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Mechanism of Activation of Phenylalanine and Synthesis of P^1,P^4-Bis(5′-adenosyl) Tetraphosphate by Yeast Phenylalanyl-tRNA Synthetase†

Sara P. Harnett, Gordon Lowe,* and Gaynor Tansley

Dyson Perrins Laboratory, Oxford University, Oxford, OX1 3QY U.K.

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ABSTRACT: The activation of L-phenylalanine by yeast phenylalanyl-tRNA synthetase using adenosine 5′-[(*S*)-α-^{17}O,α,α-$^{18}O_2$]triphosphate is shown to proceed with inversion of configuration at P_α of ATP. This observation taken together with the lack of positional isotope exchange when adenosine 5′-[β,β-$^{18}O_2$]triphosphate is incubated with the enzyme in the absence of phenylalanine and in the presence of the competitive inhibitor phenylalaninol indicates that activation of phenylalanine occurs by a direct "in-line" adenylyl-transfer reaction. In the presence of Zn^{2+}, yeast phenylalanyl-tRNA synthetase also catalyzes the phenylalanine-dependent hydrolysis of ATP to AMP and the synthesis of P^1,P^4-bis(5′-adenosyl) tetraphosphate (Ap_4A). With adenosine 5′-[(*S*)-α-^{17}O,α,α-$^{18}O_2$]triphosphate, the formation of AMP and Ap_4A is shown to occur with inversion and retention of configuration, respectively. It is concluded that phenylalanyl adenylate is an intermediate in both processes, Zn^{2+} promoting AMP formation by hydrolytic cleavage of the C–O bond and Ap_4A formation by displacement at phosphorus of phenylalanine by ATP.

Phenylalanyl-tRNA synthetase belongs to the tetrameric class of aminoacyl-tRNA synthetases and consists of non-identical subunits ($\alpha_2\beta_2$) (Söll & Schimmel, 1974; Schimmel & Söll, 1979). Like other aminoacyl-tRNA synthetases, phenylalanyl-tRNA synthetase first activates L-phenylalanine with MgATP to give enzyme-bound phenylalanyl adenylate, which then charges its cognate tRNA (Kim et al., 1977). It is not, however, necessary to have $tRNA^{Phe}$ present during the activation step:

$$E + Phe + MgATP \rightleftarrows E{\cdot}Phe{\sim}AMP + MgPP_i$$

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

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 (Spector, 1982). In order to investigate the mechanism of activation of L-phenylalanine by phenylalanyl-tRNA synthetase from yeast, the stereochemical course of nucleotidyl transfer has been investigated. Positional isotope exchange experiments with adenosine 5′-[β,β-$^{18}O_2$]triphosphate have also been undertaken. Together, these experiments indicate that the activation of L-phenylalanine occurs by a direct "in-line" adenylyl-transfer reaction.

Phenylalanyl-tRNA synthetase from yeast also synthesizes P^1,P^4-bis(5′-adenosyl) tetraphosphate (Ap_4A), especially in the presence of Zn^{2+} (Plateau et al., 1981; Plateau & Blanquet, 1982; Blanquet et al., 1982). Ap_4A, first reported in a biological system by Zamecnik et al. (1966) [for review, see Zamecnik (1983)], was shown to be ubiquitous in living cells (Zamecnik et al., 1967) and appears to play an important role in protein biosynthesis (Rapaport & Zamecnik, 1976). The intracellular level of Ap_4A is directly related to the proliferative activity of the cell and on addition to permeabilized baby hamster kidney cells arrested in the G_1 phase of the cell cycle results in stimulation of DNA synthesis (Grummt, 1978).

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Ap_4A associates tightly but noncovalently with DNA polymerase (Grummt et al., 1979) and acts as a primer for DNA replication in in vitro systems (Rapaport et al., 1981a,b; Zamecnik et al., 1982).

Many aminoacyl-tRNA synthetases possess low Ap_4A synthetase activity but only a few show enhancement of this activity by Zn^{2+} (Plateau et al., 1981; Plateau & Blanquet, 1982; Blanquet et al., 1982). Zn^{2+} also effects the conformation of Ap_4A, leading to destacking of the purine bases (Holler et al., 1983), which may be necessary for association with macromolecules such as DNA polymerase.

Until recently it was considered that the production of Ap_4A by aminoacyl-tRNA synthetases arose by the reaction of ATP with the enzyme-bound aminoacyl adenylate (Zamecnik et al., 1966), this mechanism being supported by studies on a wide variety of aminoacyl-tRNA synthetases (Goerlich et al., 1982). However, Hilderman (1983) reported recently 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. AMP was required, however, suggesting that Ap_2A may be an intermediate.

We have investigated 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 (Igloi et al., 1980), and the stereochemical course of this reaction is also reported.

