Studies on some specific Ap_4A -degrading enzymes with the use of various methylene analogues of P^1P^4 -bis-(5',5'''-adenosyl) tetraphosphate

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Six new methylenephosphonate analogues of P^1P^4 -bis-(5',5'''-adenosyl) tetraphosphate, Ap_4A , having P^2 – P^3 carbon bridges CF_2 , CCl_2 and CH_2CH_2 or P^1 – P^2 and P^3 – P^4 carbon bridges CF_2 , CCl_2 and CH_2CH_2 in the tetraphosphate chain, were examined as substrates or inhibitors for two specific Ap_4A -degrading enzymes: (asymmetrical) Ap_4A hydrolase (EC 3.6.1.17) from yellow-lupin seeds and (symmetrical) Ap_4A hydrolase (EC 3.6.1.41) from *Escherichia coli*. All analogues in which the central oxygen atom was replaced by a stable carbon bridge were hydrolysed by the asymmetrical hydrolase ($\text{CF}_2 > \text{CCl}_2 > \text{O} > \text{CHBr} > \text{CH}_2 > \text{CH}_2\text{CH}_2$). As expected, these analogues were not hydrolysed by the symmetrical hydrolase, which was also unable to act on analogues having P^1 – P^2 and P^3 – P^4 carbon bridges.

INTRODUCTION

Biochemical studies on P^1P^4 -bis-(5',5'''-adenosyl) tetraphosphate (Ap_4A or AppppA , 1) originated in the middle 1960s (Zamecnik *et al.*, 1966). It is widely agreed that the biosynthesis of this species *in vitro* results from the action of some aminoacyl-tRNA synthetases (Zamecnik *et al.*, 1966; Goerlich *et al.*, 1982; Blanquet *et al.*, 1983) or Ap_4A phosphorylase (Guranowski *et al.*, 1988a) in transferring an adenylate moiety from mixed anhydrides of AMP with amino acids or with sulphate on to P- γ of ATP. In addition to Ap_4A , other dinucleosidyl polyphosphates of general formula $\text{Np}_3\text{N}'$ and $\text{Np}_4\text{N}'$ (where N and N' are purine or pyrimidine nucleosides) are found in cells, where their normal very low concentration (approx. 10 nM) rises when the cells are subjected to stress conditions (Bochner *et al.*, 1984; Coste *et al.*, 1987). These nucleotides are substrates for non-specific enzymes such as phosphodiesterase type I or nucleotide pyrophosphatase (Robinson & Barnes, 1986). More significantly, several highly specific cleaving enzymes have been identified from many different Phylae, including an Ap_3A hydrolase (Sillero *et al.*, 1977; Jakubowski & Guranowski, 1983; Hurtado *et al.*, 1987), asymmetrical Ap_4A hydrolase (Lobatón *et al.*, 1975; Jakubowski & Guranowski, 1983; Ogilvie & Antl, 1983), symmetrical Ap_4A hydrolase (Barnes & Culver, 1982; Guranowski *et al.*, 1983) and Ap_4A (Ap_3A) phosphorylase (Guranowski & Blanquet, 1985; Guranowski *et al.*, 1988b).

There is as yet no proof that the Ap_4A -synthesizing enzymes are responsible for the accumulation of these dinucleosidyl polyphosphates *in vivo*. Equally, there is but one example of an Ap_4A -degrading enzyme affecting the concentration of the adenylate-containing dinucleosidyl polyphosphates, Ap_3N and Ap_4N , in the cell: an *Escherichia coli* strain that overproduces the symmetrical Ap_4A hydrolase shows a 10-fold-decreased concentration of Ap_4N species (Mechulam *et al.*, 1985). Moreover, suggestions that Ap_4A might be involved in the regulation of DNA replication (Grummt, 1978; Weinmann-Dorsch

et al., 1984) may need revision in the light of recent reports on variations in dinucleosidyl polyphosphate concentrations during cell cycle and growth (Garrison *et al.*, 1986; Moris *et al.*, 1987). Consequently, the most challenging question, namely 'What is the biological role of Ap_4A and other dinucleosidyl polyphosphates?', remains unanswered.

