Title
Positional isotope exchange studies on enzymes using NMR spectroscopy

Author
Matsunaga, Terry Onichi

Publication Date
1987

Peer reviewed|Thesis/dissertation

Positional Isotope Exchange Studies on Enzymes Using NMR Spectroscopy

by

Terry Onichi Matsunaga

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

Pharmaceutical Chemistry

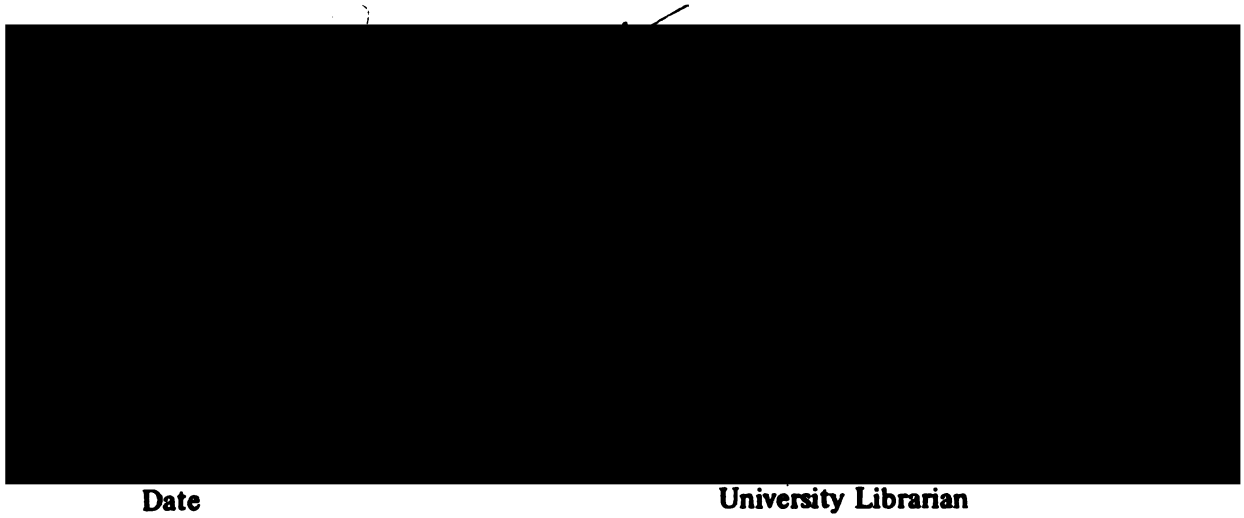
in the

GRADUATE DIVISION

of the

UNIVERSITY OF CALIFORNIA

San Francisco



Date University Librarian

Degree Conferred: . . . Jan. 4, 1987 . . . . . .

ACKNOWLEDGEMENTS

Having spent over nine years at UCSF, I have known many who have supported my efforts in both graduate and pharmacy school. The road was oftentimes long and frustrating, but never regretful or boring.

I wish to extend my sincere thanks to my research director, Professor George L. Kenyon, who patiently over the years allowed me to grow and develop the skills I needed to become a scientist. Thank you George for embodying both the qualitites of being a good mentor and a good friend as well.

Thank you to Professor Norman J. Oppenheimer, who's insight into scientific thought helped me to progress. Thank you also to Professor Paul A. Bartlett at U.C. Berkeley and Dr. Bernard L. Kam who gave me the enthusiasm to learn to appreciate research.

Thank you to all in the NMR facility, especially Drs. Vladimir Basus, Michael Moseley, Max Keniry, Ed Bubienko, Thomas Marshner, and Professor Tom James for teaching me the fine nuances neccessary to run the NMR.

Thank you to all our collaborators during all of our efforts. Special thanks to Professor Charles McKenna and William Gutheil at the University of Southern California, Professor Paul Cook at North Texas State University, Professor Richard Himes at Kansas University, and Professor George Reed for all of their help and insightfulness during all the efforts.

Thank you to all my fellow graduate students in the department for all their support. Special thanks to all in "Club 1136", George Garcia, Vince Powers, Nariman Naber, Patsy Babbitt, Mark Watanabe, Carlos Catalano, Jim Matthews, Betsy Komives, Larry Grab, Rich Blatchely and Jae Lee for their friendship. Thanks also to all in the combined Kenyon and Oppenheimer group for all of their contributions through the years.

A special thanks to all my friends, especially Dr. Richard deLeon in the Dept. of Pharmacy at the University of Michigan who helped me in my decision to return to graduate school.

