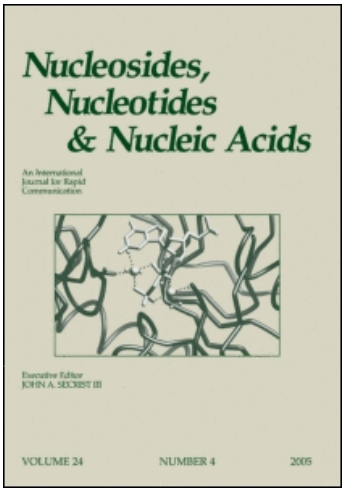

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Stereochemical Analysis of the Enzymic Synthesis and Hydrolysis of Ap_4A

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STEREOCHEMICAL ANALYSIS OF THE ENZYMIC SYNTHESIS AND HYDROLYSIS OF Ap_4A

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Abstract The stereochemical course of synthesis of P^1,P^4-Bis(5'-adenosyl) tetraphosphate (Ap_4A) from ATP catalysed by phenylalanyl-tRNA synthetase in the presence of Zn^{2+} has been shown to proceed with retention of configuration at P_α in accord with the mechanism involving phenylalanyl-adenylate as an intermediate. The hydrolysis of Ap_4A by the unsymmetrical Ap_4A phosphodiesterase from lupin seeds proceeds with inversion of configuration at P_α indicating that the hydrolysis takes place by a direct 'in line' displacement mechanism.

INTRODUCTION

P^1,P^4-Bis(5'-adenosyl)tetraphosphate (Ap_4A), which was first reported by Zamecnik *et al.*,[1] is ubiquitous in living cells.[2] It appears to play an important role in protein biosynthesis,[3] the intracellular level being directly related to the proliferative activity of the cell and results in the stimulation of DNA synthesis.[4] Moreover, Ap_4A associates tightly but non-covalently with DNA polymeraseα,[5] and acts as a primer for DNA synthesis *in vitro*.[6] In *Salmonella*, the Ap_4A concentration increases to 100 μM in response to the bacteriostatic quinone, 6-amino-7-chloro-5,8-dioxoquinoline and Ap_4A has been proposed as an "alarmone" i.e. a substance whose intracellular concentration increases dramatically in response to stress.[7]

Many aminoacyl-tRNA synthetases show weak Ap_4A synthetase activity, but for a few this is markedly enhanced by the presence of Zn^{2+}.[8] Phenylalanyl-tRNA synthetase which is a tetrameric enzyme,[9] belongs to this group. Like other aminoacyl-tRNA synthetases, phenylalanyl-tRNA synthetase first activates L-phenylalanine with MgATP to give enzyme-bound phenylalanyl-adenylate, which then normally charges its cognate tRNA.[10] It is not, however, necessary to have $tRNA^{Phe}$ present during the activation step:

$$E + Phe + MgATP \rightleftharpoons E\cdot Phe\sim AMP + MgPP_i$$

$$E\cdot Phe\sim AMP + tRNA^{Phe} \rightleftharpoons E + Phe\text{-}tRNA^{Phe} + AMP$$

It has been suggested that the mechanism of activation of amino acids by aminoacyl-tRNA synthetases should involve the initial formation of an adenylyl-enzyme intermediate.[11] We first investigated therefore the mechanism of activation of L-phenylalanine by phenylalanyl-tRNA synthetase from yeast by studying the stereochemical course of the nucleotidyl transfer raection. Positional isotope exchange experiments with adenosine 5'-[ß,ß-$^{18}O_2$]triphosphate were also undertaken.[12]

Although it has generally been considered that the production of Ap_4A by aminoacyl-tRNA synthetases arose by the reaction of ATP with the enzyme-bound aminoacyl adenylate,[1] this mechanism being supported by studies on a wide variety of aminoacyl-tRNA synthetases,[13] a recent report by Hilderman,[14] suggested that a homogeneous complex of arginyl- and lysyl-tRNA synthetase from rat liver catalyzes the lysine-independent synthesis of Ap_4A; consequently, an aminoacyl-adenylate intermediate could not be involved.

We have also investigated therefore the stereochemical course of the Zn^{2+}-stimulated production of Ap_4A by yeast phenylalanyl-tRNA synthetase and present evidence that Ap_4A synthesis proceeds by way of an aminoacyl-adenylate intermediate and not by the Hilderman pathway. Zn^{2+} also promotes the phenylalanine-dependent hydrolysis of ATP to AMP by yeast phenylalanyl-tRNA synthetase,[15] and the stereochemical course of this reaction has also been investigated.

Since Ap_4A is a pleiotropic regulator or signal nucleotide for cellular metabolism its concentration must be capable of being lowered as well as raised. Ap_4A phosphodiesterases have indeed been found and we have investigated one from lupin seeds which catalyses the hydrolysis of Ap_4A to ATP and AMP.[16]

RESULTS AND DISCUSSION

Stereochemical Course of Activation of Phenylalanine by Phenylalanyl-tRNA Synthetase.

