Measurement of Positional Isotope Exchange Rates in Enzyme-Catalyzed Reactions by Fast Atom Bombardment Mass Spectrometry: Application to Argininosuccinate Synthetase†

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Received March 25, 1985

ABSTRACT: Fast atom bombardment mass spectrometry (FAB-MS) has been used to measure positional isotope exchange rates in enzyme-catalyzed reactions. The technique has been applied to the reactions catalyzed by acetyl-CoA synthetase and argininosuccinate synthetase. The FAB technique is also able to quantitatively determine the oxygen-18 or oxygen-17 content of nucleotides on as little as 10 nmol of material with no prior derivatization. Acetyl-CoA synthetase has been shown by FAB-MS to catalyze the positional exchange of an oxygen-18 of ATP from the β-nonbridge position to the $\alpha\beta$-bridge position in the presence of acetate. These results are consistent with acetyl adenylate as a reactive intermediate in this reaction. Argininosuccinate synthetase was shown not to catalyze a positional isotope exchange reaction designed to test for the formation of citrulline adenylate as a reactive intermediate. Argininosuccinate synthetase was also found not to catalyze the transfer of oxygen-18 from [*ureido*-^{18}O]citrulline to the α-phosphorus of ATP in the absence of added aspartate. This experiment was designed to test for the transient formation of a carbodiimide as a reactive intermediate. These results suggest that either argininosuccinate synthetase does not catalyze the formation of citrulline adenylate or the enzyme is able to completely suppress the rotation of the phosphoryl groups of PP_i.

The positional isotope exchange (PIX)[1] technique, first developed by Midelfort & Rose (1976), has proven to be a valuable tool in elucidating the mechanistic pathways of many enzyme-catalyzed reactions (Wimmer et al., 1979; Raushel & Villafranca, 1980; Raushel & Garrard, 1984; DeBrosse & Villafranca, 1983). The general approach involves the measurement of the scrambling of isotopically labeled substituents in a substrate molecule which become torsionally equivalent via a reaction intermediate or product formation. The kinetic competence of a proposed reaction intermediate can thus be verified if the rearrangement can be demonstrated to occur at some reasonable rate. Furthermore, the PIX technique can be employed in a time course study to calculate the individual rate constants or ratio of rate constants in the reaction mechanism (Raushel & Villafranca, 1980; Raushel & Garrard, 1984).

The PIX technique has often been used with enzyme-catalyzed reactions involving phosphoryl transfer. Detection of the rearrangement in these cases has involved either mass spectrometry (Midelfort & Rose, 1976) of a derivatized form of the product or ^{31}P NMR (Cohn & Hu, 1978). Mass spectrometry can be performed with a small sample size and offers excellent sensitivity. It has suffered from the need to prepare a volatile derivative of the labeled compound. On the other hand, ^{31}P NMR offers a direct analysis method since Cohn & Hu (1978) have shown that the substitution of ^{18}O for ^{16}O in phosphate esters induces an $\sim$0.02 ppm upfield chemical shift in the phosphorus resonance. The weakness of this method is the reduced sensitivity compared to mass spectrometry.

Recently, Connolly et al. (1984), studying the stereochemical course of enzyme nucleotidyl transfer, used fast atom bombardment mass spectrometry (FAB-MS) to detect the presence of an α-nonbridging ^{18}O and an $\alpha\beta$-bridging ^{18}O in a sample of ATPαS. This method accomplishes the directness of the ^{31}P NMR method while obtaining the sensitivity of mass spectrometry since phosphate esters can be analyzed without derivatization by FAB techniques.

In this report we have applied the FAB-MS analysis to PIX studies of the acetyl-CoA synthetase reaction and the argininosuccinate synthetase reaction. It has previously been shown that the acetyl-CoA synthetase reaction involves the formation of an acetyl adenylate intermediate with the release of PP_i from the enzyme (Berg, 1955; Boyer et al., 1956). The partial reverse reaction that forms this intermediate from acetate and ATP (in the absence of CoA) allows positional isotope exchange within the [β,γ-$^{18}O_6$]ATP molecule. We have demonstrated that FAB-MS can be used to detect this exchange reaction and monitor its rate over an extended incubation period.

The argininosuccinate synthetase reaction has been postulated by Ratner (1973) to involve the formation of a citrulline adenylate intermediate from ATP and citrulline in the absence of aspartate. The data are consistent with this proposal but other mechanisms cannot be rigorously excluded. This enzyme therefore provides an excellent test for the use of the PIX technique and FAB-MS for the positive identification of citrulline adenylate.

Materials and Methods

Acetyl-CoA synthetase, hexokinase, and myokinase were purchased from Sigma Chemical Co. Argininosuccinate

† This work was supported by the National Institutes of Health (AM-30343 and GM-33780) and the Robert A. Welch Foundation (A-840). F.M.R. is the recipient of NIH Research Career Development Award AM-01366.

