Direct Mass Spectroscopic Method for Determination of Oxygen Isotope Position in Adenosine 5′-*O*-(1-Thiotriphosphate). Determination of the Stereochemical Course of the Yeast Phenylalanyl-tRNA Synthetase Reaction†

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ABSTRACT: Negative ion fast atom bombardment mass spectrometry has been used to distinguish between (S_p)-adenosine 5′-*O*-(1-thiotriphosphate) containing either an α-nonbridging or an α–β-bridging ^{18}O label. The method does not require any nucleotide derivatization and so avoids the excessive manipulations and purifications necessary to distinguish between the above two species using conventional mass spectroscopy. Furthermore, it is between 50 and 200 times more sensitive than other direct methods based on ^{31}P nuclear magnetic resonance spectroscopy. Routinely, 100 nmol of nucleoside phosphorothioate is ample to establish the ^{18}O isotope position by normal as well as linked scan mass spectrometry. In cases where normal mass spectrometry is considered adequate, 10 nmol of material suffices. This technique should be useful in determining the stereochemical course of enzymatic nucleotidyl transfer and nuclease-catalyzed hydrolysis reactions under conditions of limiting availability of enzyme or substrate. Yeast phenylalanyl-tRNA synthetase was used to prepare the ^{18}O-labeled adenosine 5′-*O*-(1-thiotriphosphate) species, and this enzyme was concomitantly shown to catalyze adenylyl transfer with inversion of configuration at phosphorus.

The elucidation of the stereochemical course of enzyme-catalyzed phosphoryl and adenylyl transfer has attracted the attention of a number of research groups in recent years [see reviews by Knowles (1980), Buchwald et al. (1982), Eckstein et al. (1982), Frey (1982), Frey et al. (1982), and Eckstein (1983)]. A necessary requirement for such studies is that the phosphoryl or adenylyl group enzymatically transferred be made chiral and be of known configuration. This can be achieved by the use of sulfur as an oxygen substitute, by the use of the stable isotopes of oxygen (^{17}O and ^{18}O), or by a combination of both methods. A further requisite is that the chiral phosphate containing product can be analyzed so as to determine its absolute configuration and hence give the stereochemistry of the enzyme-catalyzed reaction. Two general methods based on mass spectrometry and ^{31}P NMR are available for such an analysis. Although the mass spectrometric method has the virtue of sensitivity a great deal of further enzymatic and chemical manipulations are usually necessary both to separate the sulfur and oxygen isotope labels and to produce volatile derivatives necessary for the mass spectrometer. The ^{31}P NMR method [see a review by Tsai (1982)] is based on the observations that when ^{18}O is bonded to phosphorus, an upfield shift in the ^{31}P resonance is seen that correlates with the double bond character of the P–O bond (Cohn & Hu, 1980; Lowe et al., 1979) and that ^{17}O bonded to phosphorus causes the ^{31}P signal to be broadened beyond detection (Tsai, 1979; Lowe et al., 1979). Although the NMR method is less sensitive than the mass spectrometric method, it is more direct and much less derivatization of the initial chiral product is necessary to produce a sample suitable for measurement. This fact has made the NMR approach the method of choice in recent years.

This group has been interested in the stereochemical outcomes of the reactions catalyzed by both adenylyl transferases and nucleases. The approach we have usually used has been to utilize a chiral phosphorothioate and enzymatically introduce ^{18}O into the initial chiral product (Eckstein, 1983).

Very often [^{18}O]AMPS[1] (or the deoxy derivative) is the product of such a study. Determination of the absolute configuration of this [^{18}O]AMPS then gives the stereochemical course of the enzyme-catalyzed reaction. This configuration can be elucidated by ^{31}P NMR after stereospecific phosphorylation of the *pro-S* oxygen atom of the [^{18}O]AMPS using myokinase and pyruvate kinase (Sheu & Frey, 1977; Jaffe & Cohn, 1978). Thus S_p-[^{18}O]AMPS gives S_p-[^{18}O]ATPαS containing ^{18}O only in the α-nonbridging position whereas R_p-[^{18}O]AMPS yields S_p-[^{18}O]ATPαS containing ^{18}O in the α–β bridging position. These two possibilities can be distinguished with ^{31}P NMR spectroscopy by observing whether an upfield shift can be seen at only the α-phosphorus resonance or at both the α- and β-phosphorus resonances when referenced to unlabeled S_p-ATPαS. However, the sensitivity of the NMR approach requires that about 5 μmol of [^{18}O]AMPS is needed in order to obtain a reasonable spectrum.

In this paper we report that ATPαS species containing either an α-nonbridging ^{18}O or an α–β-bridging ^{18}O can be directly distinguished by fast atom bombardment mass spectrometry. This method combines elements of the sensitivity of the mass spectroscopic approach with the directness of the ^{31}P NMR method. In preparing the appropriately labeled [^{18}O]ATPαS derivatives, we have made use of yeast phenylalanyl-tRNA synthetase and the stereochemical course of this reaction is also reported.

