P^{α} -chiral phosphorothioate analogues of bis(5'-adenosyl)tetraphosphate (Ap_4A); their enzymatic synthesis and degradation

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ABSTRACT

Synthesis of S_p and R_p diastereomers of $Ap_4A\alpha S$ has been characterized in two enzymatic systems, the lysyl-tRNA synthetase from *Escherichia coli* and the Ap_4A α,β-phosphorylase from *Saccharomyces cerevisiae*. The synthetase was able to use both (S_p)ATPαS and (R_p)ATPαS as acceptors of adenylate thus yielding corresponding monothioanalogues of Ap_4A,(S_p) $Ap_4A\alpha S$ and (R_p)$Ap_4A\alpha S$. No dithiophosphate analogue was formed. Relative synthetase velocities of the formation of Ap_4A,(S_p) $Ap_4A\alpha S$ and (R_p)$Ap_4A\alpha S$ were 1:0.38:0.15, and the computed K_m values for (S_p)ATPαS and (R_p)ATPαS were 0.48 and 1.34 mM, respectively. The yeast Ap_4A phosphorylase synthesized (S_p)$Ap_4A\alpha S$ and (R_p)$Ap_4A\alpha S$ using adenosine 5'-phosphosulfate (APS) as source of adenylate. The adenylate was accepted by corresponding thioanalogues of ATP. In that system, relative velocities of Ap_4A, (S_p)$Ap_4A\alpha S$ and (R_p)$Ap_4A\alpha S$ formation were 1:0.15:0.60. The two isomeric phosphorothioate analogues of Ap_4A were tested as substrates for the following specific Ap_4A-degrading enzymes: (*asymmetrical*) Ap_4A hydrolase (EC 3.6.1.17) from yellow lupin (*Lupinus luteus*) seeds hydrolyzed each of the analogues to AMP and the corresponding isomer of ATPαS; (*symmetrical*) Ap_4A hydrolase (EC 3.6.1.41) from *E. coli* produced ADP and the corresponding diastereomer of ADPαS; and Ap_4A phosphorylase (EC 2.7.7.53) from *S. cerevisiae* cleaved the R_p isomer only at the unmodified end yielding ADP and (R_p)ATPαS whereas the S_p isomer was degraded non-specifically yielding a mixture of ADP, (S_p)ADPαS, ATP and (S_p)ATPαS. For all the Ap_4A-degrading enyzmes, the R_p isomer of $Ap_4A\alpha S$ appeared to be a better substrate than its S_p counterpart; stereoselectivity of the three enzymes for the $Ap_4A\alpha S$ diastereomers is 51, 6 and 2.5, respectively. Basic kinetic parameters of the degradation reactions are presented and structural requirements of the Ap_4A-metabolizing enzymes with respect to the potential substrates modified at the Ap_4A-P^{α} are discussed.

INTRODUCTION

Among different approaches trying to establish the actual role of bis(5'-adenosyl) tetraphosphate (Ap_4A or AppppA) are the studies on various enzymes which synthesize and degrade this naturally occurring nucleotide. (For short review on the occurrence of Ap_4A and related dinucleoside oligophosphates see ref. 1). Effective synthesis of Ap_4A can be carried out by some aminoacyl-tRNA synthetases (2-6) and, under certain conditions, by Ap_4A phosphorylase (7-9). Specific degradations are catalyzed by (i) (*asymmetrical*) Ap_4A hydrolase (EC 3.6.1.17) which has been found in higher eucaryotes (10-13); (ii) (*symmetrical*) Ap_4A hydrolase (EC 3.6.1.41) discovered in slime mold *Physarum polycephalum* (14) and in various bacteria (15), and (iii) Ap_4A phosphorylase (EC 2.7.7.53) revealed first in yeast (7) and then in some protozoa (1). Intriguing effects exerted by Ap_4A on some crucial biochemical processes (for the most current summary of the effects see ref. 16 and 17) encouraged chemists to produce several series of potentially useful Ap_4A analogues (16, 18-22). The $\beta\beta$-substituted, and $\alpha\beta$, $\alpha'\beta'$-disubstituted phosphonate analogues of Ap_4A were tested as inhibitors of phenylalanyl-tRNA synthetase (23) and as substrates or inhibitors of four Ap_4A-degrading enzymes: non-specific phosphodiesterase from yellow lupin seeds (24); Ap_4A hydrolase from the same lupin seeds (24, 25) and from *Artemia* embryos (16, 20); the Ap_4A hydrolase from *E. coli* (16, 24, 25), and the Ap_4A phosphorylase from yeast (24). In addition to the works on the above mentioned analogues modified at the anhydride bond(s), there exist reports on chemically synthesized fluorescent analogues of Ap_4A, P^1, P^4-bis(5'-1, N^6-ethenoadenosyl) tetraphosphate and P^1,[5'-(1,N^6-ethenoadenosyl)]-P^4-(5'-adenosyl) tetraphosphate (21) as well as on the mono- (22) and dithioderivatives of Ap_4A (20, 26). Only the α, α'-dithioderivatives were tested enzymatically (20). In this work we focus on two diastereomers R_p and S_p of P^{α}-thioderivative of Ap_4A (Fig. 1). We have first characterized the synthesis of these monothioanalogues by two enzymes: the lysyl-tRNA synthetase from *E. coli* and the Ap_4A phosphorylase from yeast. Secondly, we have characterized the cleavage of the compounds in three Ap_4A-degrading systems: the (*asymmetrical*) Ap_4A hydrolase from yellow lupin seeds, the (*symmetrical*) Ap_4A