[1] Abbreviations: PIX, positional isotope exchange; ATPαS, adenosine 5′-*O*-(1-thiotriphosphate); FAB-MS, fast atom bombardment mass spectrometry; CoA, coenzyme A; HEPES, *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid; PEP, phosphoenolpyruvate; Tris, tris(hydroxymethyl)aminomethane; ATPase, adenosinetriphosphatase.

0006-2960/85/0424-5888$01.50/0

synthetase was isolated from beef liver according to a slightly modified method described by Rochovansky et al. (1977). Carbamoyl-phosphate synthetase from *Escherichia coli* was a generous gift from Paul M. Anderson. [β,γ-$^{18}O_6$]ATP (II) was made according to the procedure of Cohn & Hu (1980). The $KH_2P^{18}O_4$ used in the synthesis of [β,γ-$^{18}O_6$]ATP was prepared following the procedure of Risley & Van Etten (1978). All of the other chemicals used were purchased from either Sigma or Fisher.

Synthesis of [ureido-^{18}O]Citrulline. Oxygen-18-labeled citrulline was synthesized from ornithine, glutamine, and bicarbonate in a reaction catalyzed by carbamoyl-phosphate synthetase and ornithine transcarbamylase. The reaction mixture contained 50 mM HEPES buffer, pH 7.5, 25 mM glutamine, 25 mM bicarbonate, 2.0 mM ATP, 10 mM Mg^{2+}, 25 mM ornithine, 50 mM PEP, and 49% $H_2{}^{18}O$ in a total volume of 2.0 mL. The mixture was incubated for 5 h at room temperature to allow for equilibration between the bicarbonate and the labeled water. Sixty units of ornithine transcarbamylase, 5 units of pyruvate kinase, and 23 units of carbamoyl-phosphate synthetase were added and allowed to react for an additional 21 h. The reaction was then quenched by centrifugation through an Amicon CF25 Centriflo ultrafiltration membrane cone, and the citrulline was used without further purification. Twenty-five micromoles of citrulline (Ceriotti, 1970) was recovered with an enrichment of oxygen-18 at the ureido position of 43%. The incorporation of oxygen-18 at the ureido position was estimated by ^{31}P NMR (Cohn & Hu, 1978) from the incorporation of oxygen-18 in the phosphate produced during the synthesis of carbamoyl phosphate (Wimmer et al., 1979).

Positional Isotope Exchange with Acetyl-CoA Synthetase. Two units of acetyl-CoA synthetase was incubated with 50 mM HEPES, pH 7.5, 5 mM acetate, 2.5 mM [β,γ-$^{18}O_6$]ATP, and 0.25 mM PP_i in a volume of 5.0 mL. Ten such samples were allowed to incubate at room temperature for times ranging from 0 to 24 h. The reactions were quenched with 5 units of hexokinase, 25 units of myokinase, and 11 mg of glucose. The AMP that was produced by the enzymatic cleavage of the γ- and β-phosphoryl groups of ATP was purified on a column of DEAE-cellulose (1 × 24 cm) with a 10–150 mM gradient of triethylamine–bicarbonate buffer, pH 7.6. Appropriate fractions were dried to a solid residue and washed twice with 10 mL of methanol. Each sample was then redissolved in 100 μL of water and analyzed by FAB-MS.

Positional Isotope Exchange with Argininosuccinate Synthetase. Twenty-four units of argininosuccinate synthetase was incubated in three separate samples, each totaling 16 mL. The first was composed of 50 mM Tris buffer, pH 8.0, 10 mM $MgCl_2$, 1.0 mM [$\beta\gamma$-$^{18}O_6$]ATP, and 1.0 mM citrulline. The second sample also contained 1.0 mM succinate. The third incubation sample was composed of 50 mM Tris buffer, pH 8.0, 10 mM $MgCl_2$, 0.5 mM ATP, and 0.5 mM [*ureido*-^{18}O]citrulline. After being incubated for 24 h at room temperature, the reaction mixtures were quenched with 5 units of hexokinase, 25 units of myokinase, and 35 mg of glucose. The AMP was then purified by chromatography on a column of DEAE-cellulose and analyzed by FAB-MS and ^{31}P NMR. The enzyme lost no activity after 24 h of incubation.

^{31}P NMR Measurements. The ^{31}P NMR spectra were obtained with a Varian XL-200 multinuclear spectrometer operating at a frequency of 81.0 MHz. Acquisition parameters included a 1000-Hz sweep width, 5.0-s acquisition time, 1.5-s delay between pulses, 7-μs pulse width, and broad-band proton decoupling.