Materials and Methods

Phenylalanyl-tRNA synthetase from baker's yeast was a gift from Dr. F. von der Haar and was prepared as described

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[1] Abbreviations: AMPS, adenosine 5′-*O*-phosphorothioate; S_p-ADPαS, the diastereomer of adenosine 5′-*O*-(1-thiodiphosphate) with the S_p configuration; S_p-ATPαS, the diastereomer of adenosine 5′-*O*-(1-thiotriphosphate) with the S_p configuration.

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by Schneider et al. (1972) and von der Haar (1973). Inorganic pyrophosphatase (yeast, 200 units/mg), hexokinase (yeast, 40 units/mg), pyruvate kinase (rabbit muscle, 200 units/mg), and adenylate kinase (rabbit muscle, 360 units/mg) were purchased from Boehringer Mannheim (West Germany). ^{18}O-Enriched water (98% isotopic enrichment) was obtained from Ventron (Karlsruhe, West Germany). The S_p isomer of ATPαS was synthesized enzymatically from AMPS (Sheu & Frey, 1977; Jaffe & Cohn, 1978). The S_p isomer of [α-^{18}O,α,β-^{18}O]ATPαS was prepared similarly from [^{18}O]AMPS as detailed by Richard & Frey (1978). Phenylalanine, isotopically enriched with ^{18}O, was prepared by acid-catalyzed oxygen exchange with $H_2{}^{18}O$. L-Phenylalanine (82.5 mg, 0.5 mmol) was dissolved in 500 μL of $H_2{}^{18}O$ and 15 μL of 100% H_2SO_4 was added. The mixture was heated at 100 °C, in a tightly stoppered tube, for 100 h. The [^{18}O]phenylalanine so produced was used, after neutralization to pH 7, without further purification. ^{31}P NMR of the [^{18}O]AMPS formed from S_p-ATPαS and this [^{18}O]phenylalanine by Phe-tRNA synthetase showed that the amino acid was 85% enriched in ^{18}O.

The purity of all the nucleotides used in this study and the course of enzyme-catalyzed reactions were routinely monitored by HPLC. A Waters Associates (Model 6000 A) liquid chromatograph fitted with a 254-nm detector (Model 440) was used. For the analysis a column (24 × 0.6 cm) packed with the reverse-phase material ODS-Hypersil (5-μm particle size, obtained from Shandon Southern Products, Ltd., Runcorn, England) and eluted with 100 mM triethylammonium acetate, pH 7, was utilized. Nucleotides were further characterized by ^{31}P NMR spectroscopy on a Bruker WP200SY with quadrature detection and ^{1}H broad band decoupling. In all cases positive δ values are chemical shifts downfield from the external standard, phosphoric acid (85%).

The mass spectra were recorded on a Kratos MS 50 S with a Kratos FAB source in the negative ion mode. The atom gun used xenon and produced a beam of neutral atoms at 8–9 kV. An aqueous solution of the triethylammonium salt of nucleotide (1–2 μL, containing approximately 20 nmol) was injected into the glycerol matrix (approximately 2 μL) present on the FAB copper probe tip. The water was removed in the direct insertion lock, and the spectra were recorded at a magnet scan rate of 300 s/decade in the linked scan B/E mode at a magnet scan rate of 100 s/decade. The resolution of the mass spectrometer for both normal and linked scan measurements was about 2000 (10% valley definition).

The stereochemical course of the reaction catalyzed by yeast phenylalanyl-tRNA synthetase was determined by using either S_p-[α-^{18}O,α,β-^{18}O]ATPαS and unlabeled phenylalanine or S_p-ATPαS and [^{18}O]phenylalanine. In both cases the reaction mixture contained, in a total volume of 10 mL, 100 mM Hepes buffer, pH 8, 200 mM KCl, 25 mM $MgCl_2$, 100 mM hydroxylamine hydrochloride (previously adjusted to pH 8 with KOH), 1 mM dithiothreitol, 10 mg of bovine serum albumin, 20 mM phosphoenolpyruvate, 10 mM S_p-ATPαS, and 40 mM phenylalanine. Inorganic pyrophosphatase (0.1 mg), pyruvate kinase (1 mg), and phenylalanyl-tRNA synthetase (1 mg) were added, and the mixture was left at room temperature for 10 h after which time HPLC showed complete conversion of S_p-ATPαS to AMPS. The mixture was applied to a column (20 × 2.5 cm) of DEAE-Sephadex A-25 and eluted with a linear gradient consisting of 800 mL each of 50 and 400 mM triethylammonium bicarbonate. The fractions that contained AMPS (which eluted at around 200 mM triethylammonium bicarbonate) were pooled and evaporated to dryness, and excess