L-Phenylalanine and adenosine 5'-[(*S*)-α-^{17}O,α,α-$^{18}O_2$]triphosphate,[17] were incubated with phenylalanyl-tRNA synthetase (yeast) in the presence of Mg^{2+} and hydroxylamine. Inorganic pyrophosphatase was also present to hydrolyze the magnesium pyrophosphate generated and so assist the overall reaction (Scheme 1). The [^{16}O,^{17}O,^{18}O]AMP was isolated and its chirality at phosphorus determined by our established procedure after cyclization and methylation.[18] The ^{31}P NMR spectrum obtained is shown in Figure 1. From the known isotopic content and enantiomeric excess of the adenosine 5'-[(*S*)-α-^{17}O,α,α-$^{18}O_2$]triphosphate, it was possible to calculate the expected relative peak intensities of the ^{31}P NMR resonances of the equatorial and axial triesters, for the reaction proceeding with retention and inversion of configuration. Comparison of the observed and calculated relative peak intensities (Table I) shows that the activation of phenylalanine by [(*S*)-α-^{17}O,α,α-$^{18}O_2$] ATP occurs stereospecifically (within experimental error) with inversion of configuration at P_α. This is most simply interpreted in terms of a direct 'in-line' displacement of magnesium pyrophosphate

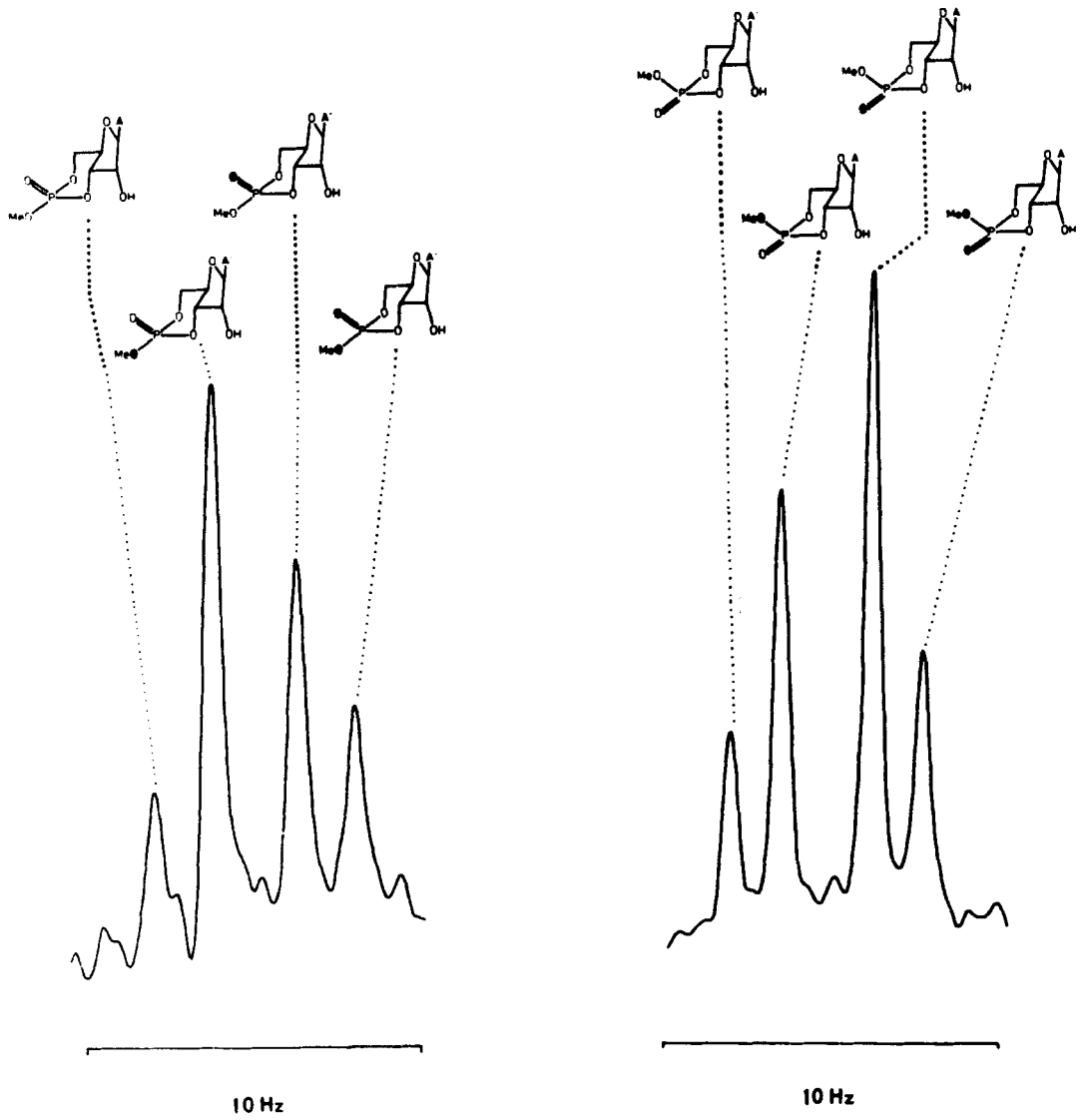
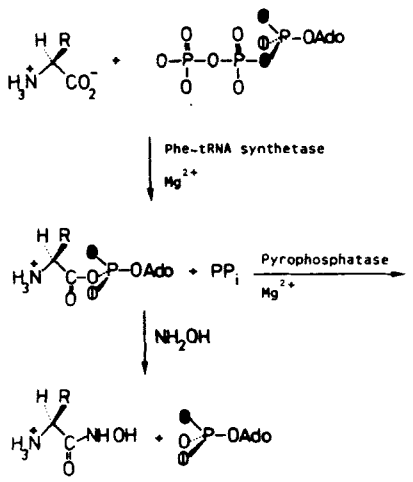


