always gives corresponding NDP as one of the reaction products (Guranowski *et al.*, 1983; Plateau *et al.*, 1985). Previous work has established that this bacterial enzyme has a preference for unmodified substrates [e.g., Ap_4A > (R_p)-Ap_SpppA > (S_p)-Ap_SpppA], (Lazewska & Guranowski, 1990). In the present studies, our objective was to see whether the enzyme introduces water into the P^2 or the P^3 phosphate relative to the preferred nucleotide residue. Analysis of products obtained in medium containing 50% $H_2{}^{18}O$ clearly demonstrates that the *E. coli* Ap_4A hydrolase acts according to the first of these alternatives. Neither AMP derived from Ap_3A nor ATP produced from Ap_5A was labeled with oxygen-18. In another experiment, using the *asymmetrical* substrate, $Ap_4A\alpha S$, we have shown that both (R_p) and (S_p) diastereomers of this compound are degraded to give [^{18}O]ADP and unlabeled

[1] The conventional nomenclature for the four phosphates of Ap_4A as $\alpha,\beta,\beta',\alpha'$ (Blackburn *et al.*, 1992) can lead to some ambiguities if used for the description of the regiospecificities of hydrolysis of Ap_4As. We have therefore chosen for this purpose alone to use the alternative P^1,P^2,P^3,P^4 nomenclature, with the added convention that P^1 is located at the more strongly bound nucleotide residue.