hydrolase from *E. coli*, and the Ap_4A phosphorylase from yeast. The data obtained shed some light on the configurational requirements of the individual enzymes with respect to substitutions at the Ap_4A-P^{α} and this increases our knowledge on the stereoselectivity of reactions catalyzed by the enzymes involved in the metabolism of Ap_4A.

MATERIALS AND METHODS

Enzymes

(*Asymmetrical*) Ap_4A hydrolase was isolated from yellow lupin (*Lupinus luteus*) seeds as described previously (12); the *E. coli* (*symmetrical*) Ap_4A hydrolase was purified according to (15), and the yeast Ap_4A phosphorylase as described by Guranowski and Blanquet (7). The lysyl-tRNA synthetase from *E. coli* was obtained from Dr. E. Holler, University of Regensburg, FRG. It was about 40% pure and the stock solution contained 9 mg protein per milliliter.

Chemicals

[2, 5, 8,-^{3}H]Adenosine 5'-triphosphate (45 Ci/mmol) and di[2,8-^{3}H]adenosine tetraphosphate (8.7 Ci/mmol) were obtained from Amersham International plc. Inorganic salts and tlc plates were from E. Merck. Adenosine, AMP, ATP, Ap_4A and adenosine 5' -phosphosulfate (APS) were from Sigma. Diastereomers of ATPαS and ADPαS were synthesized according to Goody and Isakov (27), purified on the HPLC system described below and their absolute configuration was confirmed in assays with snake venom phosphodiesterase, which degrades preferentially the diastereomer with the R_p configuration (28), and hexokinase which prefers the S_p isomer of ATPαS (29).

Chromatographic Systems

For the details of tlc and HPLC sytems used see Table I.

Enzyme assays and enzymatic synthesis of phosphorothioate analogues of Ap_4A

The activity of lysyl-tRNA synthetase was routinely assayed at 30°C. The incubation mixture (100 μl) contained 50 mM Hepes/KOH buffer (pH 8.2), 10 μM dithiothreitol, 100 mM KCl, 10 mM $MgCl_2$, 2 mM lysine, 100 μM $ZnCl_2$, 0.5 mM [^{3}H]ATP (4×10^7 cpm), 0.5 mM (S_p)ATPαS or (R_p)ATPαS and the enzyme solution (22 μg protein). The reaction was terminated by the transfer of 5-μl aliquots onto silica gel plates. The chromatograms were developed for 4 h in system A. Spots of radioactive Ap_4A or its analogues were cut out and the radioactivity counted. For estimation of the K_m values for (S_p)ATPαS and (R_p)ATPαS, the [^{3}H]ATP concentration was 4 mM and the concentration of the ATP analogues varied between 0.3 and 5 mM. To produce (S_p)[^{3}H]Ap_4AαS and (R_p)[^{3}H]Ap_4AαS, the mixture was scaled up to 1 ml and the incubation prolonged up to 6 and 15 h, respectively. Purification of the Ap_4A analogues was performed in the HPLC system (see above). The peak fractions were evaporated in the speed-vac concentrator from Savant and then the compound dissolved in a small volume of water. The analogues were at least 90% pure as judged from the chromatograms obtained in the HPLC system. In stock solutions, the concentration of the analogues was adjusted to 5 mM assuming a value for ϵ_{260} of 27×10^3 M^{-1} cm^{-1} (30). Details of the synthesis of (S_p)Ap_4AαS and (R_p)Ap_4AαS from APS and the corresponding isomer of ATPαS, catalyzed by yeast Ap_4A phosphorylase, are described in the legend to Fig. 2. Velocities of the synthesis were calculated based on the linearity between concentration of the dinucleotides and peak area.