Materials and Methods

Phenylalanyl-tRNA synthetase (yeast) was a gift form Dr. P. Rêmy (C.N.R.S., Strasbourg) and was stored at −20 °C in a buffer consisting of 50% glycerol, phosphate buffer (10 mM, pH 7.2), ethylenediaminetetraacetic acid (EDTA) (10^{-4} M), and dithioerythritol (5×10^{-4} M).

Inorganic pyrophosphatase and snake venom phosphodiesterase (*Crotalus atrox*) were obtained from Sigma Chemical Co. Ltd. (Poole, Dorset, U.K.). Deuterium oxide (99.8 atom % ^2H) was obtained from Fluorochem Ltd. (Glossop, Derbyshire, U.K.). High-grade deionized water used in the preparation of all buffers was obtained from a Milli-Q2 water purification system (Millipore Ltd., Harrow, Middlesex, U.K.).

Analysis of nucleotides was performed by ion-exchange chromatography using an FPLC system (Pharmacia, Hounslow, U.K.) and the polyanion SI resin. This system was used for the isolation of $[^{18}O_2]$ATP in the positional isotope exchange experiments.

Adenosine 5′-[(*S*)-α-^{17}O,α,α-$^{18}O_2$]triphosphate was synthesized as described by Lowe & Tansley (1984). Separate batches were used in the study of the activation of phenylalanine by phenylalanyl-tRNA synthetase (yeast) and in the study of the Zn^{2+}-dependent synthesis of Ap_4A by phenylalanyl-tRNA synthetase. Both batches had very similar isotopic labeling patterns at P_α; the first having the composition (calculated from ^{31}P NMR spectra) 11% ^{16}O, 52% ^{17}O, and 37% ^{18}O at the ^{17}O site and 92% ^{18}O per ^{18}O site. The second batch had the composition 14% ^{16}O, 50% ^{17}O, and 36% ^{18}O at the ^{17}O site and 91% ^{18}O per ^{18}O site. Both had in excess of 95% the *S* enantiomer; this was established by hydrolysis with snake venom phosphodiesterase and stereochemical analysis of the [^{16}O,^{17}O,^{18}O]AMP formed (Jarvest et al., 1981). Adenosine 5′-[β,β-$^{18}O_2$]triphosphate was prepared by the method of Lowe & Sproat (1981) and had 95% ^{18}O at the nonbridging P_β sites.

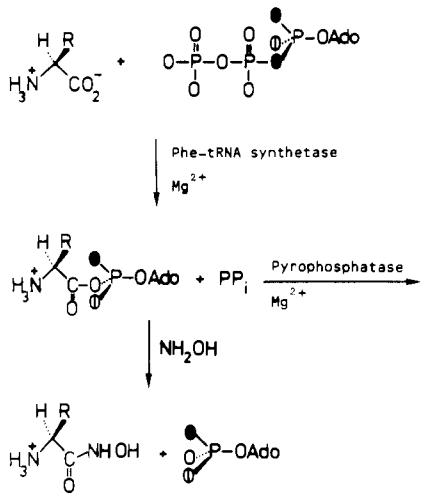
Incubation of L-*Phenylalanine, Adenosine 5′-[(S)-*α-^{17}*O,*α,α-$^{18}O_2$*]Triphosphate, and Hydroxylamine with Phenylalanyl-tRNA Synthetase and Pyrophosphatase.* Phenylalanyl-tRNA synthetase (10 μL, 14 mg/mL, 3.6 μmol min^{-1} mg^{-1}) and inorganic pyrophosphatase (0.2 mg, 500 units/mg) were added to tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl) buffer (12 mL, 100 mM, pH 8) containing L-phenylalanine (5.8 mM), adenosine 5′-[(*S*)-α-^{17}O,α,α-$^{18}O_2$]triphosphate (4.2 mM), hydroxylamine hydrochloride (1 M), magnesium acetate (4.2 mM), and dithiothreitol (10 mM). The solution was incubated at 37 °C, and the reaction was followed by the FPLC analytical system. When the reaction was complete (5.5 h), EDTA (70 μmol, pH 8) was added and the solution applied to a column (1.5 cm × 30 cm) of DEAE-Sephadex A-25 that had been equilibrated with triethylammonium bicarbonate buffer (50 mM, pH 8.0) and eluted with a linear gradient of triethylammonium bicarbonate buffer (50–200 mM, pH 8.0). The [^{16}O,^{17}O,^{18}O]AMP (45 μmol) was freed from buffer by addition and evaporation of methanol (3 × 10 mL) and then cyclized and methylated for analysis by ^{31}P NMR spectroscopy of the chirality at phosphorus (Jarvest et al., 1981).