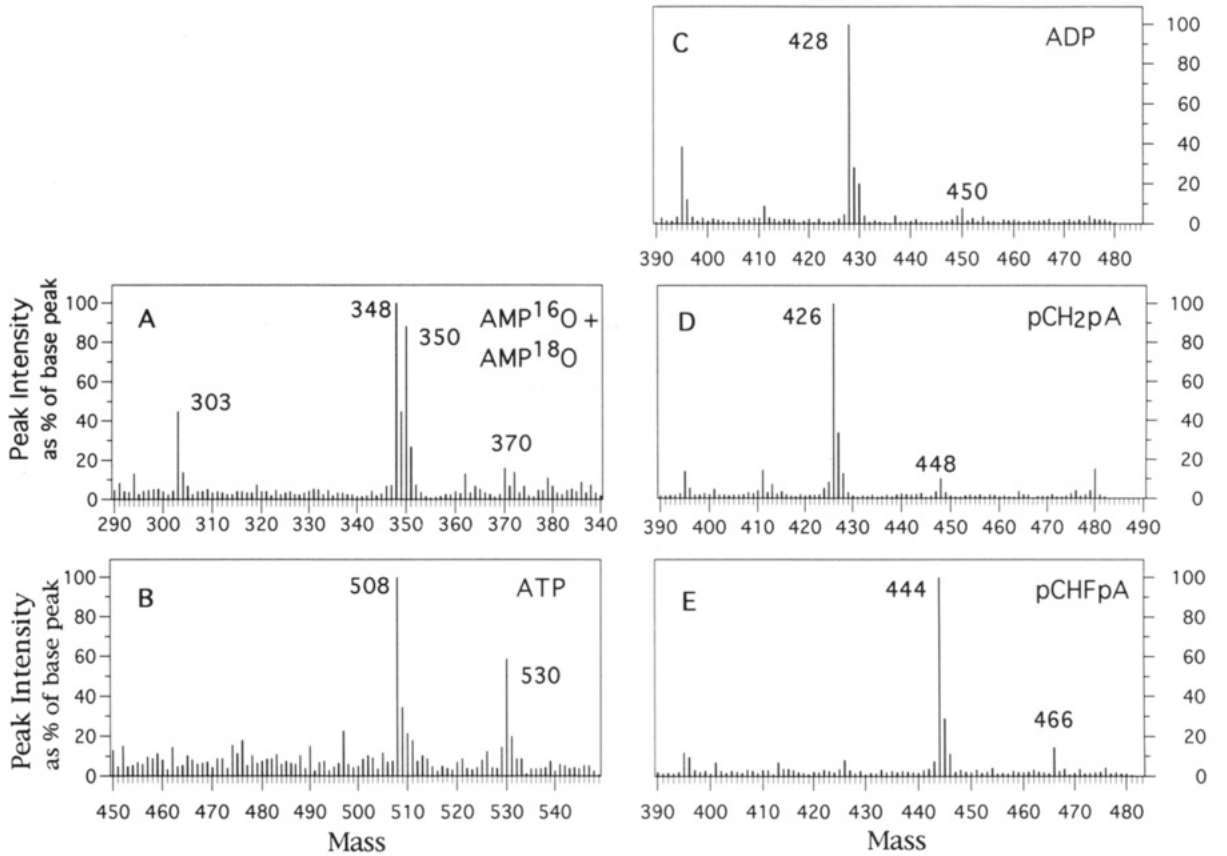

FIGURE 3: FAB MS analysis of degradation products of various substrates hydrolyzed by the Ap_3A hydrolase from lupin in 50% $H_2{}^{18}O$. Samples of the indicated nucleotides produced by the incubation of appropriate substrates with the Ap_3A hydrolase and purified then on the MonoQ column were analyzed by positive FAB MS as described in Materials and Methods. Spectra A and C, 5′-AMP and 5′-ADP, respectively, derived from hydrolyzed Ap_3A; D, pCH_2pA derived from $ApCH_2ppA$; E, pCHFpA derived from ApCHFppA; B, 5′-ATP derived from hydrolyzed Ap_4A. ($M + Na^+$) peaks can be identified in each of the spectra at 22 mass units higher.

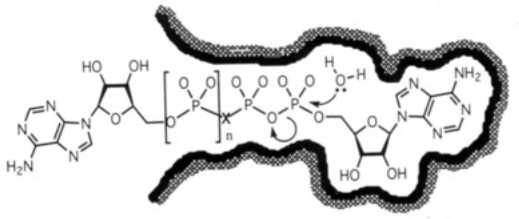

FIGURE 4: Mode of action of the Ap_3A hydrolase from yellow lupin seeds operating on Ap_3A ($n = 1$) and Ap_4A ($n = 2$).

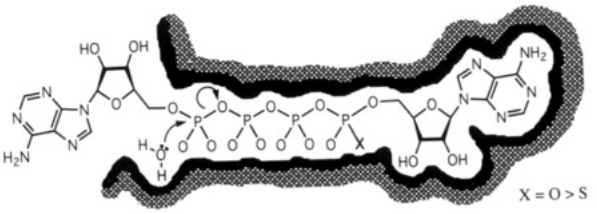

FIGURE 5: Mode of action of the (*asymmetrical*) Ap_4A hydrolase from yellow lupin seeds.

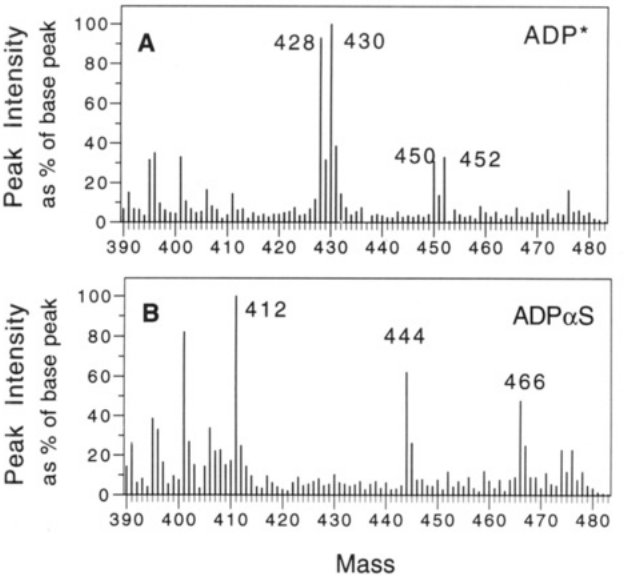

FIGURE 6: Positive ion FAB MS on the MonoQ-purified adenosine diphosphates obtained after the hydrolysis of $Ap_4A\alpha S$ catalyzed by the (*symmetrical*) Ap_4A hydrolase from *E. coli*. A, spectrum of the ADP sample; B, spectrum of the ADPαS sample. In both cases, ($M + Na^+$) peaks can be identified at 22 mass units higher.

ADPαS (Figure 6). These results show that there is discrimination against a modified phosphate at the binding site for P^1 while cleavage occurs at P^2.

It follows that this (*symmetrical*) hydrolase directs attack of water at the second phosphate from the 5′-oxygen of the more strongly recognized nucleoside moiety. A scheme illustrating this mode of action for the *E. coli* Ap_4A hydrolase is shown (Figure 7).

The general conclusion to be drawn from these studies is that each of the three specific enzymes hydrolyzing DNPPs certainly exhibits a unique mode of regiospecificity with respect to the cleavage. The relevant data are summarized in Table 1 and are incorporated into Figures 4, 5, and 7.