FIGURE 1: ^{31}P NMR spectra of the equatorial and axial triesters derived by cyclization and methylation of 5′-[^{16}O,^{17}O,^{18}O]AMP obtained by incubating phenylalanine, [(*S*)-α-^{17}O,α,α-$^{18}O_2$]ATP, hydroxylamine, yeast phenylalanyl-tRNA synthetase, and inorganic pyrophosphatase. The ratio of the $^{16}O_{ax}$,$^{18}O_{eq}$ triesters to the $^{18}O_{ax}$,$^{16}O_{eq}$ triesters shows that the 5′-[^{16}O,^{17}O,^{18}O]AMP has the S_P configuration and, hence, the reaction has proceeded with inversion of configuration at P_α of ATP as indicated in Scheme I. ● = ^{18}O; A′ = *N*-methyladenine.



[a] R = CH_2Ph. The evidence for the S_P configuration of the [^{16}O,^{17}O,^{18}O]AMP is provided in Figure 1.

Scheme I: Stereochemical Course of Activation of Phenylalanine by Adenosine 5'-[(*S*)-α-^{17}O,α,α-$^{18}O_2$]Triphosphate with Yeast Phenylalanyl-tRNA Synthetase[a]

from P_α of MgATP by phenylalanine and effectively excludes the possibility of a double-displacement mechanism involving an adenylyl-enzyme intermediate.

Positional Isotope Exchange Experiments with Phenylalanyl-tRNA Synthetase and [ß,ß-$^{18}O_2$]ATP

Inversion of configuration at phosphorus during the activation of phenylalanine by MgATP and phenylalanyl-tRNA synthetase is consistent with any odd number of displacement reactions at P_α the simplest being one. The possibility of an even number of covalent adenylyl-enzyme intermediates being involved was investigated by positional isotope exchange experiments with adenosine 5'-[ß,ß-$^{18}O_2$]triphosphate. Provided that the pyrophosphate liberated is free to rotate on the enzyme surface, the formation of an adenylyl-enzyme intermediate on incubation of Mg[ß,ß-$^{18}O_2$]ATP with phenylalanyl-tRNA synthetase would be detected by the appearance of ^{18}O label in the αβ bridging position by rotation and in the γ position by tumbling. L-Phenylalanine, [ß,ß-$^{18}O_2$] ATP and Mg^{2+} were incubated with phenylalanyl-tRNA synthetase for 21h, and as expected, the ^{31}P NMR spectrum of the recovered [$^{18}O_2$]ATP indicated that positional exchange of ^{18}O into the P_α-O-Pß bridge and into the γ position had occurred. In the absence of phenylalanine, incubation under the same conditions and for the same length of time led to no observable exchange of label. Since substrate synergism is important in catalysis of the activation step,[19,20] it is possible that, in the absence of phenylalanine,

Table I: Observed and Calculated Relative ^{31}P NMR Intensities[a]

labeled triester	equatorial triester			axial triester		
		calcd			calcd	
	obsd	retention	inversion	obsd	retention	inversion
MeO-P=O	0.28	0.28	0.28	0.29	0.28	0.28
Me●-P=O	1.00	0.67	1.00	0.64	1.00	0.67
MeO-P=●	0.70	1.00	0.67	1.00	0.67	1.00
Me●-P=●	0.43	0.38	0.38	0.41	0.38	0.38

[a] Observed relative peak intensities of the ^{31}P NMR resonances (from Figure 1) of the cyclized and methylated 5'-[^{16}O,^{17}O,^{18}O]AMP obtained from the activation of phenylalanine by [(*S*)-α-^{17}O,α,α-$^{18}O_2$]ATP and yeast phenylalanyl-tRNA synthetase. Comparison is made with the values expected for retention and inversion of configuration on the basis of the known isotopic composition of [(*S*)-α-^{17}O,-α,α-$^{18}O_2$]ATP.

the enzyme-MgATP complex is unable to adopt the correct conformation to form an adenylyl-enzyme intermediate. Therefore, phenylalaninol, a potent competitive inhibitor of phenylalanine which has a synergistic effect on the binding of MgATP to the enzyme,[21] was incubated with Mg[ß,ß-$^{18}O_2$]ATP and phenylalanyl-tRNA synthetase, again under the same conditions. No positional exchange of ^{18}O was observed.

These results indicate that an adenylyl enzyme intermediate is not formed between MgATP and phenylalanyl-tRNA synthetase in the absence of phenylalanine or in the presence of phenylalaninol.Taken together with the observed inversion of configuration at P_α of MgATP they provide strong evidence that the activation of phenylalanine catalyzed by yeast phenylalanyl-tRNA synthetase proceeds by a direct 'in-line' nucleotidyl-transfer mechanism.

Zn^{2+}-Dependent Synthesis of Ap_4A by Phenylalanyl-tRNA Synthetase.