Positional Isotope Exchange with Adenosine 5′-[β,β-$^{18}O_2$*]Triphosphate and Phenylalanyl-tRNA Synthetase.* The following solutions were incubated at 37 °C with phenylalanyl-tRNA synthetase (0.8 μL, 14 mg/mL, 3.6 mol min^{-1} mg^{-1}) for 21 h. Each experiment was conducted in Tris-HCl buffer (12 mL, 33.3 mM, pH 8) with added magnesium acetate (1.25 mM) and 2-mercaptoethanol (5 μL). To this was added the following in three separate experiments: (i) L-phenylalanine (1.8 mM) and [β,β-$^{18}O_2$]ATP (1.25 mM); (ii) [β,β-$^{18}O_2$]ATP (1.25 mM); (iii) L-phenylalaninol (6 mM) and [β,β-$^{18}O_2$]ATP (1.25 mM). The reactions were terminated by addition of EDTA (30 μmol, pH 8). [$^{18}O_2$]ATP was isolated by ion-exchange chromatography on the FPLC system, and the ^{31}P NMR spectra were determined.

Incubation of Phenylalanyl-tRNA Synthetase with AMP and ATP in the Absence of Phenylalanine. Two solutions of Tris-HCl buffer (12 mL, 20 mM, pH 8) were prepared, each containing potassium chloride (150 mM), magnesium chloride (10 mM), zinc chloride (100 μM), and adenosine triphosphate (4.2 mM). AMP (4.2 mM) was added to one of these solutions. To both were added inorganic pyrophosphatase (0.2 mg, 500 units/mg) and phenylalanyl-tRNA synthetase (10 μl, 14 mg/mL), and the solutions were incubated at 37 °C for 24 h. Each solution was analyzed by FPLC after this time.

Zinc-Dependent Synthesis of P^1,P^4*-Bis(5′-adenosyl) Tetraphosphate by Yeast Phenylalanyl-tRNA Synthetase.* Phenylalanyl-tRNA synthetase (30 μL, 14 mg/mL, 3.6 μmol min^{-1} mg^{-1}) and inorganic pyrophosphatase (0.6 mg, 500 units/mg) were added to Tris-HCl buffer (31 mL, 20 mM, pH 8) containing L-phenylalanine (2 mM), adenosine 5′-[(*S*)-α-^{17}O,α,α-$^{18}O_2$]triphosphate (4.2 mM), zinc chloride (160 μM), magnesium chloride (10 mM), and potassium chloride (150 mM). The solution was incubated at 37 °C and the reaction followed by the analytical FPLC system. After 4 h 20 min, EDTA (375 μmol, pH 8) was added and the solution applied to a column (1.5 cm × 30 cm) of DEAE-Sephadex A-25 that had been equilibrated with triethylammonium bicarbonate buffer (200 mM, pH 8.0) and eluted with a linear gradient of triethylammonium bicarbonate buffer (200–400 mM, pH 8.0). The isolated products, [^{16}O,^{17}O,^{18}O]AMP (65 μmol), isotopically labeled ADP (28 μmol), and isotopically labeled Ap_4A (15 μmol), were freed from buffer by addition and evaporation of methanol (3 times 10 mL). The

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]



[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.

[^{16}O,^{17}O,^{18}O]AMP was then cyclized and methylated for analysis by ^{31}P NMR spectroscopy of the chirality at phosphorus (Jarvest et al., 1981).

Incubation of Isotopically Labeled Ap_4A with Snake Venom Phosphodiesterase. Snake venom phosphodiesterase (1 mg, 0.34 units/mg) was added to Tris-HCl buffer (10 mL, 25 mM, pH 8.2) containing the isotopically labeled Ap_4A (15 μmol, 1.3 mM) from the previous reaction and magnesium chloride (10 mM). The solution was incubated at 37 °C, and the reaction, which was followed by the FPLC system, was complete after 1 h 20 min. EDTA (200 μmol, pH 8) was added and the solution applied to a column (1 cm × 12 cm) of DEAE-Sephadex A-25 that had been equilibrated with triethylammonium bicarbonate buffer (50 mM, pH 8.0) and eluted with a linear gradient of triethylammonium bicarbonate buffer (50–200 mM, pH 8.0). The [^{16}O,^{17}O,^{18}O]AMP (29 μmol) was freed from buffer by addition and evaporation of methanol (3 times 10 mL) and then cyclized and methylated for analysis by ^{31}P NMR spectroscopy of the chirality at phosphorus (Jarvest et al., 1981).

^{31}P NMR Spectra. ^{31}P NMR spectra were recorded at 101.256 MHz on a Bruker AM 250 Fourier-transform spectrometer with quadrature detection. Signal averaging was performed by an Aspect 2000 computer interfaced with the spectrometer. Field-frequency locking was provided by the deuterium resonance of D_2O or CD_3CN. Parameters used for the spectra of the methyl ester of cyclic AMP were sweep width 1500 Hz, acquisition time 2.73 s, pulse width 6 μs, broad-band proton decoupling, Gaussian multiplication (line broadening in the range −0.6 to −0.4 Hz, Gaussian broadening about 0.4) of the FID in 8K, and Fourier transform in 32K.