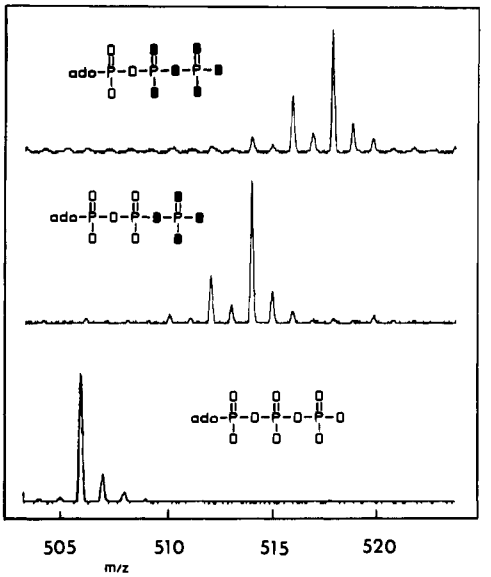


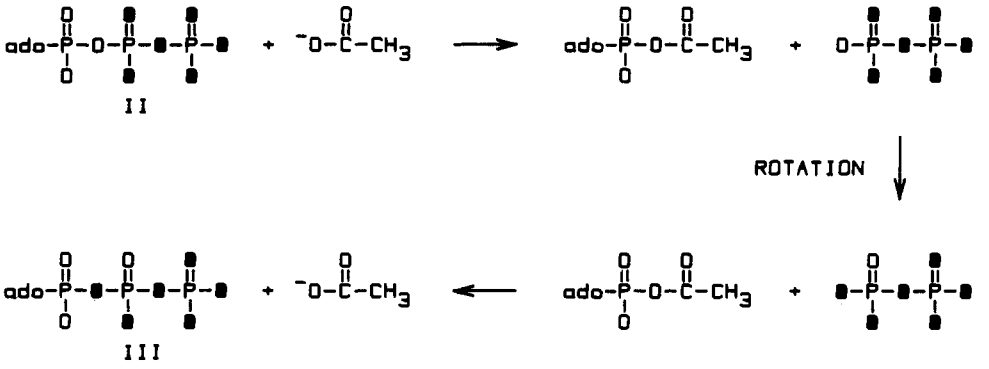
FIGURE 1: Fast atom bombardment mass spectra of ATP labeled with various amounts of oxygen-18.

Fast Atom Bombardment Mass Spectrometry. A Kratos MS-50TA (triple analyzer) high-resolution mass spectrometer was used for these studies. The instrument is equipped with a standard fast atom bombardment (FAB) ion source. The argon neutral beam was produced by an Ion Tech FAB11NF saddle field source. The FAB gun was operated at a potential of 6 kV and a neutral density corresponding to approximately 20 μA. The secondary ions formed by FAB ionization were accelerated by a potential of 8 kV. To obtain the negative ion mass spectrum, the instrument was scanned over a mass range from m/z 800 to 50 at a rate of 100 s/decade. The instrument was tuned for a mass resolution ($m/\Delta m$) of approximately 2000. The mass spectra were recorded by a Bryans-Southern UV oscillograph recorder and counted manually. The spectra shown in Figures 1–4 were obtained in the peak-matching mode with data collection signal averaged by a Nicolet 1170 signal averager. In this operating mode a mass range of about 10 amu can be observed without mass discrimination. Samples were prepared by placing 10 μL of a 10 mM aqueous sample on a gold-plated copper probe tip. The sample was dried in an external vacuum chamber prior to mixing with approximately 5 μL of glycerol.

RESULTS

Fast Atom Bombardment Mass Spectrometry of Nucleotides. In order to demonstrate the utility of FAB-MS for the detection of PIX in enzyme-catalyzed reactions, the mass spectra of some standard ATP samples were obtained. Shown in Figure 1 are the mass spectra of three samples of ATP containing a maximum of zero, four, and six atoms of oxygen-18 per molecule. The predominant ion for the unlabeled ATP in the negative ion mode occurs at m/z 506 for the M – H species. As expected, the M – H ion for the sample of [γ-$^{18}O_4$]ATP (I) appears at 514 mass units. Also apparent is an ion at m/z 512 that corresponds to the species containing only three atoms of oxygen-18. The ratio of peak heights at 514 and 512 mass units indicates that the oxygen-18 content in the four oxygen atoms directly bonded to the γ-P is 94%. This is identical with the value obtained from an NMR analysis (94%) of the same sample (Raushel & Villafranca, 1980). The third sample contains molecules having four, five, or six atoms of oxygen-18. The oxygen-18 content of this sample in the γ- and β-phosphoryl groups is calculated to be

Scheme I



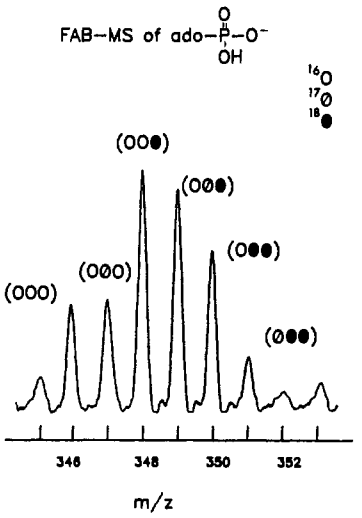


FIGURE 2: Fast atom bombardment mass spectrum of AMP labeled with oxygen-18 and oxygen-17.

92% from the ratio of peak heights at 518, 516, and 514 mass units. This is consistent with the value obtained by ^{31}P NMR at 202 MHz. The smallest amount of ATP that could be applied to the probe tip while retaining a suitable spectrum for these analyses was found to be about 10 nmol.