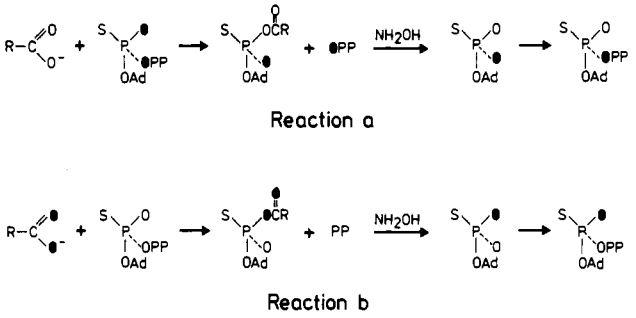

FIGURE 1: Reaction of phenylalanine with ATPαS: Ad, adenosine; PP, pyrophosphate; ●, oxygen-18.

triethylammonium bicarbonate was removed by repeated coevaporation from methanol. Typically 70 μmol (70%) of AMPS was obtained. This AMPS was converted to S_p-ATPαS with pyruvate kinase and adenylate kinase. The reactions were performed in 10 mL of 100 mM Tris, pH 8, containing 100 mM KCl, 10 mM $MgCl_2$, 10 mM phosphoenolpyruvate, 1 mM ATP, and 6 mM AMPS. Pyruvate kinase (0.5 mg) and adenylate kinase (2.5 mg) were added, and HPLC showed that the reaction was complete after 16 h. The S_p-ATPαS so produced was purified by ion-exchange chromatography over a column (20 × 2.5 cm) of DEAE-Sephadex A-25 eluted with a gradient consisting of 1 L each of 100 and 600 mM triethylammonium bicarbonate. The fractions that contained product (eluted at around 500 mM triethylammonium bicarbonate) were pooled and dried down as above. Usually 40 μmol (67%) of S_p-ATPαS was obtained.

Results

S_p-ATPαS is a reasonable substrate for yeast phenylalanyl-tRNA synthetase (von der Haar et al., 1977; Connolly et al., 1980) and so can be used to determine the stereochemical course of the reaction providing appropriate ^{18}O substitution is also used. This has been separately achieved by using either S_p-[α^{18}O,α,β-^{18}O]ATPαS together with unlabeled phenylalanine or [^{18}O]phenylalanine in the presence of unlabeled S_p-ATPαS. As is shown in Figure 1 phenylalanyl-tRNA synthetase catalyzes the formation of an adenylate between phenylalanine and the S_p-ATPαS, which is further cleaved with hydroxylamine to the appropriately ^{18}O-labeled AMPS. Inorganic pyrophosphatase must be added to the reaction mixtures to cleave the pyrophosphate formed and so drive the reversible adenylation reaction to completion. Additionally, pyruvate kinase and phosphoenolpyruvate were added to rephosphorylate any S_p-ADPαS formed by slow chemical hydrolysis of S_p-ATPαS. As the ^{18}O label was independently introduced from both S_p-[α-^{18}O,α,β-^{18}O]ATPαS (Figure 1, reaction a) and [^{18}O]phenylalanine (Figure 1, reaction b), the two [^{18}O]AMPS species shown in Figure 1 must necessarily be of opposite configurations regardless of the stereochemical course of the enzymatic reaction. The absolute configurations of both [^{18}O]AMPS's can be determined by ^{31}P NMR spectroscopy after phosphorylation to [^{18}O]ATPαS using pyruvate kinase and adenylate kinase. This method relies on the stereospecificity of adenylate kinase, which phosphorylates only the *pro-S* oxygen atom of prochiral AMPS yielding, after a further phosphorylation with pyruvate kinase, only S_p-ATPαS (Sheu & Frey, 1977; Jaffe & Cohn, 1978). Figure 2 shows the α- and β-phosphorus regions of the product S_p-[^{18}O]ATPαS mixed with an equivalent amount of unlabeled S_p-ATPαS. In this experiment the ^{18}O label was derived from S_p-[α-^{18}O,α,β-^{18}O]ATPαS (Figure 1, reaction a). The α-region consists of two sets of doublets separated by 1.9 Hz. The