Enzymatic degradation of phosphorothioate analogues of Ap_4A

Incubation mixtures (50 μl) were slightly modified with respect to those used previously (12, 15 and 7, respectively) and contained: for (*asymmetrical*) Ap_4A hydrolase – 50 mM Hepes/KOH buffer (pH 8.0), 20 μM dithiothreitol, 10 mM $MgCl_2$ and 0.5 mM[^{3}H](S_p)Ap_4AαS (2.4×10^4 cpm) or [^{3}H](R_p)Ap_4AαS (3.3×10^4 cpm); for (*symmetrical*) Ap_4A hydrolase 97 the mixture was as above except 10 mM $MgCl_2$ was substituted with 0.5 mM $MnCl_2$; for Ap_4A phosphorylase – 50 mM Hepes/KOH buffer (pH 8.0), 20 μM dithiothreitol, 5 mM K_2HPO_4, 5 mM $MgCl_2$ and either 0.5 mM radiolabelled S_p or R_p diastereomer of Ap_4A. Reactions were initiated by addition of rate-limiting amounts of the appropriate enzyme. Incubations were carried out at 30°C. At intervals (usually after 10, 20, 30 and 60 min) the 5-μl aliquots were transfered onto chromatograms, the appropriate standards of AMP, ADP, (S_p)ADPαS, (R_p)ADPαS, (S_p)ATPαS and (R_p)ATPαS were added, and developed in system A or B. Spots of AMP in the case of (*asymmetrical*) Ap_4A hydrolase, or ADP in the case of (*symmetrical*) Ap_4A hydrolase, and Ap_4A phosphorylase, were visualized under ultraviolet light, cut out, immersed in 5 ml of scintillation mixture and counted for radioactivity.

RESULTS AND DISCUSSION

Evaluation of the developed chromatographic systems

As shown (Table I), the combination of two tlc systems allows the unequivocal separation of mono- and di-nucleotides, i.e. substrates and products or vice versa, depending which enzymatic system is considered. System A appeared to be particularly useful since separation of the nucleotides is not impaired by the components of the incubation mixtures applied onto the chromatograms. System B was prefered only when assaying the degradation of radioactive analogues catalyzed by the (*asymmetrical*) Ap_4A hydrolase from lupin, because of the better separation of the product, AMP, from the substrates, (S_p)Ap_4AαS or (R_p)Ap_4AαS. Satisfactory separation of the compounds studied was also achieved in the HPLC system (Table I, Fig. 2). It was particularly useful to distinguish S_p and R_p isomers of ATPαS and ADPαS in one mixture. For quantitative measurements, we used the HPLC system only for studying the synthetic activities of the yeast Ap_4A phosphorylase due to lack of radiolabelled substrates, APS, (S_p)ATPαS and (R_p)ATPαS.

Synthesis of phosphorothioate analogues of Ap_4A catalyzed by the lysyl-tRNA synthetase from *Escherichia coli*

The synthetase was demonstrated to produce, in addition to Ap_4A, various adenylylated derivatives by transferring the adenylate from lysyl-adenylate to any compound containing a pyrophosphate moiety (31, 32). Therefore we expected to obtain at least one phosphorothioate analogue of Ap_4A when incubating ATP with the appropriate isomer of ATPαS in the presence of the enzyme. In fact the enzyme only formed Ap_4A and the corresponding monothioanalogue of Ap_4A. No α, α'-dithio-analogue of Ap_4A was formed either in the mixtures mentioned

above or in those containing ATPαS as the sole nucleotide. That observation indicates that the enzyme cannot use the thioanalogue of ATP for amino-acid activation. The ATP analogues, however, appeared to be good acceptors of the adenylate. As depicted in Fig. 3, in the presence of the S_p isomer, the velocity of formation of (S_p)$Ap_4A\alpha S$ reached 38% of that for Ap_4A synthesized in the mixture containing only ATP. The relative velocity for the R_p isomer is 15%. It should be noted that the velocity of the formation of Ap_4A synthesized simultaneously with any of the phosphorothioate analogues (Fig. 3, the dashed line) is about half that observed in the mixture without ATPαS. That points to the inhibitory effect exerted by the ATPαS isomers at the lysine-activation-center of the synthetase. Undoubtedly, the aminoacyl-activation-center of those synthetases which can produce dinucleoside oligophosphates, has much stricter requirements with respect to the structure of nucleoside triphosphate than has the pyrophosphate center with respect to an adenylate acceptor. In the activation reaction, the lysyl-tRNA synthetase tolerated only the substitution of ATP with dATP yielding dAp_4dA. Formation of no other dinucleotides except the adenylylated ones has been reported (31, 32). Varying the concentrations of (S_p)ATPαS or (R_p)ATPαS we estimated the K_m values for the ATP analogues. They were 0.48 and 1.34 mM, respectively.