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 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 I). The [^{16}O,^{17}O,^{18}O]AMP was isolated and its chirality at phosphorus determined by our established procedure after cyclization and methylation (Jarvest et al., 1981). 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 from P_α of MgATP by phenylalanine and effectively excludes the possibility of a double-displacement mechanism involving a single adenylyl–enzyme intermediate.

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

labeled triester	equatorial triester			axial triester		
	obsd	calcd		obsd	calcd	
		retention	inversion		retention	inversion
MeO—P=O	0.28	0.28	0.28	0.29	0.28	0.28
Me^{18}O—P=O	1.00	0.67	1.00	0.64	1.00	0.67
MeO—P=^{18}O	0.70	1.00	0.67	1.00	0.67	1.00
Me^{18}O—P=^{18}O	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.

Positional Isotope Exchange Experiments with Phenylalanyl-tRNA Synthetase and [β,β-^{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′-[β,β-^{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[β,β-^{18}O$_2$]ATP with phenylalanyl-tRNA synthetase would be detected by the appearance of ^{18}O label in the α–β bridging position by rotation and in the γ position by tumbling. L-Phenylalanine, [β,β-^{18}O$_2$]ATP, and Mg^{2+} were incubated with phenylalanyl-tRNA synthetase for 21 h, 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–P_β 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 (Kosakowski & Holler, 1973; Holler et al., 1975), it is possible that, in the absence of phenylalanine, 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 (Pimmer et al., 1976), was incubated with Mg[β,β-^{18}O$_2$]ATP and phenylalanyl-tRNA synthetase, again under the same conditions. No positional exchange of ^{18}O was observed.