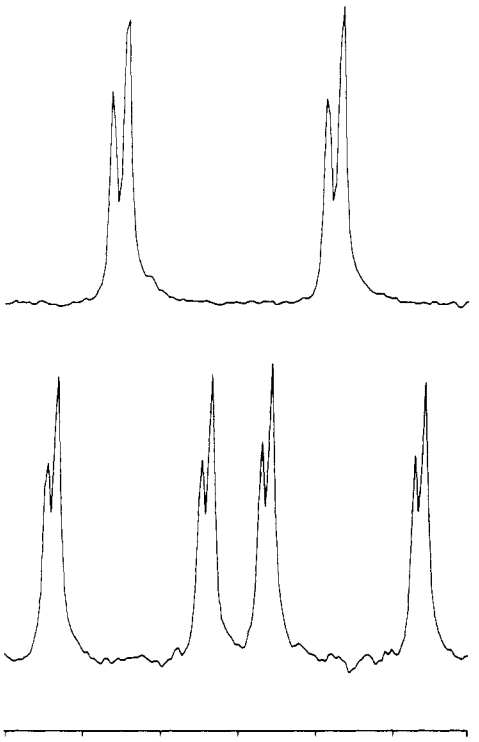

FIGURE 2: ^{31}P NMR spectrum of the α- and β-phosphorus region of S_p-[α-^{18}O,α,β-^{18}O]ATPαS in the presence of an equivalent amount of S_p-ATPαS. Upper spectrum: α-phosphorus region; the chemical shifts are 43.838, 43.815, 43.498, and 43.475 ppm. Lower spectrum: β-phosphorus region; the chemical shifts are −22.801, −22.817, −23.046, −23.062, −23.142, −23.157, −23.387, and −23.402 ppm. Amount of labeled nucleotide approximately 10 μmol; number of scans 1079; acquisition time 2.36 s; line broadening 0.43 Hz; pulse width 4.5 μs; sweep width 7042 Hz; data collection in 32K; scale 10 Hz/division.

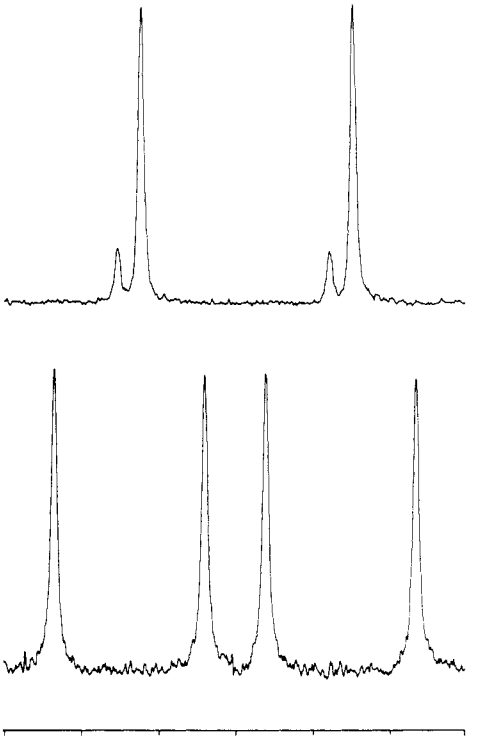

FIGURE 3: ^{31}P NMR spectrum of the α- and β-region of S_p-[α-^{18}O]ATPαS. Upper spectrum: α-phosphorus region; the chemical shifts are 43.847, 43.811, 43.507, and 43.472 ppm. Lower spectrum: β-phosphorus region; the chemical shifts are −22.641, −22.882, −22.980, and −23.221 ppm. Amount of material approximately 10 μmol of nucleotide; number of scans 341; acquisition time 16.38 s; line broadening 0.122 Hz; sweep width 500 Hz; data collection 8K with 8K zero filling; pulse width 7.0 μs; scale 10 Hz/division.

β-region contains four doublets each 1.3 Hz apart. The downfield signals in each pair are due to the unlabeled (i.e., all ^{16}O) S_p-ATPαS, whereas the upfield resonances are caused by the product S_p-[^{18}O]ATPαS. As both the α- and β-^{31}P resonances show upfield shifts, and the magnitude of the upfield shift is relatively small (1.3–1.9 Hz), typical for a single bond between P and ^{18}O, the S_p-[^{18}O]ATPαS derived from the Phe-tRNA synthetase reaction must be S_p-[α,β-^{18}O]-ATPαS in which the ^{18}O label is present only in the α–β bridge position. This assigns the absolute configuration of the starting [^{18}O]AMPS to R_p and means that the Phe-tRNA synthetase reaction proceeds with inversion of configuration. Figure 3 shows the α- and β-phosphorus regions of the S_p-[^{18}O]ATPαS produced when the ^{18}O label derives from phenylalanine (Figure 1, reaction b). In this case it was not necessary to add any unlabeled S_p-ATPαS as the phenylalanine was only 85% enriched in ^{18}O and the 15% ^{16}O-labeled amino acid gives rise to unlabeled S_p-ATPαS, which acts as an internal standard. The α-region consists of two doublets separated by 2.9 Hz, whereas only four single lines are visible in the β-region. As a relatively large upfield shift, characteristic of a double bond between P and ^{18}O, is present at only the α-phosphorus, the S_p-[^{18}O]ATPαS product must be S_p-[α-^{18}O]ATPαS containing ^{18}O in only the α-nonbridging position. This means that the [^{18}O]AMPS had the S_p configuration and once again shows that the yeast Phe-tRNA synthetase proceeds with inversion of configuration at phosphorus.