Thank you also to my bicycle riding friends, especially Dr. R. Jon Goerke, who always encouraged me to get out and ride. Thanks also to Al Kerr who made me maintain my enthusiasm by making me get over to the gym and "shoot baskets".

An additional thank you to the Earl C. Anthony Trust Fund of the Graduate Division for their generous financial support.

Thank you to Susie, my love, for all her support and patience thru the years of work and time apart.

Most of all, thank you to my brother Dennis, my sister Kay, my cousins, aunts, and uncles for all their love and support.....and last but not least, to my mother and father, whom I love and respect.

DEDICATION

To mom and dad, family and friends.....

.....and to my Uncle George, ...we miss you.

ABSTRACT

The isotopically enriched compounds, ^{18}O-β,γ-ATP and ^{18}O bridge-labeled pyrophosphate, synthesized previously in this laboratory, were used to investigate and measure the exchange vs. turnover of substrates and products from their central complexes in four selected enzyme systems. Using hi-field ^{31}P NMR, we were able to differentiate between ^{18}O labeled in the bridge vs. the non-bridge positions by virtue of the isotope shift upon the phosphorus nuclei. The bridge to non-bridge scrambling of the label was quantitated and the exchange vs. turnover ratios under a variety of conditions was determined.

Using the substrate inhibitor carboxycreatinine, PIX experiments with ^{18}O-β,γ-ATP and creatine kinase were conducted . It was shown that carboxycreatinine and creatine kinase promoted exchange of the ^{18}O label as determined by NMR. We have concluded that carboxycreatinine is either a substrate that catalyzes very slow turnover or it catalyzes exchange by a dissociative (SN_1P) type of mechanism.

PIX was studied in the enzyme pyrophosphate-dependent phosphofructokinase with the substrate ^{18}O-bridge-labeled pyrophosphate. In addition, PIX was studied in the enzymes nitrogenase and formyltetrahydrofolate synthetase with the substrate ^{18}O-β,γ-ATP. Under a variety of conditions, we could not find evidence for the exchange reaction. We have thus set upper limits to the amount of exchange vs. turnover for these three enzyme systems based upon the signal : noise ratios from the NMR spectra. The lack of PIX has been attributed to an array of "commitment factors" which favored turnover to products with subsequent release from the enzyme complex.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF SCHEMES AND FIGURES

INTRODUCTION

ATP functions as the major carrier of chemical energy in cells of all living species. It participates in a variety of phosphoryl transfer reactions in which the occurrence of a phosphorylated intermediate is proposed as a component of the reaction (Lehninger, 1972). Oftentimes, however, the lifetime of the intermediate is too short to be isolated or chemically trapped. Positional isotope exchange, or PIX, is a term originally adopted by Midelfort and Rose to describe a method for the detection of transient intermediate (enzyme: ADP: P-X) formation from ^{18}O-ATP labeled in the beta, gamma (β, γ) bridge (Midelfort and Rose, 1976). Upon formation of the intermediate complex, the $P_\beta O$ - $P\gamma$ bond is broken, allowing symmetrical torsional rotation about the P_β-O group. Should the cleavage be reversible, then the ^{18} O should randomly scramble between the two β non-bridge and the β, γ bridge positions of the ATP pool (see Scheme 1). The quantity of scrambling of ATP undergoing the reverse reaction should reflect a statistical distribution between the bridge and non-bridge species, 2/3 ending up in the non-bridge position and 1/3 in the bridge position. By quantitating the bridge to non-bridge ratio relative to turnover to products, one can determine the relative reverse to forward rate constants in ATP - utilizing enzymes.

Although ingenious, the Midelfort and Rose method was very laborious because once PIX occurred, the analysis required the following: 1) β, γ interchange of ^{18}O with acetyl CoA synthetase, 2) transfer of the $P\gamma$ to dihydroxyacetone with glycerokinase 3) nucleophilic displacement of inorganic orthophosphate (P_i) with $^-$OH, 4) derivatization with diazomethane, and 5) mass spectrometric analysis of the permethylated phosphate (see Scheme 2).