The FAB-MS method is also suitable for the detection of oxygen-17 in phosphate esters. Shown in Figure 2 is the mass spectrum of a sample of AMP that has been synthesized with the incorporation of the three isotopes of oxygen.[2] An analysis of the respective peak heights indicates the ratio of ^{16}O:^{17}O:^{18}O as 1.6:0.4:1.0. These values are consistent with the ratios expected from the isotopic composition of the water used in the synthesis of the AMP.

Positional Isotope Exchange with Acetyl-CoA Synthetase. Since acetyl-CoA synthetase is known to catalyze an ATP–PP_i exchange reaction in the presence of acetate and to form an acetyl adenylate intermediate, this enzyme provided a good example in which to test for positional isotope exchange within [β,γ-$^{18}O_6$]ATP by FAB-MS. These reactions are illustrated in Scheme I. Incorporation of oxygen-18 into the $\alpha\beta$-bridge position of ATP (III) is only possible if the bond to the α-P of ATP is broken by the enzyme and the β-phosphoryl group of the resulting PP_i is free to rotate. In order to use FAB-MS to measure the extent of the exchange reaction, the γ- and β-phosphoryl groups of ATP must be enzymatically removed by hexokinase and adenylate kinase to form AMP. Since these

[2] This AMP was obtained during the determination of the stereochemical course at phosphorus of the argininosuccinate synthetase reaction. Experimental details will be reported at a later date (Tamara Hess, unpublished results).

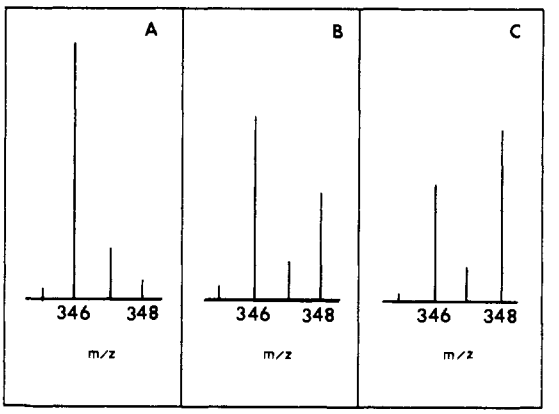


FIGURE 3: Fast atom bombardment mass spectra of AMP. Acetyl-CoA synthetase was incubated with acetate and [β,γ-$^{18}O_6$]ATP. At the indicated times the reaction was quenched with hexokinase, adenylate kinase, and glucose. The AMP was isolated by chromatography on DEAE-cellulose. (A) 0 h; (B) 1.5 h; (C) 24 h.

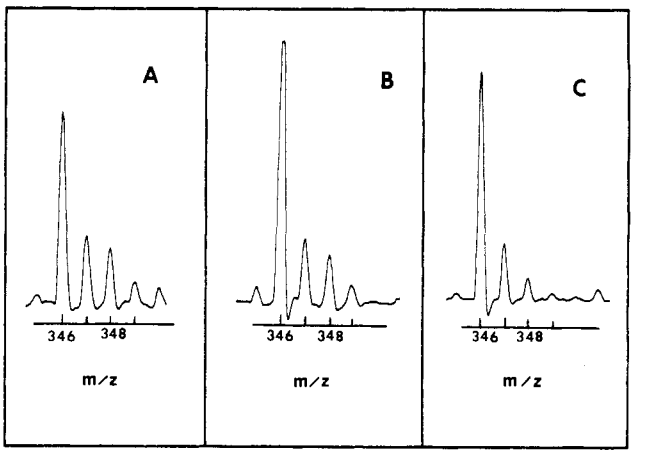


FIGURE 4: Fast atom bombardment mass spectra of AMP. (A) Argininosuccinate synthetase was incubated with citrulline and [β,γ-$^{18}O_6$]ATP. (B) Argininosuccinate synthetase was incubated with citrulline, succinate, and [β,γ-$^{18}O_6$]ATP. (C) Argininosuccinate synthetase was incubated with ATP and [*ureido*-^{18}O]citrulline. All reactions were incubated for 24 h and then quenched by the addition of hexokinase, adenylate kinase, and glucose. The AMP was isolated by chromatography on DEAE-cellulose. Additional details are given in the text.

reactions cleave ATP between the β-P and the $\alpha\beta$-bridge oxygen, the $\alpha\beta$-bridge oxygen remains with the AMP. Therefore, the extent of the exchange reaction is determined by the height of the signal at m/z 348. Shown in Figure 3 are the results when [β,γ-$^{18}O_6$]ATP, acetyl-CoA synthetase, acetate, and PP_i have been incubated for various lengths of time before the reaction was quenched with glucose, hexokinase, and adenylate kinase. At time zero there is no evidence

for any oxygen-18 incorporation into the AMP as determined by FAB-MS. After 1.5 and 24 h of incubation the amount of oxygen-18 incorporation has steadily increased to 36% and 59%, respectively.