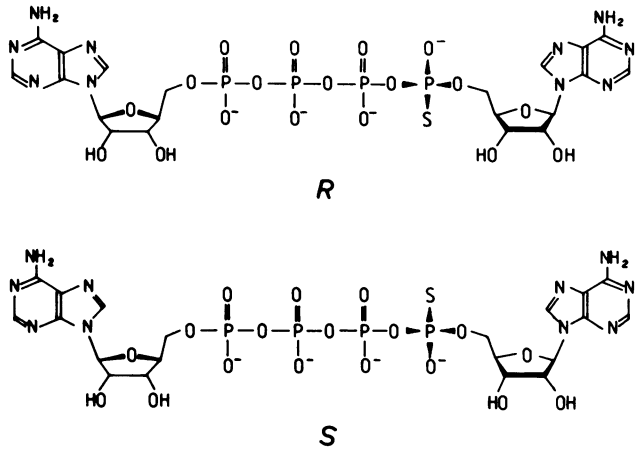

Figure 1. Diastereomers of $Ap_4A\alpha S$.

Table I. Retention times (RT) on high-performance-liquid chromatography column and R_f values on thin-layer chromatography plates for Ap_4A, its phosphorothioate analogues and possible products of their degradations.

Compound	RT (min)[a]	R_f values system A[b]	system B[c]
AMP	7.57	0.65	0.39
ADP	8.40	0.24	0.30
(S_p)ADPαS	9.70	0.47	0.12
(R_p)ADPαS	10.46	0.46	0.11
ATP	8.66	0.12	0.14
(S_p)ATPαS	11.07	0.21	0.06
(R_p)ATPαS	16.88	0.23	0.05
Ap_4A	13.60	0.46	0.26
(S_p)$Ap_4A\alpha S$	17.40	0.52	0.12
(R_p)$Ap_4A\alpha S$	19.60	0.54	0.11

[a] Standards of indicated compounds were chromatographed on reverse-phase Zorbax C_{18} column (4.6 mm×25 cm) combined with LDC Milton Roy chromatograph equipped with a detector operating at 260 nm. The column was equilibrated with 150 mM triethylammonium bicarbonate (pH 7.6) containing 5% acetonitrile. Then a linear gradient of acetonitrile from 5 to 20% in the same buffer was applied at the flow rate of 1.5 ml min.

[b] System A: aluminium plates precoated with silica gel containing fluorescent indicator were developed for 2 h at 20°C in dioxane/ammonia/water/ (6:1:4, v/v).

[c] System B: plastic sheets precoated with poly(ethyleneimine)-cellulose containing fluorescent indicator were developed first for 20 min in 75% methanol and then for 1 h in 0.85 M LiCl.

Synthesis of phosphorothioate analogues of Ap_4A catalyzed by the Ap_4A phosphorylase from *Saccharomyces cerevisiae*

As was shown earlier (9), the Ap_4A phosphorylase is able to catalyze the synthesis of Ap_4A from adenosine 5'-phosphosulfate (APS), which acts as the adenylate donor, and ATP. It was also demonstrated that some ATP analogues like