The potential for using high-field nuclear magnetic resonance (NMR) as a tool for studying isotopic shifts in enzymatic reactions was shown by the work of Cohn and Hu who were able to measure the ^{18}O - induced isotopic shifts in the ^{31}P spectrum of P_i and adenine nucleotides (Cohn and Hu, 1978). Using enzymatically labeled β - ^{18}O ADP and chemically labeled ^{18}O P_i, Cohn and Hu reported an isotopic shift of the ^{31}P NMR spectrum corresponding to 0.0206 ppm relative to the non-isotopic species. Additionally, these authors reported that in a randomized sample containing 50% ^{18}O, all five ^{16}O - ^{18}O species of inorganic orthophosphate could be completely resolved at 145.7 MHz Hence, the authors concluded that isotopic shifts in the ^{31}P nmr spectrum could be used to follow phosphate exchange reactions. Using polynucleotide phosphorylase, Cohn and Hu demonstrated the ability to follow the enzymatic exchange reaction. Starting with unlabeled ADP and P_i ($^{18}O_4$), the authors could follow the exchange reaction by NMR showing the emergence of the α - ($^{16}O_3{}^{18}O$) and β - ($^{18}O_4$) ADP resonances, thereby demonstrating that phosphate exchange occurs with bond cleavage between the Pα and the α, β bridge oxygen.

Lowe and Sproat refined the ^{31}P nmr method by assigning the relative chemical shift between the ^{18}O bridging (P_α -P_β) and non-bridging (P_α and P_β) species of ADP (Lowe et al., 1979). In addition, Lowe and Sproat discovered that the isotopic shift showed a linear relationship with respect to the square of the frequency of the stretching mode of the phosphate group, indicating that the magnitude of the isotopic shift is related to the force constant of the phosphorus-oxygen bond. That is, the isotope-induced chemical shift correlated well with the bond order of the P - O bond.

Cohn and Hu subsequently assigned the isotopic chemical shift of ADP and ATP with ^{18}O placed in various positions (Cohn and Hu, 1980). Of

importance to our present work was the fact that one could distinguish between ^{18}O-β, γ bridge induced isotopic shift vs the ^{18}O non-bridge induced shift. The bridge species, possessing only a single bond order, shifts the P_β resonance of ATP upfield 0.0165 ppm relative to unlabeled ATP. The non-bridge ^{18}O species, possessing an ***average*** bond order of 1.5, shifts the P_β resonance upfield 0.0281 ppm relative to the ^{16}O substituted species. Since PIX involves the scrambling of a bridge oxygen to a non-bridge position, one can take advantage of the small yet discernable difference in the ^{31}P resonances in going from bridge to non-bridge species.

The first application of ^{31}P NMR to examine PIX in an enzymatic system was by Raushel and Villafranca (Raushel and Villafranca, 1980). Using the enzyme carbamoyl-phosphate synthetase from E. coli and the compound Pγ-$^{18}O_4$ ATP, they were able to demonstrate catalysis of the β, γ bridge : β non-bridge exchange of the label. In addition, analysis of PIX along with rapid quench data enabled them to measure the individual rate constants for partitioning of the intermediates in the reaction. Thus, it was demonstrated that PIX can be used to determine relative reverse and forward kinetic constants.

Reynolds et al. refined the use of ^{31}P NMR to determine PIX by designing a selective synthesis of ^{18}O-β, γ -ATP (Reynolds et al, 1983). In addition, the same method was used to synthesize ATP with ^{17}O enrichment. Importantly, ^{17}O directly bonded to ^{31}P induces such large line broadening that the resonance intensity of the phosphorus nuclei is essentially undetectable. This phenomenon, "referred to as scalar coupling of the second kind" by Abragam is dependent upon the quadrupolar relaxation time T_q of the ^{17}O nucleus and the spin-spin coupling constant between the ^{31}P and ^{17}O nuclei (Abragam, 1961). In this particular case, the ^{31}P linewidth is inversely proportional to the spin-spin relaxation time, T_2:

$$1/T_2 = 4p^2J^2I\,(I + 1)T_q / 3$$

Where $I = 5/2$

The ^{17}O-β, γ bridge labeled ATP allowed Reynolds and coworkers to follow the PIX reaction catalyzed by glutamine synthetase by observing the regeneration of the ^{31}P resonance of the P_γ relative to P_β when the ^{17}O label scrambled from β, γ bridge to the β non-bridge position.

We have since used the highly enriched ^{18}O-β, γ -ATP to examine the PIX reaction for the following enzymes: nitrogenase, creatine kinase, and formyl tetrahydrofolate synthetase. In addition, we have used the selectively ^{18}O bridge-labeled pyrophosphate to investigate the PIX reaction in pyrophosphate-dependent phosphofructokinase.