Positional Isotope Exchange with Argininosuccinate Synthetase. Shown in Figure 4 are the results when argininosuccinate synthetase has been incubated with [β,γ-$^{18}O_6$]ATP or [*ureido*-^{18}O]citrulline for 24 h at pH 8.0. When 24 units of argininosuccinate are incubated with 1.0 mM [β,γ-$^{18}O_6$]-ATP and citrulline, these is no significant increase in the incorporation of oxygen-18 into the $\alpha\beta$-bridge position of ATP as determined FAB-MS (Figure 4A). The maximum rate of positional isotope exchange catalyzed by this enzyme under these conditions is therefore calculated to be ≤ 0.0001 s^{-1}. When succinate is added to the reaction mixture in an effort to induce the enzyme into a conformational state that would catalyze the expected exchange reaction, no oxygen-18 is detected in the AMP after the quench. The calculated exchange rate is also ≤ 0.0001 s^{-1}. No significant exchange from the ureido oxygen of citrulline into the α-phosphoryl group of ATP is detected in the absence of aspartate (Figure 4C). Similar results in all experiments were obtained when the oxygen-18 content was analyzed by ^{31}P NMR. Moreover, no PIX reactions were observed when the incubations were conducted at pH 6, 7, or 9. Negative results were also obtained when the initial citrulline concentration was reduced to 0.10 mM.

Discussion

Fast Atom Bombardment Mass Spectrometry. A number of methods have been utilized for the analysis of positional isotope exchange rates in enzyme-catalyzed reactions. Midelfort & Rose (1976) originally developed a mass spectroscopic approach that required both enzymatic and chemical derivatization of the ATP in order to ascertain whether positional scrambling of oxygen in ATP was catalyzed by glutamine synthetase in the presence of glutamate. More recently, Cohn & Hu (1978) discovered that oxygen-18, when directly bonded to phosphorus, caused an $\sim$0.02 ppm upfield shift in the ^{31}P NMR spectrum of phosphate esters and anhydrides. This observation permitted the direct analysis of the positional integrity of the labeled oxygens in ATP or other phosphate-containing molecules (Raushel & Villafranca, 1980). The NMR method appears to have become the method of choice for the analysis of PIX rates because of its simplicity and the possibility of obtaining results on a continuous and real-time basis. The major disadvantage of the NMR method relative to the MS method is sensitivity. At least 100 times more material is required for the NMR analysis.

The advent of FAB-MS suggested to us that this technique could potentially be used to monitor PIX rates in underivatized samples of ATP or other nucleotides (Eagles et al., 1984). While this work was in progress, Connolly et al. (1984) elegantly demonstrated that FAB-MS could be used to conveniently determine the stereochemical outcome of enzyme-catalyzed adenylyl transfer in thionucleotide substrates. We have now extended these previous findings to the analysis of positional isotope exchange reactions catalyzed by enzymes that direct nucleophilic attack at the α-P of ATP.

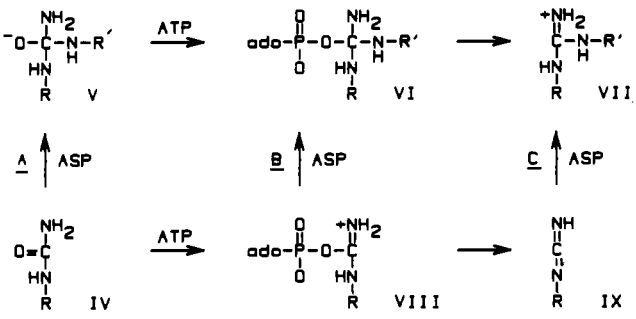
Figures 1 and 2 clearly indicate that the oxygen-17 and oxygen-18 content of nucleotides can be quantitatively determined by FAB-MS. The minimum amount of material required for such an analysis (10 nmol) is approximately 1000 times smaller than that required for a comparable NMR analysis. Furthermore, the content of ^{17}O and ^{18}O can be measured in the same experiment. Thus far, we have been unable to induce the fragmentation of ATP at a position that would enable us to directly determine whether positional exchange occurred from a β-nonbridge position to the $\alpha\beta$-bridge position in ATP. This problem was conveniently overcome by quenching the exchange reaction at various times with hexokinase and myokinase in the presence of glucose. This set of reactions produces AMP with the $\alpha\beta$-bridge oxygen retained by the AMP moiety. The oxygen-18 content of the AMP is therefore a direct measure of the positional isotope exchange rate. Mass spectrometry should once again become the method choice for the determination of oxygen isotopes in nucleotide molecules. A chemical and/or enzymatic degradation scheme would also need to be devised to use FAB-MS in the analysis of positional exchange of the $\beta\gamma$-bridge oxygen into the β-nonbridge position. This is the exchange reaction common to most kinases.