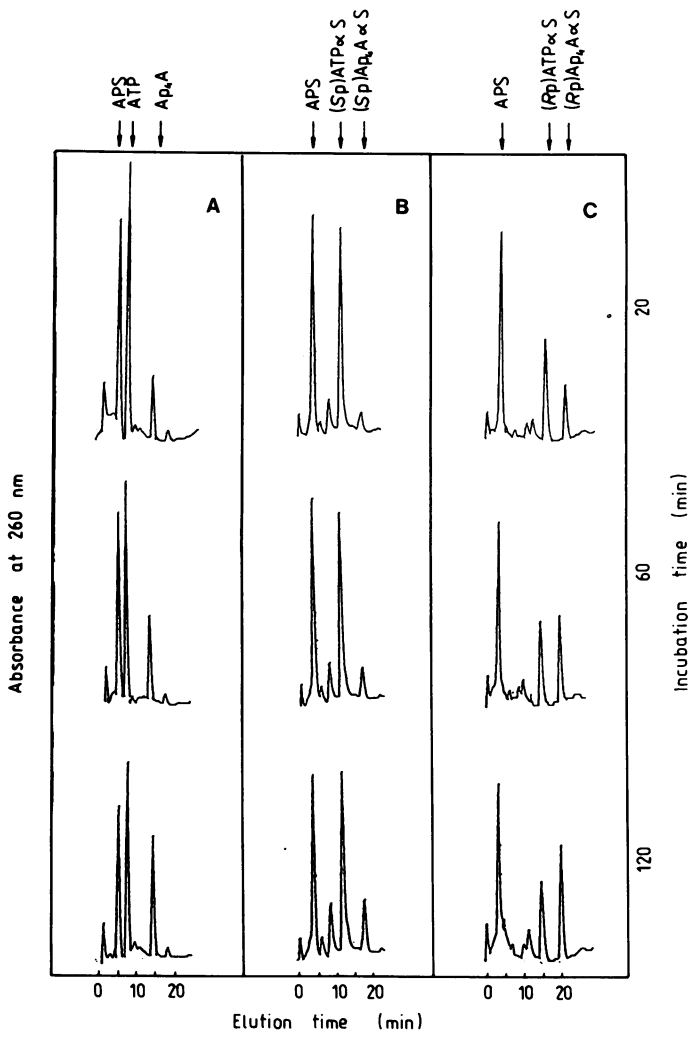

Figure 2. Demonstration by high-performance liquid chromatography that yeast Ap_4A phosphorylase catalyzes synthesis of Ap_4A, (S_p) $Ap_4A\alpha S$ and (R_p)$Ap_4A\alpha S$ from APS and either ATP, (S_p) ATPαS or (R_p)ATPαS, respectively. The assay mixture (50 μl final volume) contained 50 mM Hepes/KOH (pH 8.0), 0.5 mM APS, 100 μM dithiothreitol, 0.5 mM $MgCl_2$, 2 mM nucleoside triphosphate, (ATP, (S_p) ATPαS or (R_p)ATPαS) and the yeast Ap_4A phosphorylase (0.2 μM final concentration). Incubation was carried out at 30°C. At indicated times, the 10-μl aliquots were transferred into 40 μl of hot water, heated for 3 min in boiling water, chilled, centrifuged and 10-μl portions of the supernatant were analyzed on the reverse-phase Zorbax column (see Table I). Panels A, B and C show elution profiles of the reaction mixtures in which Ap_4A, (S_p)$Ap_4A\alpha S$ and (R_p)$Ap_4A\alpha S$ were produced, respectively.

adenosine 5'-tetraphosphate, GTP, adenosine 5'-(α, β-methylene-triphosphate) and adenosine 5'-(β, γ-methylenetriphosphate) are also substrates for the Ap_4A phosphorylase and accept the adenylate residue from APS with the formation of Ap_5A, Ap_4G, $AppCH_2pA$ and $AppCH_2ppA$, respectively. In this study, we tested two diastereomers of ATPαS as substrates for this reaction. Figure 2 illustrates synthesis of Ap_4A and two Ap_4A analogues, (S_p) $Ap_4A\alpha S$ and $(R_p)Ap_4A\alpha S$, monitored in HPLC system. The initial velocities of the formation of those dinucleotides measured in mixtures containing the corresponding adenosine triphosphate at 2 mM concentration are at the ratio 1:0.15:0.60, respectively. Based on the previous calculations (9), the rate constants of the $(S_p)Ap_4A\alpha S$ and $(R_p)Ap_4A\alpha S$ synthesis are 1.8 s^{-1} and 0.45 s^{-1}, respectively. In contrast to the lysyl-tRNA synthetase, of the two P^{α}-thioisomers of ATP, the Ap_4A phosphorylase prefers the R_p one as an adenylate acceptor. It is evident that substitution of oxygen with sulfur at P^{α} of ATP does not change substantially the adenylate-acceptor-properties of that purine nucleoside triphosphate.

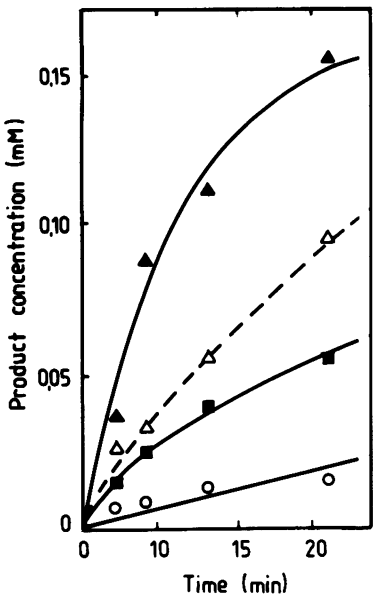

Figure 3. Time course of formation of Ap_4A, $(S_p)Ap_4A\alpha S$ and $(R_p)Ap_4A\alpha S$ catalyzed by lysyl-tRNA synthetase from *E. coli*. The incubation mixture was described in the Materials and Methods. The incubation was carried out at 30°C and at indicated time intervals, 5-μl aliquots were withdrawn and analyzed by tlc in system A (see the legend to Table I). Unlabelled markers were used to localize the products. Concentrations of Ap_4A, in the absence (Δ) or presence (Δ) of either S_p or R_p isomer of 0.5 mM ATPαS, and of $(S_p)Ap_4A\alpha S$ ([]) and $(R_p)Ap_4A\alpha S$ (O) were determined by counting of the radioactivity in the individual spots of the reaction products.