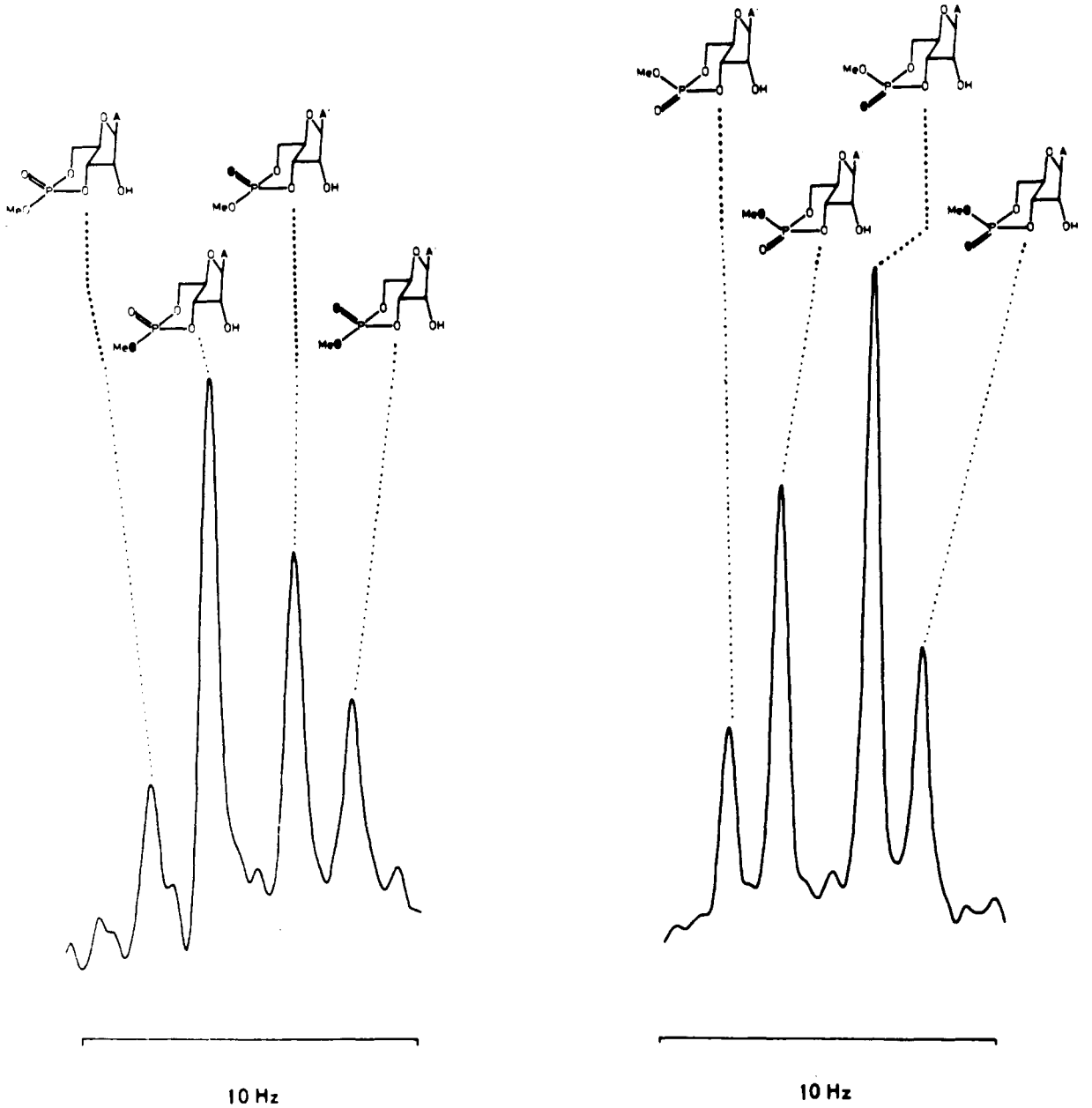


FIGURE 1: ^{31}P NMR spectra of the equatorial and axial triesters derived by cyclization and methylation of 5′-[^{16}O,^{17}O,^{18}O]AMP obtained by incubating phenylalanine, [(*S*)-α-^{17}O,α,α-$^{18}O_2$]ATP, hydroxylamine, yeast phenylalanyl-tRNA synthetase, and inorganic pyrophosphatase. The ratio 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.

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. These observations taken together with the observed inversion of configuration at P_α of MgATP 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 (1983) 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.

Phenylalanyl-tRNA synthetase was incubated with L-phenylalanine, magnesium chloride, zinc chloride, and adenosine 5′-[(*S*)-α-^{17}O,α,α-$^{18}O_2$]triphosphate. The production of isotopically labeled Ap_4A, ADP, and AMP was followed by FPLC analysis. The reaction 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*)-α-^{17}O,α,α-$^{18}O_2$]ATP by phenylalanyl-tRNA synthetase [see Igloi et al., (1980)]. The ^{31}P NMR spectrum (Figure 2) obtained after cyclization and methylation of this [^{16}O,^{17}O,^{18}O]AMP (Jarvest et al., 1981) shows that the hydrolysis of [(*S*)-α-^{17}O,α,α-$^{18}O_2$]ATP by yeast phenylalanyl-tRNA synthetase occurs with inversion of configuration at P_α.