The S_p-[α-^{18}O]ATPαS and S_p-[α,β-^{18}O]ATPαS produced by the Phe-tRNA synthetase reaction can also be directly distinguished by negative-ion FAB mass spectroscopy.

The negative-ion FAB mass spectroscopy of ATPαS, S_p-[α,β-^{18}O]ATPαS and S_p-[α-^{18}O]ATPαS shows besides the

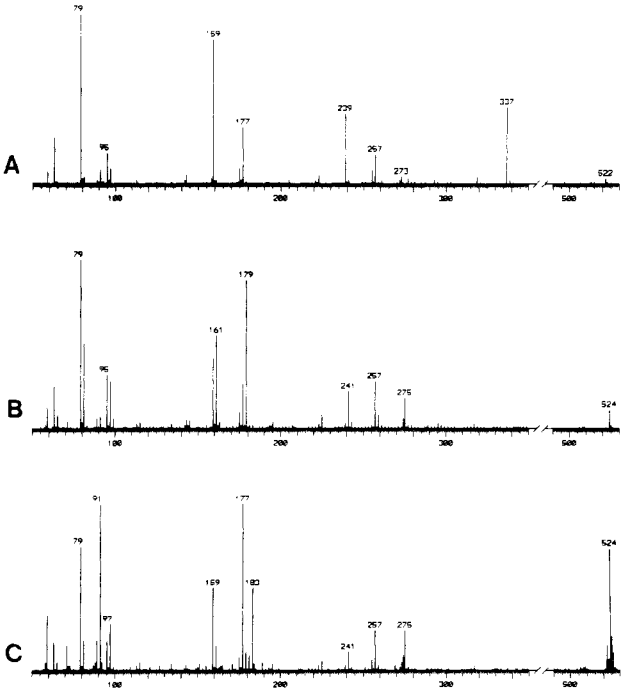

FIGURE 4: Negative ion FAB mass spectra of ATPαS, S_p-[α,β-^{18}O]ATPαS, and [α-^{18}O]ATPαS. (A) ATPαS; (B) S_p-[α,β-^{18}O]-ATPαS; (C) S_p-[α-^{18}O]ATPαS. The region between 350 and 500 d has been left out since no signals are present there.

deprotonated molecular ion at 522 d (unlabeled) and 524 d (^{18}O labeled) characteristic mono-, di-, and triphosphate sequence ions (97, 117, and 273 d for ATPαS; 97, 179, and 275 d for S_p-[α,β-^{18}O]ATPαS; 97, 177, and 275 d for S_p-[α-^{18}O]ATPαS) (Figure 4).

Table I: Results of Linked Scan Mass Spectra of Tri- and Diphosphate Sequence Ions[a]

nucleotide	start signal of triphosphate sequence ion	transition signals	start signal of diphosphate sequence ion	transition signals
S_p-ATPαS[b]	273	225 ($-H_2O$) 239 ($-H_2S$)	177	159 ($-H_2O$)
S_p-[α-^{18}O]ATPαS[b]	275	257 ($-H_2O$) 255 ($-H_2{}^{18}O$) 241 ($-H_2S$)	177	159 ($-H_2O$)
S_p-[α,β-^{18}O]ATPαS[c]	275	257 ($-H_2O$) 241 ($-H_2S$) 177 ($-PO_4H_3$) 175 ($-PO_3{}^{18}OH_3$) 161 ($-PO_3SH_3$) 159 ($-PO_2{}^{18}OSH_3$)	179	161 ($-H_2O$) 159 ($-H_2{}^{18}O$) 97 ($-PO_3H_3$) 95 ($-PO_2{}^{18}OH_3$) 81 ($-PO_4H_3$) 79 ($-PO_3{}^{18}OH_3$)
	start signal of monophosphate sequence ion			
	97	95 (−2H) 79 ($-H_2O$)		

[a] All masses are given in daltons. [b] Only the important masses are given. [c] The data are taken from the spectra reproduced in Figure 5.