Degradation of phosphorothioate analogues of Ap_4A by the specific Ap_4A-degrading enzymes

(Asymmetrical) Ap_4A hydrolase from yellow lupin seeds. This enzyme was shown to degrade the substrates, releasing always a nucleoside triphosphate as one of the reaction products (10, 12, 13). Therefore it is not surprising that the phosphorothioate analogues of Ap_4A studied were also cleaved according to the same pattern. It is interesting, however, that the enzyme recognized in the both isomers exclusively the unmodified $P^{\alpha'}$, which is attacked by water to yield AMP and the corresponding isomer of ATPαS (Table II), and did not produce the adenosine 5'-phosphorothioate and ATP. In the case of Ap_4A, $(S_p)Ap_4A\alpha S$ and $(R_p)Ap_4A\alpha S$ assayed in the lupin system (Table II), the relative velocities of hydrolysis are 1:0.09:0.92, respectively. Also in the case of dithioanalogues of Ap_4A with a methylene bridge between β and β'-phosphates tested in the *Artemia* system (20), the S_p, S_p isomer appeared to be more (5-fold) resistant than the R_p, R_p one. However, both analogues were highly resistant to cleavage. Compared to Ap_4A hydrolysis there was a 2500- and 500-fold slower degradation, respectively. For the lupin Ap_4A hydrolase, the R_p isomer of $Ap_4A\alpha S$ is 10-fold less resistant than the S_p counterpart and it is degraded only at a slightly slower rate than Ap_4A. In general, the substitution of O with S only at one end of Ap_4A (at P^{α}) does not slow the hydrolysis of the modified molecule as drastically as in the case when the substitutions are introduced at the both ends of the Ap_4A molecule. The phosphorothioate analogues with modifications at P^{α} and $P^{\alpha'}$ proved to be completely resistant to asymmetrical degradation catalyzed by *Artemia* hydrolase (20). In the light of recent data concerning the mechanism of hydrolysis catalyzed by the *(asymmetrical)* Ap_4A hydrolases from yellow lupin (26) and *Artemia* (33), as well as from the observations described above, it appears that the unmodified form of the phosphate residue at which the nucleophilic attack can proceed is crucial for the recognition of the substrate molecule by the enzyme.

(Symmetrical) Ap_4A hydrolase from E. coli. This enzyme hydrolyzes Ap_4A by splitting the anhydride bond between β and β' phosphates (15). In previous studies on phosphonate analogues of Ap_4A (16, 24, 25) it was demonstrated that the *(symmetrical)* Ap_4A hydrolase has much stricter requirements with respect to the structure of the oligophosphate chain in the potential substrates than the *(asymmetrical)* Ap_4A hydrolases, and it is unable to degrade such analogues as $ApCH_2ppCH_2pA$ and $ApCH_2pppA$

Table II. Cleavage of Ap_4A and its phosphorothioate analogues by specific enzymes degrading Ap_4A; mode of cleavage and kinetic parameters.

Enzyme, systematic number, source	Substrate	Products	V_{rel} (%)	K_m (μM)	Rel. spec. (%)
(asymmetrical) Ap_4A hydrolase	Ap_4A	AMP, ATP	100	1	100
(EC 3.6.1.16)	$(S_p)Ap_4A\alpha S$	AMP, (S_p)ATPαS	9.5	1470	0.006
yellow lupin (*Lupinus luteus*) seeds	$(R_p)Ap_4A\alpha S$	AMP, (R_p)ATPαS	92	300	0.306
(symmetrical) Ap_4A hydrolase	Ap_4A	ADP	100	27	100
(EC 3.6.1.41)	$(S_p)Ap_4A\alpha S$	ADP, (S_p)ADPαS	18	1040	0.467
Escherichia coli	$(R_p)Ap_4A\alpha S$	ADP, (R_p)ADPαS	75	710	2.8
Ap_4A phosphorylase	Ap_4A	ADP, ATP	100	60	100
(EC 2.7.7.53)	$(S_p)Ap4A\alpha S$	ADP, (S_p)ADPαS			
yeast (*Saccharomyces cerevisiae*)		ATP, (S_p)ATPαS	3.8	1850	0.123
	$(R_p)Ap_4A\alpha S$	ADP, (R_p)ATPαS	20	3800	0.316

despite the fact that the $\beta\,\beta'$ bond in these compounds remains unchanged. The phosphorothioate analogues of Ap_4A studied here were active as substrates (Table II). The velocity of hydrolysis of $(R_p)Ap_4A\alpha S$ was 75% of that estimated for Ap_4A and the S_p isomer was 4-fold less susceptible to hydrolysis than the R_p one. Both isomers were hydrolyzed symmetrically yielding ADP as one of the reaction products. It would be interesting to estimate the actual site of cleavage in these compounds and to check, using $H_2{}^{18}O$ and mass spectroscopy analysis, whether the hydrolysis proceeds randomly or whether the unmodified end is attacked by water. It is worth mentioning here that exclusively ADP was labeled when Ap_3A and Ap_5A were hydrolyzed by the same enzyme in the presence of $H_2{}^{18}O$ (Guranowski and Blackburn, manuscript in preparation).