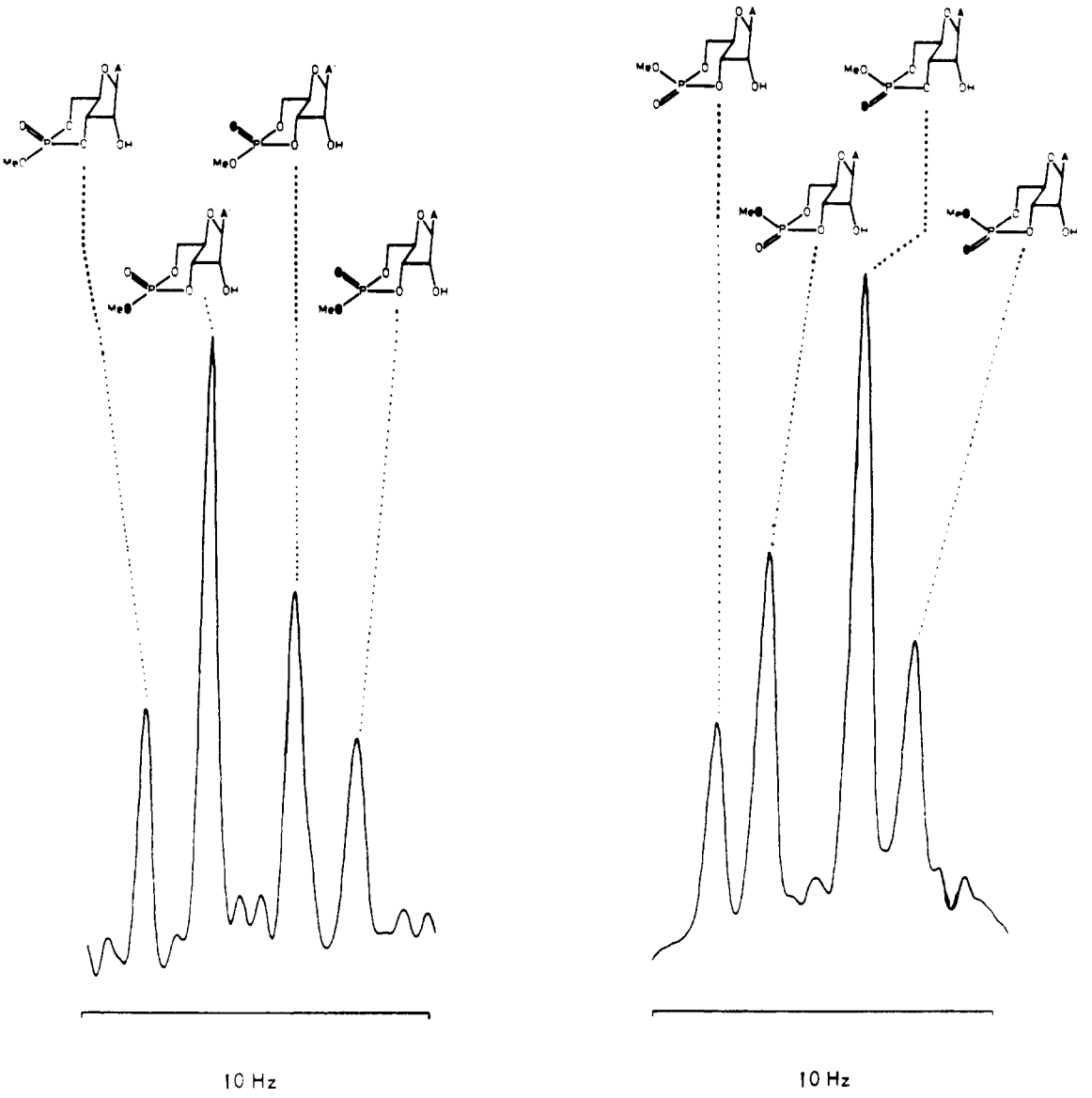


FIGURE 2: ^{31}P NMR spectra of the equatorial and axial triesters derived by cyclization and methylation of 5′-[^{16}O,^{17}O,^{18}O]AMP produced by Zn^{2+}- and phenylalanine-dependent hydrolysis of [(*S*)-α-^{17}O,α,α-$^{18}O_2$]ATP catalyzed by yeast phenylalanyl-tRNA synthetase. The ratio of the $^{16}O_{ax}$,$^{18}O_{eq}$ 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.

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 Igloi et al. (1980) have shown that the hydrolysis of ATP is dependent on phenylalanine as well as Zn^{2+}, 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 (Jarvest & Lowe, 1981; Mehdi & Gerlt, 1981). 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 (Figure 3) with the calculated values (Table III) showed that the [^{16}O,^{17}O,^{18}O]AMP had the R_P 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

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^{18}O—P=O	1.00	0.59	1.00	0.59	1.00	0.59
MeO—P=^{18}O	0.58	1.00	0.59	1.00	0.59	1.00
Me^{18}O—P=^{18}O	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.