Since Hilderman reported a lysine-independent but AMP-dependent synthesis of Ap_4A by a homogeneous complex of arginyl- and lysyl-tRNA synthetases from rat liver, apparently eliminating the involvement of an aminoacyl-adenylate intermediate with this enzyme, preliminary experiments were undertaken to see if Ap_4A was synthesized by phenylalanyl-tRNA synthetase in the absence of phenylalanine, but in the presence of Zn^{2+}. No production of Ap_4A was observed, either when ATP alone or when ATP and AMP were incubated with phenylalanyl-tRNA synthetase (yeast) in the presence of Mg^{2+} and Zn^{2+}. Consequently, the Hilderman mechanism did not appear to be the mechanism for Ap_4A production by phenylalanyl-tRNA synthetase.

When phenylalanyl-tRNA synthetase was incubated with L-phenylalanine, magnesium chloride, zinc chloride, and adenosine 5'-[(*S*)-$\alpha^{17}O$,α,α-$^{18}O_2$] triphosphate, isotopically labeled Ap_4A, ADP, and AMP were formed. The reaction

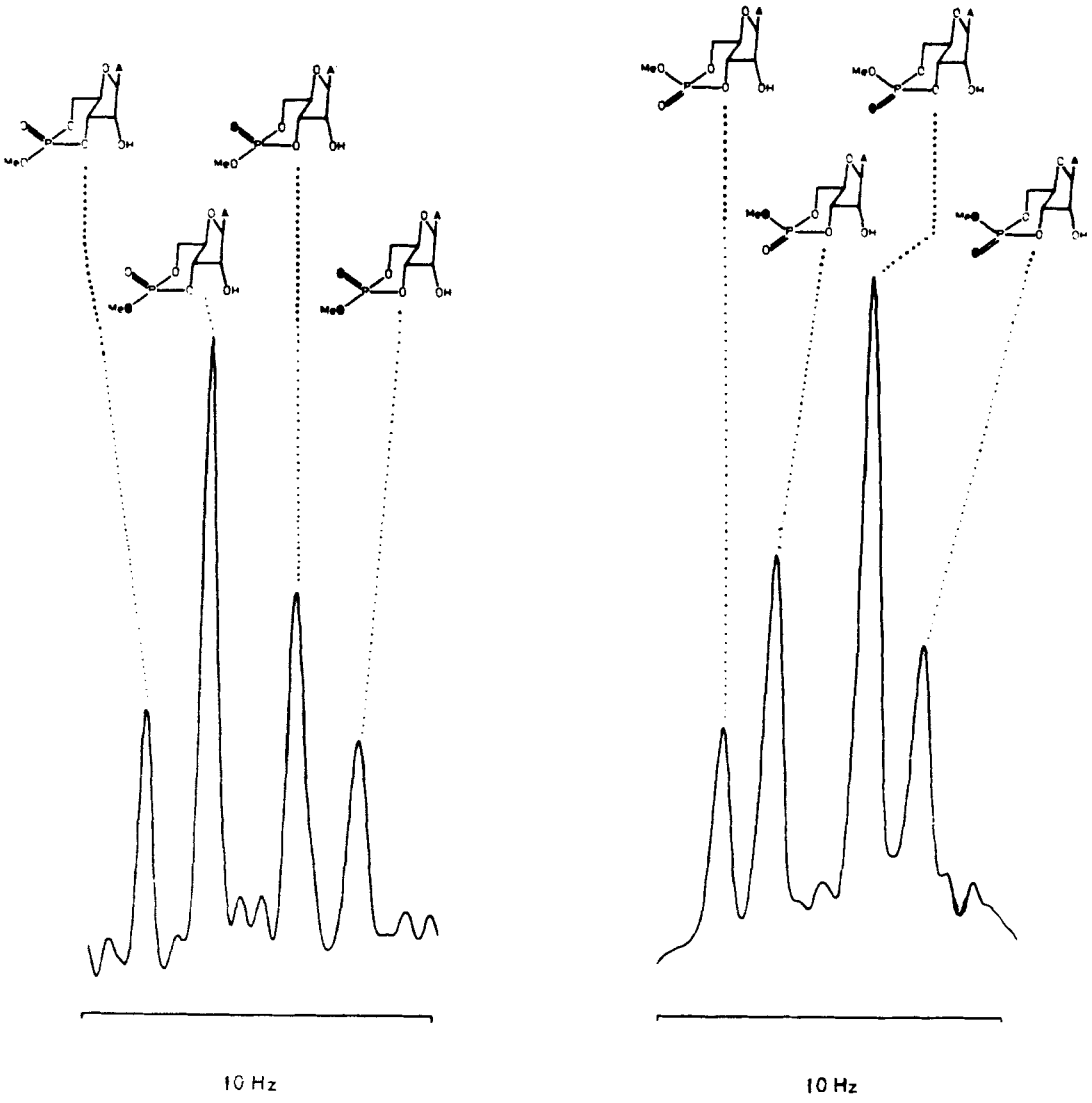


FIGURE 2: ^{31}P NMR spectra of the equatorial and axial triesters derived by cyclization and methylation of 5′-[^{16}O,^{17}O,^{18}O]AMP produced by Zn^{2+}- and phenylalanine-dependent hydrolysis of [(*S*)-α-^{17}O,α,α-$^{18}O_2$]ATP catalyzed by yeast phenylalanyl-tRNA synthetase. The ratio of the $^{16}O_{ax}$,$^{18}O_{eq}$ triesters to the $^{18}O_{ax}$,$^{16}O_{eq}$ triesters shows that the 5′-[^{16}O,^{17}O,^{18}O]AMP has the S_P configuration and, hence, that hydrolysis has occurred with inversion of configuration as indicated in Scheme II. ● = ^{18}O; A′ = *N*-methyladenine.