We have therefore chosen to approach the elucidation of the biological role of various dinucleosidyl polyphosphates and of Ap_4A in particular through the effects of various analogues of these nucleotides on the enzymes involved in the biosynthesis or degradation of Ap_4A . Hitherto, three methylene analogues, AppCH_2ppA (5), ApppCH_2pA and $\text{ApCH}_2\text{ppCH}_2\text{pA}$ (9), have been studied as inhibitors of reactions catalysed by the phenylalanyl-tRNA synthetase (Biryukov *et al.*, 1987), which is one of the most effective producers of Ap_4A , and as substrates or inhibitors of four Ap_4A -degrading enzymes (Guranowski *et al.*, 1987). Also, some stereochemically defined phosphonate and thiophosphate analogues of Ap_4A have been investigated with a non-specific phosphodiesterase and the specific asymmetrical Ap_4A hydrolase from *Artemia salina* (Blackburn *et al.*, 1987a,b). Most recently, cells of *Dictyostelium discoideum* have been shown to produce significant quantities of AppCH_2ppA when incubated in the presence of toxic methylenebisphosphonate (Klein *et al.*, 1988). In this present study, we have employed isopolar/isosteric, isopolar/non-isosteric and non-isopolar/non-isosteric analogues (Blackburn, 1981) of Ap_4A to study the degradation of Ap_4A by the specific asymmetrical cleavage enzyme from lupin and the symmetrical hydrolase from *E. coli*.

EXPERIMENTAL

The Ap_4A -degrading enzymes asymmetrical Ap_4A hydrolase (EC 3.6.1.17) from yellow-lupin (*Lupinus luteus*) seeds (Jakubowski & Guranowski, 1983) and symmetrical Ap_4A hydrolase from *Escherichia coli* K-12 (EC 3.6.1.41) (Guranowski *et al.*, 1983) and nucleoside

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phosphotransferase (EC 2.7.1.77) from yellow-lupin seedling cotyledons (Guranowski, 1979), used here as an auxiliary enzyme to release P_i from AMP (see below), were prepared as described previously. Chromatographic methods, enzyme assays and the method used for the measurement of *K_i* values were the same as described before (Guranowski *et al.*, 1987). Ap₄A analogues were synthesized by the condensation of $\beta\gamma$ -substituted analogues of ATP or of the parent bisphosphonate with adenosine 5'-phosphomorpholidate (Blackburn *et al.*, 1987a) and by dimerization of $\alpha\beta$ -substituted analogues of ADP. Details of these preparations are provided (Table 1). Structures of analogues were authenticated by ¹H, ¹⁹F and ³¹P n.m.r. and m.s. analysis (Taylor, 1989).

The concentrations of nucleosides in solution were calculated from light absorption measured at 260 nm assuming a value for ϵ_{260} of $27 \times 10^3 \text{ M}^{-1} \cdot \text{cm}^{-1}$ (Holler *et al.*, 1983).

The relative rates of degradation of AppppA and of those analogues that are substrates for the lupin Ap₄A hydrolase [analogues (2), (3) and (6)] were measured at 30 °C by using the following protocol: incubation mixture, 400 μl total, containing Bicine/KOH buffer, pH 8.0 (50 μM), MgCl₂ (5 mM), dithiothreitol (20 μM), substrate (Ap₄A or analogue, 500 μM) and a rate-limiting quantity of the lupin Ap₄A hydrolase. At various time intervals (usually 0, 10, 20 and 60 min) portions (60 μl) were withdrawn, kept at 100 °C (4 min) and then chilled in an ice bath. The resulting precipitate was removed by centrifugation, and the supernatant (50 μl) was supplied with a solution of nucleoside phosphotransferase (10 munits; 2 μl). The released P_i was then determined

by the modified Fiske-SubbaRow colorimetric method (Chen *et al.*, 1956). Control experiments established that none of the other possible nucleotide hydrolysis products of the Ap₄A analogues were substrates for the nucleoside phosphotransferase under these conditions.