Positional Isotope Exchange Catalyzed by Acetyl-CoA Synthetase. The methodology for monitoring positional isotope exchange rates by FAB-MS in enzyme-catalyzed reactions was developed with acetyl-CoA synthetase. This enzyme catalyzes the formation of acetyl-CoA from acetate, CoA, and ATP. It has been previously shown that acetyl adenylate is a kinetically competent intermediate in this reaction (Berg, 1955). The enzyme also catalyzes an ATP–PP_i exchange reaction in the presence of acetate alone. These results indicate that PP_i is able to dissociate from the E–acetyl adenylate–PP_i complex. Therefore, we were virtually assured that the enzyme would catalyze a PIX reaction with [β,γ-$^{18}O_6$]ATP in the presence of acetate as indicated in Scheme I. The results presented in Figure 3 clearly indicate that a positional isotope exchange reaction is catalyzed by acetyl-CoA synthetase and further demonstrate the utility of FAB-MS for these measurements.

After 24 h of incubation the relative heights of the M − H and M − H + 2 species are approximately equivalent. The expected final equilibrium ratio of labeled and unlabeled AMP is 5:1 because the PP_i is able to interchange the phosphoryl groups upon dissociation and reassociation from the E–PP_i–acetyl adenylate complex. At least three factors can be cited in explanation for the reduced level of the exchanged material. First, the labeled ATP was not completely [β,γ-$^{18}O_6$]ATP as indicated by the mass spectrum in Figure 1. Significant amounts of ATP containing only four and five atoms of oxygen-18 were also present. Second, unlabeled PP_i was added to the incubation mixture to ensure an adequate initial concentration of PP_i in solution so that the reverse reaction (reformation of ATP) would be kinetically favorable. Third, a contaminating ATPase activity was detected in the commercial preparation of acetyl-CoA synthetase. This enzyme would cleave the γ-phosphoryl group from a fraction of the labeled ATP and thus prevent it from undergoing the partial reverse reaction catalyzed by acetyl-CoA synthetase. The AMP that is formed from this ADP (through the action of myokinase and hexokinase/glucose) is indistiguishable from the AMP formed from ATP that had been involved in the reaction catalyzed by acetyl-CoA synthetase. Nevertheless, these results clearly demonstrate the utility of FAB-MS for the detection of positional isotope exchange reactions in ATP.

Positional Isotope Exchange Catalyzed by Argininosuccinate Synthetase. The positional isotope exchange technique was applied to the reaction catalyzed by argininosuccinate synthetase in an attempt to identify the intermediates that may be involved in the reaction mechanism. Three potential mechanisms for this reaction are illustrated in Scheme II. The problem is to decide which structure actually reacts with the α-amino group of aspartate. In mechanism A as-

Scheme II

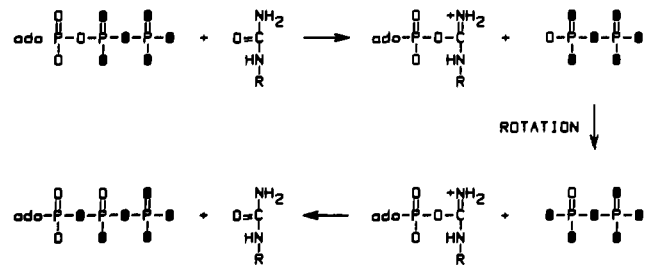


partate reacts with the ureido carbon of citrulline (IV) to generate a tetrahedral adduct (V) which is then adenylated by ATP to form intermediate VI. This intermediate then eliminates AMP to produce the ultimate product, argininosuccinate (VII). Mechanism B is initiated by a nucleophilic attack of the ureido oxygen on the α-P of ATP to form citrulline adenylate (VIII) and pyrophosphate. Citrulline adenylate then reacts with aspartate to form argininosuccinate. This transformation presumably would go through the same tetrahedral intermediate (VI) as mechanism A. The last mechanistic possibility also involves the formation of citrulline adenylate. However, prior to the attack by aspartate there is carbon–oxygen bond cleavage to form a cyanamide or carbodiimide (IX) intermediate and AMP. Aspartate would then react directly with this intermediate to form argininosuccinate.

Ratner (1973) and colleagues have provided evidence to suggest that citrulline adenylate is the reactive intermediate in the argininosuccinate synthetase reaction. They were able to demonstrate that the ureido oxygen of citrulline is quantitatively transferred to AMP during the reaction (Rochovansky & Ratner, 1961). However, oxygen transfer from citrulline to AMP is required by all three mechanisms and thus provides no information as to the activation process. In one of the earliest pulse–chase experiments conducted in mechanistic enzymology it was shown that enzyme-bound radiolabeled citrulline in the presence of ATP was nearly quantitatively transformed into argininosuccinate during the first turnover after the addition of aspartate and an excess of unlabeled citrulline (Rochovansky & Ratner, 1967). A comparable experiment with radiolabeled aspartate yielded no radiolabeled argininosuccinate during the first turnover. These experiments suggested that an intermediate was formed from ATP and citrulline prior to the addition of aspartate. However, Rose et al. (1975) have shown that this type of experiment does not require the prior formation of an intermediate. All that is required is a slow rate of dissociation of citrulline from E–citrulline–ATP relative to the k_{cat} for the formation of argininosuccinate. We have recently shown that argininosuccinate synthetase has a sequential kinetic mechanism that proceeds by the ordered addition of ATP, citrulline, and aspartate. In such a mechanism it is required that all of the initially bound citrulline be transformed into product at saturating aspartate (Meek et al., 1982). Therefore, the pulse–chase experiments provide no additional evidence for the formation of citrulline adenylate.