Table II: Observed and Calculated Relative ^{31}P NMR Intensities[a]

labeled triester	equatorial triester			axial triester		
		calcd			calcd	
	obsd	retention	inversion	obsd	retention	inversion
MeO-P=O	0.39	0.36	0.36	0.34	0.36	0.36
Me●-P=O	1.00	0.59	1.00	0.59	1.00	0.59
MeO-P=●	0.58	1.00	0.59	1.00	0.59	1.00
Me●-P=●	0.34	0.34	0.34	0.46	0.34	0.34

[a] Observed relative peak intensities of the ^{31}P NMR resonances (from Figure 2) of the cyclized and methylated 5'-[^{16}O,^{17}O,^{18}O]AMP obtained by the Zn^{2+}- and phenylalanine-dependent hydrolysis of [(*S*)-α-^{17}O,α,α-$^{18}O_2$]ATP catalyzed by yeast phenylalanyl-tRNA synthetase. Comparison is made with the values expected for retention and inversion of configuration on the basis of the known isotopic composition of [(*S*)-α-^{17}O,α,α-$^{18}O_2$]ATP.

was terminated when all the ATP had been consumed and the isotopically labeled Ap_4A, ADP, and AMP were isolated.

[^{16}O,^{17}O,^{18}O]AMP is produced by Zn^{2+}-dependent hydrolysis of [(*S*)-$\alpha^{17}O$,α,α-$^{18}O_2$]ATP by phenylalanyl-tRNA synthetase.[15] The ^{31}P NMR spectrum (Figure 2) obtained after cyclization and methylation of this [^{16}O,^{17}O,^{18}O]AMP,[18] shows that the hydrolysis of [(*S*)-$\alpha^{17}O$,α,α-$^{18}O_2$] ATP by yeast phenylalanyl-tRNA synthetase occurs with inversion of configuration at P_α.

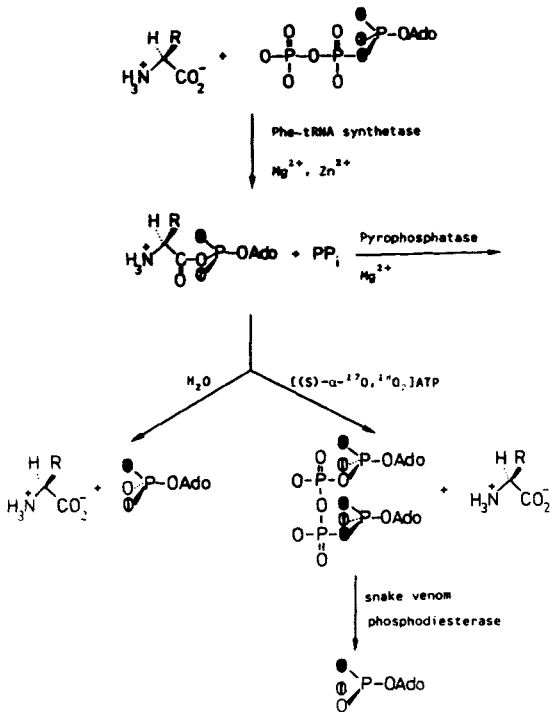
Comparison of the observed and calculated relative peak intensities (Table II) shows that [^{16}O,^{17}O,^{18}O] AMP is produced stereospecifically (within experimental error). Since it has been shown that the hydrolysis of ATP is dependent on phenylalanine as well as Zn^{2+},[15] we interpret this result to mean that hydrolysis of the phenylalanyl-adenylate intermediate, which we now know to be formed with inversion of configuration at phosphorus, occurs by C-O bond cleavage.

The isotopically labeled Ap_4A was incubated with snake venom phosphodiesterase, which hydrolyzes phosphate diesters with retention of configuration at phosphorus.[22] The [^{16}O,^{17}O,^{18}O] AMP produced was cyclized and methylated. Comparison of the ratios of the peak intensities in the ^{31}P NMR spectrum (not shown) with the calculated values (Table III) showed that the [^{16}O,^{17}O,^{18}O] AMP had the *Rp* configuration at phosphorus, and hence, the Zn^{2+}-dependent synthesis of Ap_4A by phenylalanyl-tRNA synthetase occurs with overall retention of configuration. If the reaction had proceeded with overall inversion of configuration, [^{16}O,^{17}O,^{18}O]AMP racemic at phosphorus would have been formed on incubation of the isotopically labeled Ap_4A with snake venom phosphodiesterase. Since the formation of phenylalanyl-adenylate is now known to proceed with inversion of configuration at phosphorus (in the absence of Zn^{2+}), the simplest