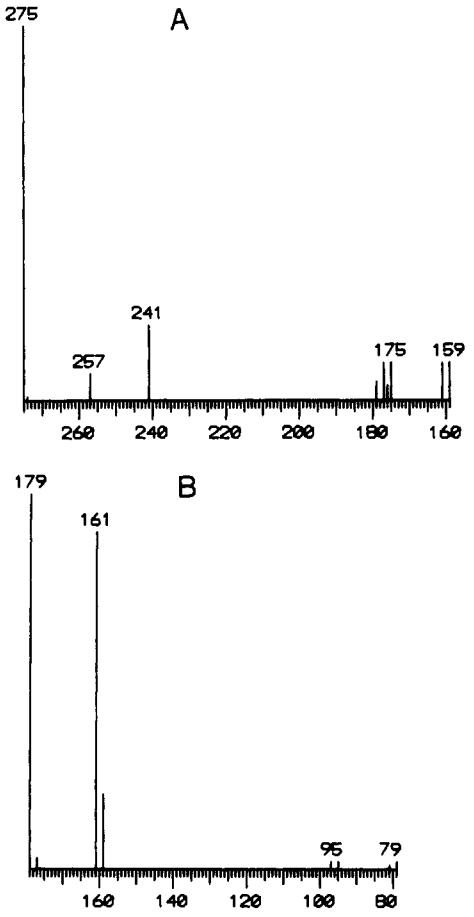

FIGURE 5: Linked scan B/E spectra of the tri- and diphosphate sequence ions of S_p-[α,β-^{18}O]ATPαS. (A) Spectrum of mass 275. The vertical scale is multiplied by a factor of 10 from mass 274 and 100 from mass 179. (B) Spectrum of mass 179. The vertical scale is multiplied by a factor of 100 from mass 177. The origins of the various peaks are explained in Table I (lower entry).

The mass spectrum of ATPαS shows an impurity at 337 d that, however, does not interfere with the interpretation of the spectrum. The matrix ions at 91, 183, and 275 d are largely suppressed in the spectra of ATPαS and [α,β-^{18}O]-ATPαS but are clearly visible in the spectrum of [α-^{18}O]-ATPαS. At the resolution of 2000 used in all these measurements reported here, the sequence ion at 275 d could be

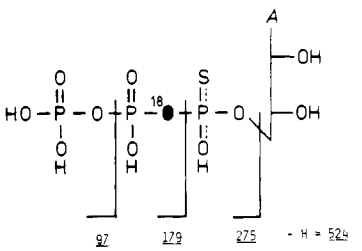

FIGURE 6: Breakdown of S_p-[α,β-^{18}O]ATPαS into tri-, di-, and monophosphate sequence ions.

clearly distinguished from the matrix ion at 275 d. The intensity of the matrix ions is very dependent on the exact pH and buffer conditions of the applied sample. About 15% of [α-^{18}O]ATPαS are not labeled by ^{18}O.

Linked scan measurements were performed on the deprotonated molecular ion as well as the tri-, di-, and monophosphate sequence ions. Of these the most important in determining the ^{18}O isotope position are those of the tri- and diphosphate sequence ions, which are represented for [α,β-^{18}O]ATPαS as an example in Figure 5. The origin of the sequence ions is shown schematically in Figure 6. For the measurement of [α-^{18}O]ATPαS glycerol-d_5 was used as the matrix because the matrix ion of glycerol at 275 d could not be completely suppressed. The results of the linked scan measurements are summarized in Table I. A more detailed interpretation of the mass spectrometry data is given under Discussion.

Discussion

Using S_p-ATPαS together with appropriate ^{18}O labeling, we have shown that the yeast Phe-tRNA synthetase catalyzed reaction proceeds with inversion of configuration at the α-phosphorus atom. This is no great surprise as all the tRNA synthetase (Langdon & Lowe, 1979; Lowe et al., 1983a,b) as well as most of the nucleotidyltransferase-catalyzed reactions so far determined [see a review by Eckstein (1983) for an extensive list] also proceed with inversion of configuration. This result is most simply interpreted by assuming a single nucleophilic substitution reaction by an in-line mechanism with a trigonal-bipyramidal transition state for the reaction catalyzed by this enzyme. This result eliminates a covalent enzyme–adenylyl species as a kinetically competent intermediate although some doubts have been raised (Mehdi & Gerlt, 1982)

as to whether the existence of a covalent intermediate involving a carboxyl group of the enzyme will always lead to overall retention of configuration as has been observed for the sarcoplasmatic reticulum ATPase (Webb & Trentham, 1981). However, the elucidation of the Phe-tRNA synthetase reaction stereochemistry described here serves mainly as an example for the demonstration that fast atom bombardment mass spectrometry is a very sensitive, yet simple, method to determine such stereochemical reaction courses.