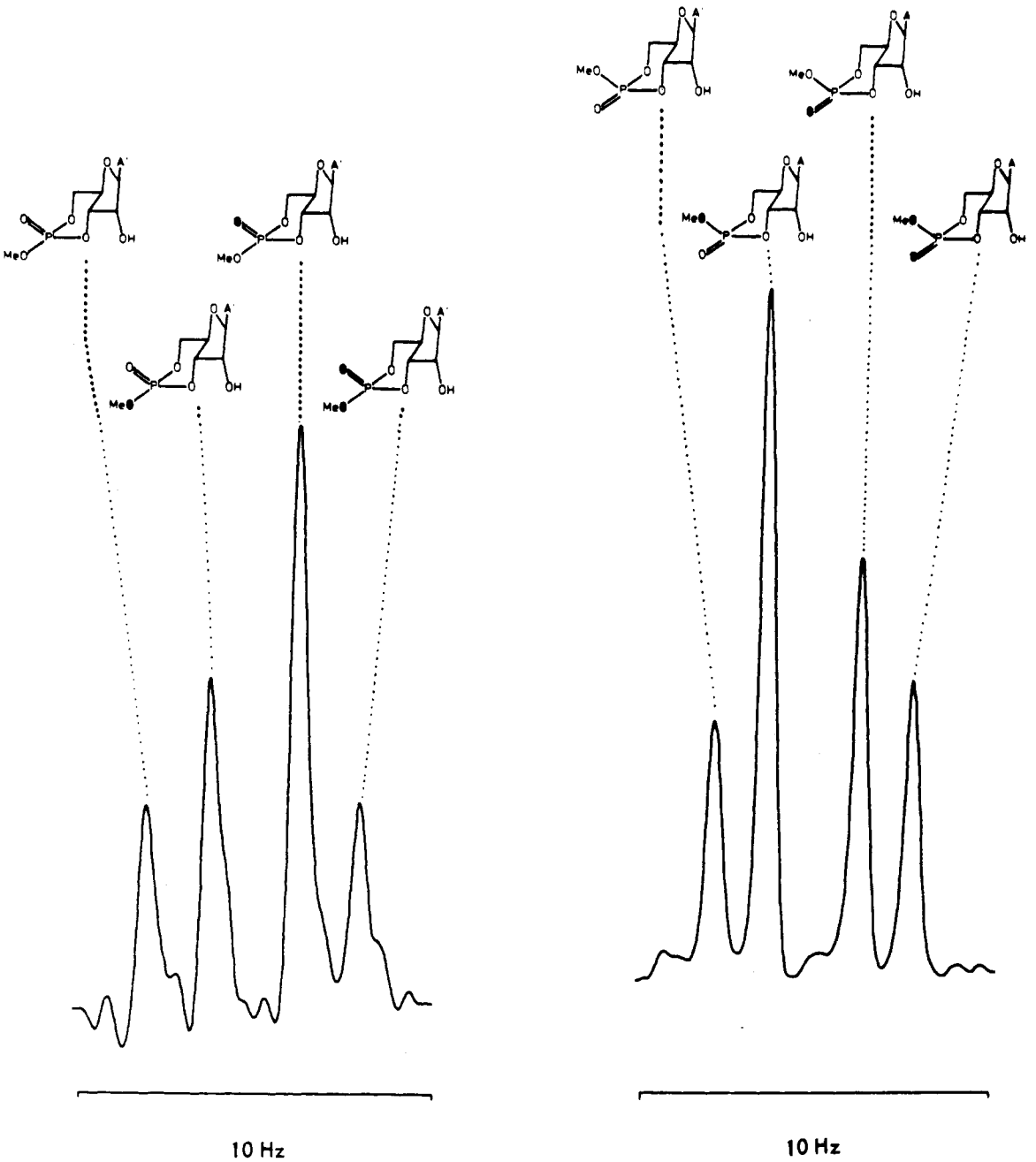


FIGURE 3: ^{31}P NMR spectra of the equatorial and axial triesters derived by cyclization and methylation of 5′-[^{16}O,^{17}O,^{18}O]AMP obtained by hydrolyzing isotopically labeled Ap_4A with snake venom phosphodiesterase (which is known to occur with retention of configuration). The ratio of the $^{16}O_{ax}$,$^{18}O_{eq}$ triester to the $^{18}O_{ax}$,$^{16}O_{eq}$ triesters shows that the 5′-[^{16}O,^{17}O,^{18}O]AMP has the R_P configuration and, hence, the Zn^{2+}- and phenylalanine-dependent formation of Ap_4A by yeast phenylalanyl-tRNA synthetase and [(*S*)-α-^{17}O,α,α-$^{18}O_2$]ATP occurs with overall retention of configuration as indicated in Scheme II. ● = ^{18}O; A′ = *N*-methyladenine.

to proceed with inversion of configuration at phosphorus (in the absence of Zn^{2+}), the simplest interpretation of this stereochemical observation is that the isotopically labeled Ap_4A is produced by nucleophilic substitution with [(*S*)-α-^{17}O,α,α-$^{18}O_2$]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-type 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 (Hilderman, 1983).

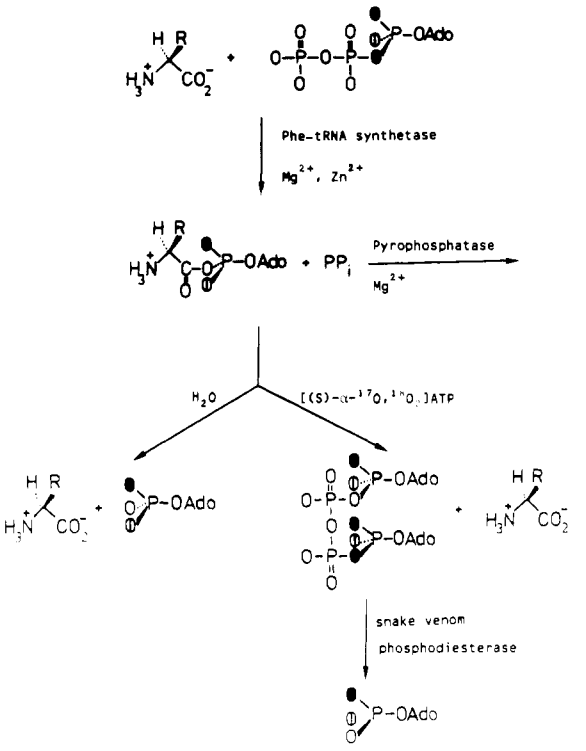
In the experiment with [(*S*)-α-^{17}O,α,α-$^{18}O_2$)ATP, the AMP required for production of Ap_4A by the Hilderman pathway would have to arise by hydrolysis of [(*S*)-α-^{17}O,α,α-$^{18}O_2$]ATP, which is now known to occur with inversion of configuration. This [^{16}O,^{17}O,^{18}O]AMP would then have to react with [(*S*)-α-^{17}O,α,α-$^{18}O_2$]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*)-α-^{17}O,α,α-^{18}O]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

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^{18}O—P=O	0.57	0.59	1.00	1.00	1.00	0.59
MeO—P=^{18}O	1.00	1.00	0.59	0.61	0.59	1.00
Me^{18}O—P=^{18}O	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.

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]



[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.

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*)-α-^{17}O,α,α-$^{18}O_2$]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*)-α-^{17}O,α,α-$^{18}O_2$]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.

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.

Since this investigation was completed, the stereochemical course of activation of phenylalanine by yeast phenylalanyl-tRNA synthetase using adenosine 5′-*O*-[1(*S*)-thiotriphosphate] has been reported to occur with inversion of configuration (Connolly et al., 1984). It is gratifying to note that the stereochemical course followed by this unnatural analogue of ATP is the same as that reported in this paper with [(*S*)-α-^{17}O,α,α-$^{18}O_2$]ATP.