Table III: Observed and Calculated Relative ^{31}P NMR Intensities[a]

labeled triester	equatorial triester			axial triester		
		calcd			calcd	
	obsd	retention	inversion	obsd	retention	inversion
MeO-P=O	0.37	0.36	0.36	0.35	0.36	0.36
Me●-P=O	0.57	0.59	1.00	1.00	1.00	0.59
MeO-P=●	1.00	1.00	0.59	0.61	0.59	1.00
Me●-P=●	0.34	0.34	0.34	0.43	0.34	0.34

[a] Observed relative peak intensities of the ^{31}P NMR resonances (from Figure 3) of the cyclized and methylated 5′-[^{16}O,^{17}O,^{18}O]AMP obtained by snake venom phosphodiesterase catalyzed hydrolysis of the isotopically labeled Ap_4A derived by the Zn^{2+}- and phenylalanine-dependent activity of yeast phenylalanyl-tRNA synthetase in the presence of [(*S*)-α-^{17}O,α,α-$^{18}O_2$]ATP. Comparison is made with the values expected for retention and inversion of configuration on the basis of the known isotopic composition of [(*S*)-α-^{17}O,α,α-$^{18}O_2$]ATP.



[a] R = CH_2Ph. The evidence for the S_P configuration of the [^{16}O,^{17}O,^{18}O]AMP obtained by hydrolysis is provided in Figure 2. The evidence for the R_P configuration of the [^{16}O,^{17}O,^{18}O]AMP obtained by snake venom phosphodiesterase catalyzed hydrolysis (which occurs with retention of configuration at phosphorus) of the isotopically labeled Ap_4A is shown in Figure 3.

Scheme II: Stereochemical Course of Hydrolysis of [(*S*)-α-^{17}O,α,α-$^{18}O_2$]ATP and Synthesis of Isotopically Labeled Ap_4A by Yeast Phenylalanyl-tRNA Synthetase in the Presence of Zn^{2+}, Phenylalanine, and Inorganic Pyrophosphatase[a]

interpretation of this stereochemical observation is that the isotopically labeled Ap_4A is produced by nucleophilic substitution with [(*S*)-$\alpha^{17}O,\alpha,\alpha$-$^{18}O_2$] ATP on phenylalanyl-adenylate displacing phenylalanine with inversion of configuration at phosphorus, leading to overall retention of configuration at P_α (Scheme II).

The above evidence is not consistent with the Hilderman mechanism for the AMP-dependent (amino acid independent) formation of Ap_4A as observed with the rat liver arginyl/lysyl-tRNA synthetase complex, which seems to require Ap_2A as an enzyme-bound intermediate which is not free to reorientate. Subsequent attack by ATP would need to occur, regiospecifically displacing AMP that had originated from ATP, in order to account for the presence of equimolar amounts of ^{32}P and ^{3}H in the Ap_4A derived from [^{3}H]AMP and [γ-^{32}P]ATP.[14]

In the experiment with [(*S*)-$\alpha^{17}O,\alpha,\alpha$-$^{18}O_2$] ATP the AMP required for production of Ap_4A by the Hilderman pathway would have to arise by hydrolysis of [(*S*)-$\alpha^{17}O,\alpha,\alpha$-$^{18}O_2$]ATP, which is now known to occur with inversion of configuration. This [$^{16}O,^{17}O,^{18}O$]AMP would then have to react with [(*S*)-$\alpha^{17}O,\alpha,\alpha$-$^{18}O_2$] ATP, giving three isotopomeric Ap_4A species since the [$^{16}O,^{17}O,^{18}O$]AMP could react through each of its peripheral oxygens with equal probability (neglecting the kinetic isotope effect). Attack by the γ-phosphate of [(*S*)-$\alpha^{17}O,\alpha,\alpha$-$^{18}O_2$] ATP must occur at the phosphorus atom of Ap_2A derived from the [$^{16}O,^{17}O,^{18}O$]AMP , thus displacing three differently labeled species of AMP and giving three isotopomeric species of Ap_4A. Since the isotopically labeled AMP is being formed and reused throughout the incubation period by the Hilderman mechanism, the isotopically labeled AMP isolated would be quite different from that expected by the hydrolysis of [(*S*)-$\alpha^{17}O,\alpha,\alpha$-$^{18}O_2$] ATP by way of phenylalanyl-adenylate. In fact, the isotopically labeled AMP isolated had the same isotopic composition (within experimental error) as that obtained by hydrolyzing the [(*S*)-$\alpha^{17}O,\alpha,\alpha$-$^{18}O_2$] ATP by snake venom phosphodiesterase (Table IV). This effectively eliminates the Hilderman-type mechanism for the production of Ap_4A by yeast phenylalanyl-tRNA synthetase and is consistent with its formation by the enzyme-catalyzed displacement of phenylalanine from the enzyme-bound phenylalanyl-adenylate by the γ-phosphate of ATP.