As outlined in the introduction and also briefly under Results, the evaluation of the stereochemical course of the reactions catalyzed by nucleotidyltransferases and nucleases, using phosphorothioates, requires the determination of the absolute configuration of [^{18}O]AMPS. After enzymatic and stereospecific phosphorylation of this [^{18}O]AMPS to S_p-ATPαS, the problem reduces to distinguishing between ATPαS containing an α-nonbridging ^{18}O and ATPαS containing an α–β-bridging ^{18}O. These two possibilities can be distinguished by ^{31}P NMR as demonstrated in this paper and many other publications. Although the NMR method is direct and gives an answer by observation of the actual ATPαS produced, it is rather insensitive, requiring micromole quantities of material. Usually we attempt to produce 5 μmol of the appropriately labeled ATPαS and feel confident of obtaining reasonably good spectra with this quantity. Mass spectrometry offers a way of increasing the sensitivity of the above determination, but with this method the ^{18}O-labeled ATPαS cannot be studied directly but must be further derivatized. Midelfort & Sarton-Miller (1978) have used mass spectrometry in a study of the stereochemical mechanism of acetyl-CoA synthetase. The ^{18}O-labeled ATPαS obtained was further treated with lysyl-tRNA synthetase in order to separate the α-nonbridging oxygens into AMPS and the α–β-bridging oxygen into pyrophosphate, which was simultaneously degraded to phosphate by contaminating inorganic pyrophosphatase. After chromatographic separation the phosphate was converted to trimethyl phosphate with diazomethane and then subjected to mass spectrometry. The AMPS was further degraded to phosphate by using sodium periodate followed by base treatment and, after chromatographic purification, methylated and analyzed as above. In a more general method to determine the ^{18}O position in phosphorothioates, Richard et al. (1978) degraded the nucleoside triphosphorothioate to tripolyphosphorothioate using sodium periodate followed by base. The tripolyphosphorothioate was then reacted with diazomethane to give the permethylated derivative. This was hydrolyzed in water at 100 °C and the products were further methylated to give trimethyl phosphate and *O,O,S*-trimethyl phosphorothioate. These two species were subjected to mass spectrometry, and the distribution of the ^{18}O between them revealed its position in the original nucleotide.

It occurred to us that fast atom bombardment mass spectroscopy, in which the nucleotide is subjected to mass spectrometry without derivatization, could offer both the ease and directness of the NMR method and the sensitivity associated with mass spectrometry. That this mass spectrometric method is suitable for underivatized nucleotides has been shown by its recent application to ATP (Barber et al., 1981) and to oligonucleotides up to 10 monomers long (Grotjahn et al., 1982). Using the S_p-[α-^{18}O]ATPαS and the S_p-[α,β-^{18}O]-ATPαS produced by Phe-tRNA synthetase and characterized by ^{31}P NMR, we show here that fast atom bombardment mass spectrometry can indeed distinguish between bridging and nonbridging ^{18}O atoms in nucleoside phosphorothioates. The negative-ion FAB mass spectrum of ATPαS shows a molecular ion peak at 522 d whereas that of both [α,β-^{18}O]ATPαS and [α-^{18}O]ATPαS gives a molecular ion peak at 524 d, clearly indicating the presence of one ^{18}O label (Figure 4). The peak at 522 d for [α-^{18}O]ATPαS is due to the 15% unlabeled ATPαS present in this sample. To distinguish between the two ^{18}O-labeled ATPαS species, the diphosphate sequence ion, arising from cleavage of the P_α–$O_{\alpha,\beta}$ bond, is of most interest. The breaking of this bond efficiently separates oxygen atoms in the α-nonbridging and the α–β-bridging position. This ion has a mass of 177 d for unlabeled ATPαS and [α-^{18}O]ATPαS whereas it occurs at 179 d for [α,β-^{18}O]ATPαS. This simple analysis is somewhat complicated by the presence of extra masses in the region of the diphosphate ion. Thus, the spectrum of [α,β-^{18}O]ATPαS shows a peak at 177 d and that of [α-^{18}O]ATPαS also shows a resonance at 179 d. However, these side peaks are smaller than those of the appropriate diphosphate sequence ions that comprise the major signals in this area. If it is assumed that Phe-tRNA synthetase catalyzes displacement at phosphorus with either 100% inversion or retention of configuration, then observation of the diphosphate ion peak is enough to give the stereochemical reaction course. Should the major signal occur at 177 d, then the ^{18}O label is bridging, whereas if this signal is at 179 d, then the ^{18}O label is nonbridging. This information gives the configuration of the starting [^{18}O]AMPS and hence the stereochemical mechanism of the enzyme reaction. The assumption of a clean enzymatic stereochemical outcome is necessary because it means that the small extra mass peaks (i.e., at 179 d when 177 d is the major signal and at 177 d when 179 d predominates) can be ignored as not being caused by a degree of racemization in the enzyme reaction. As no enzyme, of the 50 or so thus far studied, has been found to cause any racemization at phosphorus during catalysis [see Eckstein (1983) for a complete listing], this assumption would seem to be valid. This simple FAB mass spectroscopic method requires about 10 nmol of substance. The stereochemical analysis can be further refined by observing the linked scan FAB mass spectra of the triphosphate sequence ion (derived from cleavage of the bond between the sugar C5′ atom and the oxygen bridging C5′ and P) and the diphosphate sequence ion. Daughter ions are generated from these two species by loss of H_2O in which the lost oxygen atom is derived from nonbridging but not from bridging positions. When sulfur is present in the sequence ion, H_2S is also lost. All these possibilities are illustrated in Figure 5 for [α,β-^{18}O]ATPαS. The triphosphate sequence ion (275 d) loses H_2O to give a peak at 257 d and H_2S to give a peak at 241 d. This ion contains ^{18}O only in a bridging position, and thus $H_2{}^{18}O$ loss, which would give a peak at 255 d, is not observed. The diphosphate sequence ion (179 d) contains ^{18}O in a nonbridging position and so loses both H_2O and $H_2{}^{18}O$, giving signals at 161 and 159 d, respectively. No sulfur is present in this ion and therefore no peak at 145, corresponding to H_2S loss, is observed. Table I shows that [α,β-^{18}O]ATPαS and [α-^{18}O]ATPαS can be unequivocally distinguished by the linked scan approach. Thus, [α,β-^{18}O]ATPαS loses $H_2{}^{18}O$ from the diphosphate but not the triphosphate sequence ion, whereas the situation is reversed with [α-^{18}O]ATPαS with $H_2{}^{18}O$ loss from the tri- but not the diphosphate ion. This approach requires about 100 nmol of nucleotide, compared with the 10 nmol needed for the simple FAB mass spectrum. However, it is not dependent on any assumptions about the stereochemistry of the enzyme-catalyzed reaction as is the direct FAB method.