RESULTS AND DISCUSSION

As shown (Table 2), the t.l.c. systems employed enabled us in each case to obtain a satisfactory separation of Ap₄A analogues, the potential substrates, from the possible products of enzymic hydrolysis of Ap₄A (1) or its analogues. The ability of both the lupin and *E. coli* enzymes to degrade the Ap₄A analogues was investigated by incubations with a large excess of enzyme (sufficient fully to cleave 0.5 mM-Ap₄A within 1 min) at 30 °C for 16 h. Analysis of the incubation mixtures by t.l.c. in both systems described established that only the lupin asymmetrical Ap₄A hydrolase decomposed any of the analogues. This enzyme hydrolysed AppCF₂ppA (2), AppCCl₂ppA (3) and AppCH₂CH₂ppA (6) with velocities 0.7, 0.3 and 0.3 relative to Ap₄A itself. Previous work (Guranowski *et al.*, 1987) has established rather lower rates of hydrolysis for AppCHBrppA (5) (0.16), AppCH₂ppA (5) (0.06) and ApppCH₂pA (0.02). As expected, this enzyme did not degrade the P¹-P² doubly bridged analogues ApCF₂ppCF₂pA (7), ApCCl₂ppCCl₂pA (8) or ApCH₂ppCH₂pA (9), behaviour that is consistent with its characteristic cleavage of the P¹-O-P² bridge of Ap₄A by the lupin enzyme to give ATP and AMP (Jakubowski & Guranowski, 1983).

The strength of binding of the P²-P³-bridged com-

Table 1. Conditions for the synthesis of analogues of Ap₄A

All reactions were performed in pyridine solution. pCFp is difluoromethylenebisphosphonic acid; pCH₂CH₂p is ethane-1,2-diylbisphosphonic acid; Morph-p-Ado is adenosine 5'-phosphomorpholidate; DCCD is dicyclohexylcarbodi-imide.

Nucleotide		Precursor	Condensing agent	Time (temperature)	Yield (%)
AppCF ₂ ppA	(2)	pCF ₂ ppAdo*	Morph-p-Ado	24 h (60 °C)	36
AppCCl ₂ ppA	(3)	pCCl ₂ ppAdo*	Morph-p-Ado	24 h (60 °C)	46
AppCH ₂ CH ₂ ppA	(6)	pCH ₂ CH ₂ p	Morph-p-Ado	24 h (60 °C)	18
ApCF ₂ ppCF ₂ pA	(7)	pCF ₂ pAdo	DCCD	4 days (4 °C)	69
ApCCl ₂ ppCCl ₂ pA	(8)	pCCl ₂ pAdo	DCCD	4 days (4 °C)	39
ApCH ₂ CH ₂ ppCH ₂ CH ₂ pA	(10)	pCH ₂ CH ₂ pAdo	DCCD	4 days (4 °C)	20

* Blackburn *et al.* (1984).

Table 2. T.l.c. characteristics of nucleotide analogues

System I: plastic sheets precoated with polyethyleneimine-cellulose (E. Merck) developed in methanol/water (3:1, v/v, 20 min), then in aqueous LiCl (0.85 M, 1 h). System II: aluminium sheets precoated with silica gel (E. Merck) developed (2 h) in dioxan/conc. ammonia (sp. gr. 0.88)/water (6:1:4, by vol.). Abbreviation: N.D., not determined.