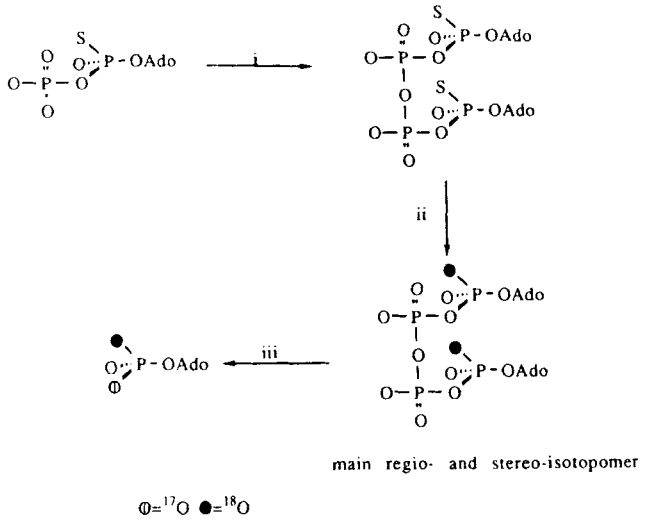
Synthesis of the Stereoisomers of P^1,P^4-Bis(5'-adenosyl)-1,4-dithiotetraphosphate

(*Sp*)-ADPαS was prepared by the method of Sheu and Frey,[23] and self coupled using diphenylphosphorochloridate to give (*Sp,Sp*)-P^1,P^4-Bis(5'-adenosyl) 1,4-dithiotetraphosphate (Scheme III) which was separated from a small amount of the (*Rp,Sp*)stereoisomer chromatographically. It is possible that the (*Rp,Sp*)-stereoisomer arose by dismutation of the ADPαS to AMPS and ATPαS which on coupling would give rise to both the (*Sp,Sp*)- and (*Rp,Sp*)-stereoisomers. When a mixture of (*Rp*)- and (*Sp*)-ADPαS,[24] were coupled in the same way, all three stereoisomers were formed.

Table IV: Comparison of ^{18}O Labeling of Isotopically Labeled AMP Observed by ^{31}P NMR Spectroscopy

labeled AMP	sv phosphodiesterase hydrolysis[a]	Zn^{2+}- and Phe-dependent hydrolysis[b]
$^{18}O,^{16}O_3$	0.47	0.46
$^{18}O_2,^{16}O_2$	1.00	1.00

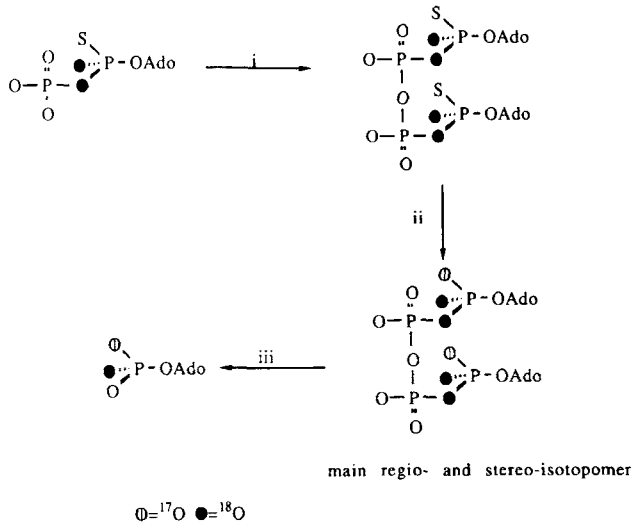
[a] From sv phosphodiesterase hydrolysis of [(*S*)-α-^{17}O,α,α-$^{18}O_2$]ATP. [b] By Zn^{2+}- and Phe-dependent hydrolysis of [(*S*)-α-^{17}O,α,α-$^{18}O_2$]ATP with yeast phenylalanyl-tRNA synthetase.



Scheme III. **Synthesis and stereochemical analysis of (S_p,S_p)-P^1,P^4-bis(5'-adenosyl)-1,4[$^{18}O_2$]tetraphosphate.** Reagents: *i*, $(PhO)_2POCl$; *ii*, CNBr/[^{18}O]water; *iii*, snake venom phosphodiesterase/[^{17}O]water.

Replacement of Sulphur by ^{18}O in (Sp,Sp)-P1,P4-Bis(5'-adenosyl)-1,4-dithiotetraphosphate

Bromine-water and N-bromosuccinimide both reacted slowly with (*Sp,Sp*)-P1,P4-bis(5'-adenosyl) 1,4-dithiotetraphosphate and gave a mixture of products which contained only a small amount of Ap4A and some P1,P4-bis(5'adenosyl) 1-thiotetraphosphate. Cyanogen bromide in the absence of buffer gave a much cleaner product and after 9 minutes about 70% Ap4A was formed, the minor products being identified as P1,P4-bis(5'-adenosyl) 1-thiotetraphosphate, Ap3A, ATP, AMPS and AMP. When the cyanogen bromide reaction was run in [^{18}O]-water the [$^{18}O_2$]Ap4A contained 70% of the label at P1 and P4, and 30% at P2 and P3. The P1,P4-bis(5'adenosyl)[^{18}O]-1- thiotetraphosphate was found to contain 78% label at P4, 8% at P3, and 14% at P2. Clearly the central phosphate



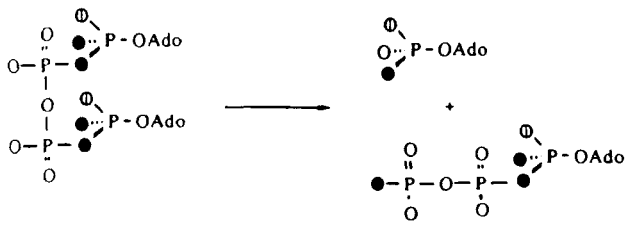
Scheme IV. **Synthesis and stereochemical analysis of (R_p,R_p)-P^1,P^4-bis(5′-adenosyl)-1[^{17}O,$^{18}O_2$],4[^{17}O,$^{18}O_2$]tetraphosphate.** Reagents: *i*, $(PhO)_2POCl$; *ii*, CNBr/[^{17}O]water; *iii*, snake venom phosphodiesterase/water.