If citrulline adenylate and PP_i are formed upon the addition of ATP and citrulline to the enzyme active site, then an isotopic exchange between ATP and PP_i should be measurable if the PP_i is able to be released from the active site into the bulk solution. Rochovansky & Ratner (1961) have shown that in the absence of aspartate there is no detectable citrulline-dependent exchange between ATP and PP_i. These results demonstrate that if citrulline adenylate is a reactive intermediate then PP_i is not released until after the aspartate site is occupied. This conclusion is supported by the kinetic mechanism recently determined by Raushel & Seiglie (1983). However, a citrulline-dependent ATP–PP_i exchange was observed when α-methyl aspartate was added to the reaction solution (Rochovansky & Ratner, 1967). In our hands, α-methyl aspartate appears to be a slow substrate for argininosuccinate synthetase, and thus the exchange reaction between ATP and PP_i could have been the result of reversal of the whole reaction and not necessarily the formation of citrulline adenylate.[3] Although the formation of citrulline adenylate might be favored on chemical grounds, none of the presently available data can eliminate the other possible mechanisms. For these reasons we initiated a series of positional isotope exchange experiments to help differentiate among the three possible mechanisms.

Scheme III

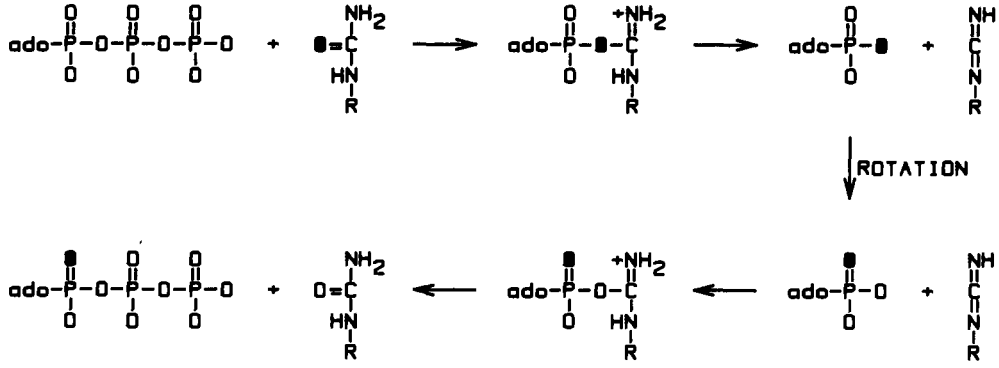


Shown in Scheme III is an outline for a positional isotope exchange experiment designed to determine whether citrulline adenylate is a reactive intermediate in the argininosuccinate synthetase reaction. If pyrophosphate and citrulline adenylate are formed upon the incubation of enzyme, ATP, and citrulline, then positional scrambling of the oxygens of the β-phosphoryl group in PP_i can occur. Re-formation of ATP and citrulline would result in a 67% probability that an oxygen-18 would now be present in the $\alpha\beta$-bridge position. Shown in Scheme IV is an outline for a positional isotope exchange experiment designed to determine whether a carbodiimide or cyanamide is the reactive intermediate. The formation of citrulline adenylate and a carbodiimide would enable the oxygen-18 that was originally on the ureido group of citrulline to ultimately be found in the α-phosphoryl group of ATP.

These two positional isotope exchange experiments should permit a distinction among the three mechanisms. If a citrulline adenylate intermediate is formed via route B or C, then a PIX reaction in the absence of aspartate should be observed as illustrated in Scheme III. If a carbodiimide structure is formed, then a transfer of oxygen-18 from citrulline to ATP should also be observed as illustrated in Scheme IV. If mechanism A or a concerted reaction is operating, then no exchange reaction would occur unless all three substrates were added to the reaction mixture. Although the outcome appears clear-cut, at least three factors could be responsible for no positional isotope exchange reaction even though the mechanism would involve a citrulline adenylate and/or a carbodiimide intermediate. First, since the kinetic mechanism is ordered and citrulline binds after ATP, then high concentrations of citrulline can totally prevent the exchange of ATP from the enzyme active site and artificially suppress the PIX reaction even though citrulline adenylate is formed. Second, the enzyme may catalyze the formation of the intermediate only in the presence of the third substrate, aspartate. Aspartate

[3] The substrate activity of α-methyl aspartate is reported in the following paper (Ghose & Raushel, 1985).

Scheme IV



may be required to induce the enzyme into a proper conformation required for activity. Third, the enzyme may prevent the rotation of the phosphoryl groups of pyrophosphate or AMP and thus suppress the positional exchange even though the intermediates may be formed.