Nucleotide		R_F (system I)	R_F (system II)
AppppA	(1)	0.17	0.61
pA		0.39	0.65
ppA		0.24	0.24
pppA		0.07	0.14
AppCF ₂ ppA	(2)	0.16	0.48
pCF ₂ ppA		0.08	0.08
AppCCl ₂ ppA	(3)	0.15	0.35
pCCl ₂ ppA		0.06	0.04
AppCH ₂ CH ₂ ppA	(6)	0.40	0.75
pCH ₂ CH ₂ ppA		N.D.	0.25
ApCF ₂ ppCF ₂ pA	(7)	0.11	0.46
pCF ₂ pA		0.18	0.20
ApCCl ₂ ppCCl ₂ pA	(8)	0.16	0.28
pCCl ₂ pA		0.18	0.14
ApCH ₂ CH ₂ ppCH ₂ CH ₂ pA	(10)	0.55	0.76
pCH ₂ CH ₂ pA		0.50	0.34

pounds (2–6) to the lupin enzyme was determined from their inhibition of [³H]Ap₄A hydrolysis as described previously (Guranowski *et al.*, 1987). Four of these compounds [(2)–(5)] constitute a group of analogues isosteric (Blackburn, 1981) with Ap₄A. Their relative specificity constants ($V_{rel.}/K_m$) for the hydrolysis reaction showed a marked and progressive increase with the electronegativity of the P²–P³ carbon bridge in the series CF₂ (2.33) > CCl₂ (1.5) > CHBr (0.5) > CH₂ (0.24) (Table 3). As it has been demonstrated that the hydrolysis of Ap₄A (1) by the lupin enzyme involves the addition of water to the P¹ atom rather than to the P² atom of Ap₄A (Dixon & Lowe, 1989), it is reasonable to conclude that these data are more in accord with a transition state for cleavage of Ap₄A by the lupin enzyme that involves

breakdown of a five-co-ordinate intermediate, and where there is a significant degree of breaking of the P¹–O(P²) bond, rather than a transition state involving addition of water to generate the five-co-ordinate species at the P¹ atom. A rather similar situation has been observed recently with a range of Ap₄A analogues as substrates for the asymmetrical hydrolase from *Artemia* (McLennan *et al.*, 1989b), where cleavage has also been shown to involve scission of the P¹–O(P²) bond (McLennan *et al.*, 1989a).

It thus appears that the effects of a family of isosteric methylenephosphonate substitutions on reactions involving enzymic cleavage or formation of adjacent P–O–P bonds are capable of providing valuable information concerning the nature of their transition states, and that they may also be applicable to a wide range of enzyme-catalysed processes utilizing NTPs and dNTPs.

It is noteworthy that the non-isosteric analogue (6) also shows quite good substrate selectivity (0.17). This could be consistent either with the enzyme binding the dinucleotide in a hairpin conformation, leaving the location of the phosphate residues essentially unchanged, or with a relatively limited stereospecificity of recognition of the four anionic charges of these nucleotides. This apparent tolerance for extension of the polyphosphate chain might well be further explored by the examination of analogues of bisadenosyl pentaphosphate such as ApCH₂pppCH₂pA etc.

The symmetrical hydrolase from *E. coli* appears to be unable to cleave the P²–P³ carbon-bridged analogues [(2), (3) and (6)] even under conditions with excess enzyme, behaviour that is in line with the normal mode of P²–P³ cleavage of Ap₄A by the *E. coli* enzyme. [We estimate the lower limit of detection at 0.1 %, at which level there is some evidence for very slow cleavage in the case of analogue (5) to give pA and pCH₂ppA (A. Guranowski & H. Jakubowski, unpublished work)]. All the analogues (2)–(6) proved to be competitive inhibitors of the hydrolysis of Ap₄A (Table 4). The isosteric analogues (2)–(5) bind more strongly to the enzyme than does Ap₄A, through there is no simple correlation between binding and the electronegativity of the carbon bridge (Table 3). The non-isosteric analogue (6) also binds well, thus showing comparable behaviour towards the bacterial and plant hydrolases.