The (*symmetrical*) Ap_4A hydrolase from *E. coli* identifies two phosphates from the adenosine recognition site and cleaves the P^2–O–P^3 bond by addition of water to P^2. If this cleavage

Table 1: Regiospecificity of Action of Some Specific Enzymes Hydrolyzing Various Diadenosine Polyphosphates in Oxygen-18-Enriched Water

enzyme (systematic number) source	experimental data: substrate reacting with $H_2{}^{18}O$	experimental data: products	general features: no. of phosphates in chain	general features: cleavage site[a]
Ap_3A hydrolase (EC 3.6.1.29)	ApppA	[^{18}O]AMP + ADP	3	P-1
	AppppA	[^{18}O]AMP + ATP	≥3	P-1
yellow lupin (*L. luteus*)	$ApCH_2ppA$	[^{18}O]AMP + pCH_2ppA	≥3	P-1
	ApCHFppA	[^{18}O]AMP + pCHFppA	≥3	P-1
Ap_4A hydrolase (EC 3.6.1.17) yellow lupin	AppppA	[^{18}O]AMP + ATP	4	P-4
	AppppppA	[^{18}O]ADP + ATP	≥4	P-4
Ap_4A hydrolase (EC 3.6.1.41)	ApppA	[^{18}O]ADP + AMP	3	P-2
	AppppA	[^{18}O]ADP + ADP	4	P-2
E. coli	AppppppA	[^{18}O]ADP + ATP	≥4	P-2
	Ap_spppA	[^{18}O]ADP + p_spA	4	P-2

[a] Arabic number assignation of phosphates starts from the nucleoside moiety which is bound by the enzyme recognition site.

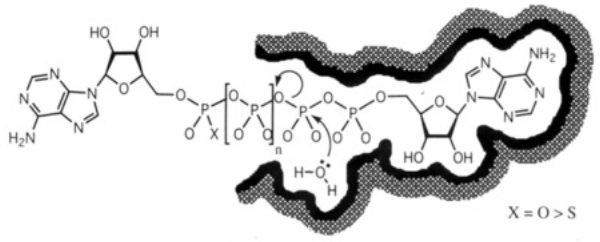

FIGURE 7: Mode of action of the (*symmetrical*) Ap_4A hydrolase from *E. coli* operating on substrates Ap_3A ($n = 0$), Ap_4A ($n = 1$), and Ap_5A ($n = 2$).

occurs with "in-line" stereochemistry at phosphate, as predominantly is the case for phosphoryl transfer enzymes (Knowles, 1980), then this mode of action corresponds to an *endo* pattern of attack of the nucleophilic water molecule (Figure 7). While throughout this simplified analysis we have assumed an extended conformation for the tetraphosphate chain, three pieces of evidence suggest that there is secondary recognition of P^4 and probably also of P^3 by the enzyme. Firstly, it is known that the (R_p)-Ap_spppA is cleaved at P^2 4-fold faster than is the (S_p) diastereomer. Secondly, the Ap_sppp_sA species are not substrates for the *E. coli* Ap_4A hydrolase (Lazewska & Guranowski, 1990, and this study). Lastly, the P^3,P^4-methylene analogue $ApCH_2pppA$ is not a substrate for this enzyme (Guranowski *et al.*, 1987). These observations are in accord either with the binding of the substrate in a hairpin-like conformation with interactions from both P^1 and P^4 and the essential metal (either cobalt or manganese) or with the use of binding from both adenylates being required to effect a conformational change of the enzyme to a closed form, as is the case for adenylate kinase (Gerstein *et al.*, 1993).

The (*asymmetrical*) Ap_4A hydrolase from lupin shows a completely different pattern of regiospecificity. It clearly recognizes four phosphate residues and cleaves by addition of water at P^4 with scission of the P^4–O–P^3 for Ap_4A and also at P^4 with cleavage of the P^4–O–P^3 bond for Ap_5A. Moreover, unlike the bacterial enzyme, it tolerates a thiophosphate residue in the P^1 binding site. The known inversion stereochemistry of action of this enzyme (Dixon & Lowe, 1989) permits definition of an *exo* pattern of attack of water (Figure 5). As before, in this analysis we assume an extended conformation for the polyphosphate chain though we can not exclude a folded form. Lastly, this analysis explains the failure of this enzyme to cleave Ap_3A, which is unable to meet the strict requirement for a fourth phosphate to occupy the S^4 cleavage site of the enzyme.

It is evident that the lupin has coped with the need to control these two different nucleotides because it has an Ap_3A hydrolase, whose regiospecificity is described by *endo* attack of water at P^1 with cleavage of the P^1–O–P^2 bond. It operates with both Ap_3A and Ap_4A as substrates although Ap_5A is cleaved very slowly. It does not accept a thiophosphate in the P^1 site and is unable to cleave Ap_sPp_sA (A. Guranowski and G. M. Blackburn, unpublished work).

This investigation of the linear regiospecificity of these three Ap_nA-hydrolyzing enzymes will lead to further analysis of the mechanism of action of these enzymes and the design of specific inhibitors.

In conclusion, the overall picture that emerges is one of three independent regiospecificities for the three Ap_nA pyrophosphohydrolases investigated. We anticipate that future studies relating these substrate specificities to enzyme structure will further illuminate these differences.

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