residues can participate in the reaction leading to *cyclo*-triphosphate and *cyclo*-diphosphate as transient intermediates. If the label at P^1 and P^4 was introduced exclusively by way of these cyclic intermediates overall retention of stereochemistry would be expected.[25]

The [$^{18}O_2$]Ap$_4$A was hydrolysed with snake venom phosphodiesterase in ^{17}O-water and the AMP analysed for chirality at phosphorus after cyclization and methylation.[18] The ^{31}P n.m.r. spectrum (not shown) shows it to be predominantly of the (*Sp*)-configuration. Since snake venom phosphodiesterase is known to hydrolyse phosphate diesters with retention of configuration at phosphorus,[22] the sulphur displacement has occurred with participation of the neighbouring phosphate groups leading to predominant retention of configuration, as was observed with adenosine 5′[(*S*)α-thio-γ-benzyl]triphosphate.[25]

Replacement of Sulphur by ^{17}O in (Sp,Sp)-P^1,P^4-Bis(5′-adenosyl) 1[$^{18}O_2$,thio], 4[$^{18}O_2$,thio]-tetraphosphate

Ap$_4$A chirally labelled at P^1 and P^4 with both ^{17}O and ^{18}O, was synthesized. (*Sp,Sp*)-P^1,P^4-Bis(5′-adenosyl)1[$^{18}O_2$,thio],4[$^{18}O_2$,thio]-tetraphosphate was prepared from (Sp)-[α-$^{18}O_2$]ADPαS 5,[27] by the same procedure used for the unlabelled material and purified chromatographically. On treatment with cyanogen bromide in ^{17}O-water it gave predominantly (*Rp,Rp*)-P^1,P^4-bis(5′-adenosyl)1[^{17}O,$^{18}O_2$],4[^{17}O,$^{18}O_2$]-tetraphosphate.Hydrolysis



Scheme V . **The stereochemical course of hydrolysis by Ap_4A phosphodiesterase from lupin seeds.** The reaction proceeds with inversion of configuration at phosphorus.

TABLE V

Comparison of the observed peak intensities of the ^{31}P NMR resonances of the diastereoisomeric triesters derived by cyclization and methylation of the 5'-[^{16}O,^{17}O,^{18}O]AMP obtained by hydrolysing (S_p,S_p)-P^1,P^4-bis(5'-adenosyl)-1-[^{17}O,$^{18}O_2$],4-[^{17}O,$^{18}O_2$]-tetraphosphate with snake venom phosphodiesterase (SVPDE) and Ap_4A phosphodiesterase from lupin seeds (Ap_4APDE) in ordinary water

Labeled triester	Equatorial triester		Axial triester	
	SVPDE	Ap_4APDE	SVPDE	Ap_4APDE
MeO-P=O	0.77	0.95	0.77	0.84
Me●-P=O	1.00	0.76	0.77	1.00
MeO-P=●	0.76	1.00	1.00	0.67
Me●-P=●	0.20	0.30	0.24	0.16

with snake venom phosphodiesterase in water gave [^{16}O,^{17}O,^{18}O]AMP which had predominantly the (*Sp*)-configuration (Scheme IV), confirming that the displacement of sulfur occurred with predominant retention of configuration.

The Stereochemical Course of Hydrolysis of Ap_4A with Ap_4A Phosphodiesterase (from Lupin Seeds)

(*Rp*,*Rp*)-P^1,P^4-bis(5'-adenosyl)1[^{17}O,$^{18}O_2$],4[^{17}O,$^{18}O_2$]-tetraphosphate (note it has *predominantly* the (*Rp*)-configuration at P^1 and P^4) was hydrolysed with Ap_4A phosphodiesterase in water to give labelled ATP and [^{16}O,^{17}O,^{18}O] AMP (Scheme V). After purification the [^{16}O,^{17}O,^{18}O] AMP was analysed for chirality by ^{31}P n.m.r. spectroscopy (not shown) after cyclization and methylation.[18] From the ratio of the intensities of the two mono-^{18}O isotopomers of the axial and equatorial triesters it is clear that the [^{16}O,^{17}O,^{18}O] AMP has predominantly the (*Rp*)-configuration. Comparison of the relative intensities of the resonances derived from the [^{16}O,^{17}O,^{18}O] AMP obtained by hyrolysis of (*Rp*,*Rp*)-P^1,P^4-bis(5'-adenosyl)1[^{17}O,$^{18}O_2$],4[^{17}O,$^{18}O_2$]-tetraphosphate with snake venom phosphodiesterase (Table V) shows that Ap_4A phosphodiesterase catalyses

the hydrolysis of Ap_4A with inversion of configuration at P_α since snake venom phosphodiesterase is known to catalyse the hydrolysis of phosphodiesters with retention.[22] This result indicates that the Ap_4A phosphodiesterase catalyses the hydrolysis of Ap_4A at P^1 by a direct 'in-line' mechanism.[28]

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