Table 3. Kinetic data for the interaction of Ap₄A and its phosphonate analogues with the specific asymmetrical hydrolase from *Lupinus luteus*

Nucleotide		$V_{rel.}$ (%) [*]	K_m or K_i † (μ M)	V_m/K_m (%)
AppppA	(1)	100	1.0	100
AppCF ₂ ppA	(2)	70	0.3	233
AppCCl ₂ ppA	(3)	30	0.2	150
AppCHBrppA	(4)†	16	0.32	50
AppCH ₂ ppA	(5)†	6	0.25	24
AppCH ₂ CH ₂ ppA	(6)	30	1.8	17
ApCF ₂ ppCF ₂ pA	(7)	0	3.6	–
ApCCl ₂ ppCCl ₂ pA	(8)	0	3.8	–
ApCH ₂ ppCH ₂ pA	(9)†	0	10.9	–
ApCH ₂ CH ₂ ppCH ₂ CH ₂ pA	(10)	0	8.1	–

^{*} ± 3 %.

† ± 10 %.

‡ Data from Guranowski *et al.* (1987).

Table 4. Data for the interaction of Ap₄A and its analogues with the specific symmetrical hydrolase from *E. coli*

Nucleotide		$V_{rel.}$ (%) [*]	K_m or K_1 [†] (μ M)
AppppA	(1)	100	25
AppCF ₂ ppA	(2)	0	17
AppCCl ₂ ppA	(3)	0	11
AppCHBrppA	(4) [‡]	0	1.7
AppCH ₂ ppA	(5) [‡]	0	3.3
AppCH ₂ CH ₂ ppA	(6)	0	16
ApCF ₂ ppCF ₂ pA	(7)	0	18
ApCCl ₂ ppCCl ₂ pA	(8)	0	18
ApCH ₂ ppCH ₂ ppA	(9) [‡]	0	8.1
ApCH ₂ CH ₂ ppCH ₂ CH ₂ pA	(10)	0	> 300

* $\pm 3\%$.† $\pm 10\%$.‡ Data from Guranowski *et al.* (1987).

The P¹-P² and P³-P⁴ doubly bridged analogues [(7)-(10)] also are resistant to cleavage and, with the exception of the non-isosteric analogue (10), they bind to the bacterial enzyme somewhat more tightly than does Ap₄A itself (Table 4). This is an unexpected result, which contrasts markedly with the less-surprising behaviour of the lupin enzyme. It suggests that at least one of the P¹-P²-bridging oxygen atoms adjacent to the site of P-O-P cleavage is essential for enzyme activity whereas neither substrate binding nor leaving-group ability of the α,β -bridged ADP analogue seems capable of explaining the stability to cleavage of the analogues (7)-(9). If, on the one hand, the symmetrical hydrolysis is an associative, S_N2(P), process, this result indicates either that a P¹-P² carbon bridge inhibits attack of the (unidentified) nucleophilic species at P² or that it impedes breakdown of the five-co-ordinate species for reasons other than its effect on the pK_a of the leaving group. On the other hand, if the hydrolysis is essentially dissociative, S_N1(P), in character then the carbon substituent at P² must have a destabilizing influence on the metaphosphate species. Neither of these postulates appears very satisfactory, and a more detailed investigation of the mode of action of the *E. coli* hydrolase is clearly required.

Taken together, the studies on these examples of an asymmetrical and a symmetrical hydrolase suggest that the single most effective phosphonate analogue for the simultaneous inhibition of enzymes of both classes of specific hydrolases ought to be a P¹-P² and P³-P⁴ doubly bridged analogue of Ap₄A. There appears to remain considerable scope for exploring the optimization of substituents on the one-carbon bridges, though present results might indicate a preference for a single halogen substituent. Whether this analysis can be extended to effective inhibition of the specific Ap₄A phosphorylase, as from yeast, must be a subject for further investigation.

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