We were unable to find any reaction conditions that would indicate that argininosuccinate synthetase catalyzes a positional isotope exchange reaction in the absence of aspartate. the upper limit for the PIX reaction (0.0001 s^{-1}) is 10000 times slower than k_{cat} (1 s^{-1}) for the forward reaction. This would suggest either that argininosuccinate synthetase does not utilize citrulline adenylate or the carbodiimide as reactive intermediates or that one of the above-mentioned factors was artificially suppressing the exchange reaction. Although the actual dissociation constant for citrulline from the E–ATP–citrulline complex is unknown, the concentration of citrulline (1.0 mM) used in the PIX experiment does not appear to be too high. Experiments at 0.1 mM citrulline and alternate pH values of 6, 7, and 9 were unsuccessful. Succinate was added to the reaction mixture in an attempt to induce the enzyme into the proper conformational state necessary for intermediate formation. Succinate is a competitive inhibitor vs. aspartate and is, unlike α-methyl aspartate, unable to be a slow substrate. As indicated under Results, these experiments were unsuccessful.

Therefore, these studies serve to indicate either that argininosuccinate synthetase does not catalyze the formation of citrulline adenylate in the absence of aspartate or, alternatively, that the enzyme binds the pyrophosphate so tightly as to totally suppress the rotation of the β-phosphoryl group. There has been only one reported case of such restrictive bond rotation in an enzyme-catalyzed reaction. The enzyme farnesylpyrophosphate synthetase has been shown by Mash et al. (1981) not to catalyze a positional isotope exchange reaction with oxygen-18-labeled geranyl pyrophosphate even though independent experiments indicated that a geranyl cation intermediate was formed (Poulter et al., 1981). These results were rationalized on the basis of a tight ion pair between the geranyl cation and pyrophosphate. Static and dynamic quench experiments reported in the following paper (Ghose & Raushel, 1985) indicate quite clearly that argininosuccinate synthetase catalyzes the formation of citrulline adenylate in the absence of aspartate. Therefore, the enzyme must somehow prevent the rotation of the β-phosphoryl group of PP_i.

In summary, we have shown that FAB-MS can be used to measure the oxygen-18 and oxygen-17 content of underivatized nucleotides. The method is rapid, simple, and significantly more sensitive than NMR analysis. This technique has been applied to the measurement of PIX rates in enzyme-catalyzed reactions. The enzyme argininosuccinate synthetase was found not to catalyze a positional isotope exchange reaction designed to test for the formation of citrulline adenylate as a reactive intermediate.

Acknowledgments

We thank Chandra Ghose for the generous supply of purified argininosuccinate synthetase.

References

Berg, P. (1955) *J. Am. Chem. Soc. 77*, 3163–3164.

Boyer, P. D., Koeppe, O. J., & Luchsinger, W. W. (1956) *J. Am. Chem. Soc. 78*, 356–357.

Ceriotti, G. (1970) in *Methods of Enzymatic Analysis* (Bergmeyer, H. U., Ed.) Vol. I, p 654, Academic Press, New York.

Cohn, M., & Hu, A. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 200–203.

Connolly, B. A., Eckstein, F., & Grotjahn, L. (1984) *Biochemistry 23*, 2026–2031.

DeBrosse, C. W., & Villafranca, J. J. (1983) in *Magnetic Resonance in Biology* (Cohen, S., Ed.) pp 1–52, Wiley, New York.

Eagles, J., Javanaud, C., & Self, R. (1984) *Biomed. Mass Spectrom. 11*, 41–46.

Ghose, C., & Raushel, F. M. (1985) *Biochemistry* (following paper in this issue).

Mash, E. A., Gurria, G. M., & Poulter, C. D. (1981) *J. Am. Chem. Soc. 103*, 3927–3929.

Midelfort, C. F., & Rose, I. A. (1976) *J. Biol. Chem. 251*, 5881–5887.

Poulter, C. D., Wiggens, P. L., & Le, A. T. (1981) *J. Am. Chem. Soc. 103*, 3926–3927.

Ratner, S. (1973) *Adv. Enzymol. Relat. Areas Mol. Biol. 39*, 1–91.

Raushel, F. M., & Villafranca, J. J. (1980) *Biochemistry 19*, 3170–3174.

Raushel, F. M., & Seiglie, J. L. (1983) *Arch. Biochem. Biophys. 225*, 979–985.

Raushel, F. M., & Garrard, L. J. (1984) *Biochemistry 23*, 1791–1795.

Riseley, J. M., & Van Etten, R. L. (1978) *J. Labelled Compd. Radiopharm. 15*, 533–538.

Rochovansky, O., & Ratner, S. (1961) *J. Biol. Chem. 236*, 2254–2260.

Rochovansky, O., & Ratner, S. (1967) *J. Biol. Chem. 242*, 3839–3849.

Rochovansky, O., Kodowki, H., & Ratner, S. (1977) *J. Biol. Chem. 252*, 5287–5294.

Rose, I. A., O'Connell, E. L., Litwin, S., & Bar Tana, J. (1974) *J. Biol. Chem. 249*, 5163–5167.

Wimmer, M. J., Rose, I. A. Powers, S. G., & Meister, A. (1979) *J. Biol. Chem. 254*, 1854–1859.