This method of analysis seems to open the way to study the stereochemical courses of enzyme reactions where the amount

of product is expected to be too low for analysis by ^{31}P NMR spectroscopy. There are a number of cases where this applies. One of them is the determination of the stereochemistry of restriction endonuclease catalyzed hydrolysis where enzyme and substrate in the form of an oligonucleotide containing a phosphorothioate at the cleavage site are not available in large quantities. Another system is the aminoacyl synthetases (Schimmel & Söll, 1979), which do not catalyze pyrophosphate exchange unless tRNA is present such as glutamate (Ravel et al., 1965), glutamine (Folk, 1971), and arginine (Mehler & Mitra, 1967) synthetases. Here the amount of tRNA is limiting. Yet other examples are those where single turnover experiments are necessary. As has been discussed for the nuclease from *Staphylococcus aureus* (Mehdi & Gerlt, 1982), this seems to be important for enzymes where a carboxyl group at the active site could possibly be involved in the formation of a covalent enzyme intermediate.

Although not explicitly investigated in the context of this study, it seems likely that FAB mass spectroscopy can distinguish between β-nonbridging and β–γ-bridging oxygen atoms in ATP. We have obtained spectra (data not shown) of S_p-[α-^{18}O]ADPαS and S_p-[α,β-^{18}O]ADPαS derived from their respective trinucleotides by hexokinase-catalyzed phosphoryl transfer. It is again possible to distinguish oxygen isotope position in these nucleoside thiodiphosphates. By use of normal FAB mass spectroscopy the predominant phosphate sequence ion occurs at 97 d for [α-^{18}O]ADPαS and 99 d for [α,β-^{18}O]ADPαS. The linked scan spectra of [α-^{18}O]ADPαS shows $H_2{}^{18}O$ loss from the diphosphate but not the monophosphate sequence ion, whereas that of [α,β-^{18}O]ADPαS shows $H_2{}^{18}O$ loss only from the diphosphate ion. If, as seems likely, [α-^{18}O]ADPαS is a model for [β-^{18}O]ATP and [α,β-^{18}O]ADPαS a model for [β,γ-^{18}O]ATP, then it is probable that FAB mass spectroscopy can also distinguish between these two nucleoside triphosphates. Thus, this method should be useful in the detection of bridge to nonbridge oxygen isotope scrambling in ATP, a powerful approach for the elucidation of phosphoryl transfer mechanisms (Midelfort & Rose, 1976).

In summary, we have shown that FAB mass spectrometry is capable of distinguishing between the oxygen isotope positions in nucleoside triphosphates at least for the case of S_p-[α-^{18}O]ATPαS and S_p-[α,β-^{18}O]ATPαS. At present we estimate this technique to be some 50–200 times more sensitive than ^{31}P NMR, the only other equally direct method. We have based these numbers on the amounts of material with which we feel confident of obtaining publishable data (5 μmol for ^{31}P NMR and 100 nmol for routine and linked scan FAB-MS) and on what we consider to be the limits of both techniques (2 μmol for ^{31}P NMR and 10 nmol for routine FAB-MS without linked scan). Additionally, as ^{31}P NMR is well established in determining ^{18}O isotope position whereas this is the first use of FAB-MS for this purpose, we feel that, if anything, the sensitivity advantage of the mass spectrometric method will increase relative to that of the NMR approach. Hopefully this advantage will be put to good use tackling some of the outstanding problems detailed in this discussion.

Registry No. S_p-[α-^{18}O]ATPαS, 72237-24-2; S_p-[α,β-^{18}O]ATPαS, 72237-23-1; S_p-[α-^{18}O,α,β-^{18}O]ATPαS, 88837-46-1; S_p-ATPαS, 58976-48-0; Phe, 63-91-2; Phe-tRNA synthetase, 9055-66-7; ^{18}O, 14797-71-8.

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