Registry No. Ap_4A, 5542-28-9; adenosine 5′-[(*S*)-α-^{17}O,$^{18}O_2$]-triphosphate, 88837-44-9; L-phenylalanine, 63-91-2; phenylalanyl-tRNA synthetase, 9055-66-7; ATP, 56-65-5.

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Ubiquitin-Dependent Proteolysis of Native and Alkylated Bovine Serum Albumin: Effects of Protein Structure and ATP Concentration on Selectivity†

Anthony C. Evans, Jr., and Keith D. Wilkinson*
Department of Biochemistry, Emory University School of Medicine, Atlanta, Georgia 30322
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ABSTRACT: The susceptibility of bovine serum albumin to degradation by the ubiquitin-dependent system of proteolysis depends on the severity of the iodination conditions [Wilkinson, K. D., & Audhya, T. K. (1981) *J. Biol. Chem. 256*, 9235–9241]. To evaluate if other modifications of the protein changed its susceptibility to degradation, chemically modified derivatives of bovine serum albumin have been synthesized, characterized, and tested as substrates for the ubiquitin-dependent system. Serum albumin was reduced or reduced and alkylated with iodoacetic acid or iodoacetamide. Only the alkylated derivatives exhibit saturation kinetics. Both alkylated proteins competitively inhibit the degradation of the other. These substrates are useful for assay of the intact proteolysis system in crude extracts and in assays for other substrates using competitive alternate substrate inhibition. The physical properties of these proteins suggest that charge, denaturation, or aggregation is not correlated with the degradation rate of these proteins by this system. However, the selectivity of the ubiquitin-dependent proteolysis depends strongly on the ATP concentration. At saturating substrate concentrations, both alkylated substrates are degraded equally. At low ATP concentrations, there is a 2.4-fold difference in the degradation rates of the alkylated proteins. The results presented here indicate that the ubiquitin-dependent protein degradation system is selective and responsive to ATP concentrations and that not all abnormal proteins are equally preferred substrates. Thus, the system may be more selective than previously thought.

The rate of cellular protein turnover varies markedly, depending upon the protein being degraded. The highly selective nature of this process is presumed to arise from the cell's recognition of certain physical and chemical properties of each particular protein (Goldberg & Dice, 1974; Goldberg & St. John, 1976; Hershko & Ciechanover, 1982). For instance, large proteins generally possess shorter half-lives than small ones (Dehlinger & Schimke, 1972; Glass & Doyle, 1972; Dice et al., 1973; Dice & Goldberg, 1975). Also acidic polypeptides are degraded faster than neutral or basic ones (Dice & Goldberg, 1975; Dice et al., 1979; Duncan et al., 1980), indicating that the isoelectric point is related to the half-lives of cellular proteins. Moreover, several proteins which are turned over rapidly in vivo show an increased susceptibility to proteolytic attack by neutral or acidic proteases in vitro (Dice et al., 1973; Goldberg, 1972; Bohley et al., 1972; Segal et al., 1974; Dean, 1975a). Hydrophobicity has also been cited as a property of proteins which leads to an increased rate of breakdown (Dean, 1975b; Segal et al., 1976; Bohley & Reimann, 1977).

Although the distinct pathways by which various classes of proteins are degraded in vivo are not clear, available evidence indicates that, in general, degradation of "long-lived" proteins occurs by lysosomal autophagy, especially in times of nutritional deprivation (Mortimore et al., 1978). In contrast, abnormal proteins produced by the incorporation of amino acid analogues or puromycin are degraded with extreme rapidity by cells, mainly through energy-requiring nonlysosomal systems (Mortimore et al., 1978; Knowles & Ballard, 1976; Neff et al., 1979). Furthermore, it has been shown that most, if not all, cells require ATP for sustained protein catabolism. This energy requirement does not appear to be related to lysosomal integrity, since even cells without lysosomes exhibit the ATP requirement (Goldberg & St. John, 1976). Since proeolysis is an exergonic process, this energy requirement is suspected to impart selectivity to the proteolytic process.

One energy-dependent protein degradation system, which degrades abnormal globins in cell-free extracts of rabbit reticulocytes, has been shown to require ATP (Etlinger & Goldberg, 1977) and a small (8500 daltons) polypeptide cofactor (Ciechanover et al., 1978; Hershko et al., 1979; Ciechanover et al., 1980) identified as ubiquitin (Wilkinson et al., 1980; Wilkinson & Audhya, 1981). In this system, ubiquitin is coupled to the ϵ-amino groups of lysine residues in the substrate protein via an ATP-coupled condensation reaction requiring no less than three separate proteins (Ciechanover et al., 1981; Haas et al., 1982; Haas & Rose, 1982; Hershko et al., 1983). It is presumed that soluble proteases recognize these conjugates and degrade the attached protein (Hershko

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