Ap_4A phosphorylase from Saccharomyces cerevisiae. In addition to the synthesis of Ap_4A (7) and its different analogues (7–9 and this paper), the enzyme effectively catalyzes phosphorolysis of the dinucleoside oligophosphates, always cleaving the substrates' α, β-anhydride bond and introducing P_i into the β-position of the corresponding NDP formed (7). Whereas phosphonate analogues of Ap_4A proved to be resistant to cleavage by this enzyme (24), the P^{α} -phosphorothioates studied here behaved as substrates. As in the case of (*asymmetrical*) Ap_4A hydrolase from lupin and the (*symmetrical*) Ap_4A hydrolase from *E. coli*, the R_p isomer was shown to be a better substrate (over 5-fold) for the phosphorylase than the S_p one (Table II). Interestingly, the latter analogue was degraded randomly and all four possible products appeared in the incubation mixture. Identity of the products was confirmed by HPLC. The cleavage of $(S_p)Ap_4A\alpha S$ proceeded at the same rate at both ends of the molecule. Net retention of configuration in $(S_p)ADP\alpha S$ suggests that the phosphorolysis of $(S_p)Ap_4A\alpha S$ proceeded via covalent enzyme-adenosine 5′-phosphorothioate as an intermediate (34). This corroborates previous observations that the Ap_4A phosphorylase supports the NDP-P_i exchange reaction (35) and catalyzes displacement of sulfate from adenosine 5′-phosphosulfate either by P_i (35) or by ATP (9). In each of these cases, the formation of an NMP-enzyme intermediate had been postulated.

The usefulness of phosphorothioate analogues of nucleotides in stereochemical analysis of the reactions catalyzed by nucleotidyl transfer enzymes is widely acknowledged (34, 36, 37). Here, the analogues bearing the modification at only one end of the molecule had been synthesized enzymatically and employed mainly to evaluate the importance of the Ap_4A-P^{α} for the recognition by various Ap_4A-metabolizing enzymes. Exchange in Ap_4A of nonbridging oxygen for sulfur at one (α) phosphate group does not prevent the molecule from reacting with any of the enzymes which specifically degrade Ap_4A. In all the systems considered, that modification, however, dramatically lowers specificity for the substrates expressed as V_{max}/K_m, due to much higher K_m values for the analogues than for Ap_4A. A convenient parameter which allows the comparison of diastereomers in a given enzymatic system is stereoselectivity, defined as the ratio between specificities estimated for each individual isomer. Stereoselectivity of (*asymmetrical*) Ap_4A hydrolase, (*symmetrical*) Ap_4A hydrolase and Ap_4A phosphorylase for the $Ap_4A\alpha S$ diastereomers (R_p:S_p) is 51:6:2.5, respectively. So, it is not high, particularly if one compares these figures with the data on stereoselectivity of some kinases for adenosine thiotriphosphate diastereomers which reach several thousands (37). Anyhow, in all the Ap_4A-degrading systems, $(R_p)Ap_4A\alpha S$ is the preferred isomer. Despite their reactiveness, the phosphorothioate analogues of Ap_4A bind to all the Ap_4A-degrading enzymes more weakly than Ap_4A. This effect is probably because sulfur has only a slightly bigger atomic value than oxygen (15.5 versus 14.0) but a much lower Pauling's electronegativity (2.5 versus 3.5), (38). As concerns regiospecificity of the catalyzed conversions, we can conclude that with the exception of Ap_4A phosphorylase cleaving $(S_p)Ap_4A\alpha S$, the Ap_4A-degrading enzymes attack the substrate molecule exclusively at the unmodified end.

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REFERENCES

1. Guranowski,A., Starzyńska,E. and Wasternack,C. (1988) *Int. J. Biochem.* **20**, 449–455.
2. Zamecnik,P.C., Stephenson,M.L., Janeway,C.M. and Randerath,K. (1966) *Biochem. Biophys. Res. Commun.* **24**, 91–97.
3. Plateau,P., Mayaux,J.-F. and Blanquet,S. (1981) *Biochemistry* **20**, 4654–4662.
4. Goerlich,O., Foeckler,R. and Holler,E. (1982) *Eur. J. Biochem.* **126**, 135–142.
5. Jakubowski,H. (1983) *Acta Biochim. Polon.* **30**, 51–69.
6. Dang,C.V. and Traugh,J.A. (1989) *J. Biol. Chem.* **264**, 5861–5865.
7. Guranowski,A. and Blanquet,S. (1985) *J. Biol. Chem.* **260**, 3542–3547.
8. Brevet,A., Coste,H., Fromant,M., Plateau,P. and Blanquet,S. (1987) *Biochemistry* **26**, 4763–4768.
9. Guranowski,A., Just,G., Holler,E. and Jakubowski,H. (1988) *Biochemistry* **27**, 2959–2964.
10. Lobatón,C.D., Vallejo,C.G., Sillero,A. and Sillero,M.A.G. (1975) *Eur. J. Biochem.* **50**, 495–501.
11. Ogilvie,A. and Antl,W. (1983) *J. Biol. Chem.* **258**, 4105–4109.
12. Jakubowski,H. and Guranowski,A. (1983) *J. Biol. Chem.* **258**, 9982–9989.
13. Prescott,M., Milne,A.D. and McLennan,A.G. (1989) *Biochem.J.* **259**, 831–838.
14. Barnes,L.D. and Culver,C.A. (1982) *Biochemistry* **21**, 6123–6128.
15. Guranowski,A., Jakubowski,H. and Holler,E. (1989) *J. Biol. Chem.* **258**, 14784–14789.
16. McLennan,A.G., Taylor,G.E., Prescott,M. anad Blackburn,G.M. (1989) *Biochemistry* **28**, 3868–3875.
17. Kobayashi,Y. and Kurotomi,K. (1989) *Biochem. Biophys. Res. Commun.* **160**, 1379–1386.
18. Tarussova,N.B., Shumiyanzeva,V.V., Krylov,A.C., Karpeisky,M.Ya. and Khomutov,R.M. (1983) *Bioorg. Khim.* **9**, 838–843.
19. Tarussova,N.B., Zavgorodny,C.G. and Osipova,T.I. (1985) *Bioorg. Khim.* **11**, 802–807.
20. Blackburn,G.M., Taylor,G.E., Thatcher,G.R.J., Prescott,M. and McLennan,A.G. (1987) *Nucleic Acids Res.* **15**, 6991–7004.
21. Shumyantseva,V.V., Poletaev,A.I. and Gnuchev,N.V. (1985) *Bioorg. Khim.* **11**, 227–230.
22. Haikal,A.F., Chavis,C., Pompon,A. and Imbach,J.L. (1989) *Bull. Soc. Chim. Fr.*, pp 521–531.
23. Biryukov,A.I., Zakharova,O.D. and Lavrik,O.I. (1987) *Bioorg. Khim.* **13**, 1164–1169.
24. Guranowski,A., Biryukov,A., Tarussova,N.B., Khomutov,R.M. and Jakubowski,H. (1987) *Biochemistry* **26**, 3425–3429.
25. Guranowski,A., Starzyńska,E., Taylor,G.E. and Blackburn,G.M. (1989) *Biochem. J.* **262**, 244–246.
26. Dixon,R.M. and Lowe,G. (1989) *J. Biol. Chem.* **264**, 2069–2074.
27. Goody,R.S. and Isakov,M. (1986) *Tetrahedron Lett.* **27**, 3599–3602.
28. Burgers,P.M.J. and Eckstein,F. (1978) *Proc. Natl. Acad. Sci. USA* **75**, 4798–4800.

29. Stahl,K.W., Schlimme,E. and Eckstein,F. (1974) *FEBS Lett.* **40**, 241–246.
30. Holler,E., Holmquist,B., Vallee,B.L., Tanja,K. and Zamecnik,P.C. (1983) *Biochemistry* **22**, 4924–4933.
31. Randerath,K., Janeway,C.M., Stephenson,M.L. and Zamecnik,P.C. (1966) *Biochem. Biophys. Res. Commun.* **24**, 98–105.
32. Plateau,P. and Blanquet,S. (1982) *Biochemistry* **21**, 5273–5279.
33. McLennan,A.G., Prescott,M. and Evershed,R.P. (1989) *Biomed. Environ. Mass Spectrom.* **18**, 450–452.
34. Eckstein,F., Romaniuk,P.J. and Connolly,B.A. (1982) *Methods Enzymol.* **87**, 197–212.
35. Guranowski,A. and Blanquet,S. (1986) *J. Biol. Chem.* **261**, 5943–5946.
36. Eckstein,F. (1980) *Trends in Biochem.* **5**, 157–159.
37. Eckstein,F. (1985) *Annu. Rev. Biochem.* **54**, 367–402.
38. *The Table of Periodic Properties of the Elements*. Sargent-Welch Scientific Company (1968).