HISTORICAL BACKGROUND AND THEORY OF ISOTOPE-INDUCED SHIFTS

Theoretical calculations for the isotope-induced chemical shift change for polyatomic molecules is much more complicated than the corresponding treatise for the HD molecule (Batiz-Hernandez and Bernheiim, 1967). Because a full quantitative analysis would require extensive wave function calculations and their relation to various vibrational degrees of freedom of a molecule (with and without isotopic substitution), only semi-quantitative approaches to the isotope shift in polyatomic molecules have been addressed.

Isotopic shifts in NMR spectroscopy were first predicted by Ramsey and Purcell who proposed a relationship between the shift of the observed resonance and the difference in the zero point enegy upon isotopic substitution (Ramsey and Purcell, 1952). This was confirmed by the first experimental observation of the isotope shift by Wimett in 1953.(Wimett, 1953) Wimett determined the values for the differences in the proton magnetic shielding

constants, those being $[\sigma(D_2) - \sigma(H_2)] = 0.065 \pm 0.059 \times 10^{-7}$ and $[\sigma(D_2) - \sigma(HD)] = 0.048 \pm 0.032 \times 10^{-7}$. In 1957, Tiers first observed upfield isotope shifts in both proton and fluorine resonances of deuterated molecules (Tiers, 1957; Tiers, 1958). By comparing the spectrum of n-C_3F_7D with that of n-C_3F_7H, the author found that the fluorine nuclei in the CF_2D group are 0.60 ± 0.05 ppm upfield (more shielded) than those in the corresponding CF_2H group. This prompted the first semiquantitative treatise on isotopic shifts by Gutowsky (Gutowsky, 1959).

Gutowsky first approached the question by using an electrostatic deformation model for geminal nuclei (Gutowsky, 1958; Gutowsky, 1959). In Gutowsky's model, the change in the magnetic shielding of a nucleus by intramolecular electrostatic fields (E fields) is considered. Two major points are addressed: 1) in the presence of an electrostatic field about a neighboring nucleus, the magnetic shielding about an observed nucleus is decreased by an amount proportional to the square of the electrostatic field charge (q), and, 2) since the electrostatic field is due to a charge q on an atom in the molecule, the decrease in shielding of the resonant nucleus will also vary as the fourth power of the distance between the charge (q) and the resonant nucleus. Thus, the electrostatic deformation model predicts a decrease in shielding equal to:

$$s = -2q^2/b^4 \times 10^{-5}$$

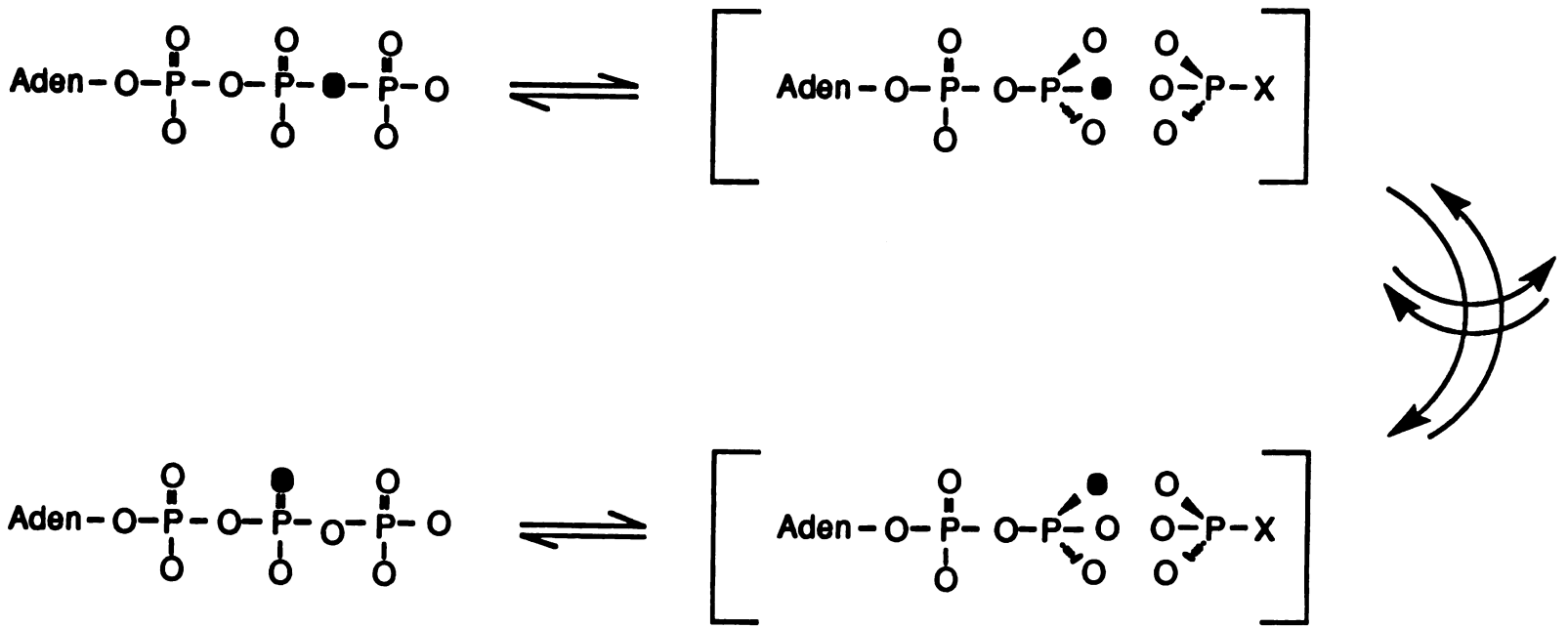
$$s = \text{shielding component}$$

Briefly, since a heavier isotope produces a smaller zero-point vibrational amplitude (and hence a *larger* charge-resonant nucleus separation, b) relative to the non-isotopic counterpart, the heavier isotope either perturbs or deforms the magnetic shielding about the resonant nucleus to a *smaller* extent, resulting in *less deshielding* and, consequently, a higher resonance frequency by NMR. In addition, the smaller amplitude results in a smaller electric field (q) at the

resonant nucleus, resulting in an effective increase (less of a decrease) in the shielding component.

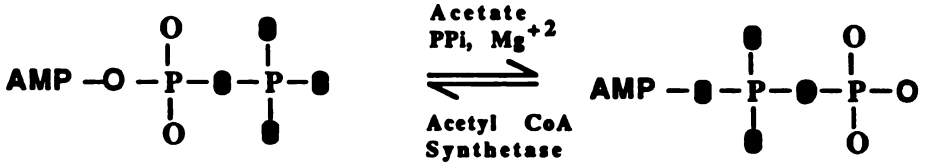
The above calculations predict an unreasonably large point charge, q; however, Gutowsky rationalizes this by allowing for the spatial distribution of the point charge, which reduces the net value of q to a more reasonable number. Presumably, similar electrostatic deformation models are analogous for other resonant nuclei with isotopic substitutions.

A second explanation for isotope-induced shifts in polyatomic molecules involves small changes in hybridization secondary to changes in vibrational potentials to anharmonic components (Ibers and Stevenson, 1958). Bernheim and Batiz-Hernandez found that the change in the diamagnetic component of the shielding (σ^d) accompanying changes in bond hybridization (due to anharmonic isotope effects relative to non-isotopic effects) was sufficient to explain the observed isotope-induced chemical shift changes (Batiz-Hernandez and Bernheim, 1967). In short, a decrease in s character of the bond would lead to an increase in the isotope shift. This inverse relationship is consistent with the later observations of Lowe and coworkers who noted the increase in the magnitude of the isotope shift with higher bond order. Paramagnetic contributions to magnetic shieldings σ^p will be influenced by the changes in hybridizations secondary to isotopic substitutions; however, it is argued that, at least for small molecules, σ^p is insignificant compared to σ^d. In addition, ionic character of the bond between the observed and isotopic nuclei appear to provide a modest effect to the calculated isotope shift (Bernheim and Batiz-Hernandez, 1966). Briefly, other theories that have been proposed which address the following: 1) temperature dependence upon shielding; (Lauterbur, 1965) 2) temperature and pressure dependence of shielding components of specific nuclei (^{59}Co) (Benedek et al., 1963; Loewenstein and Shporer, 1965);



Scheme 1. Positional Isotope Exchange (PIX).

1) β,γ interchange of ^{18}O ATP.



2) transfer of P_γ to dihydroxyacetone phosphate (DHAP) with glycerokinase.

β,γ interchanged ^{18}O-ATP ⇌ ADP + DHAP

3) Nucleophilic exchange of inorganic orthophosphate (P_i).

DHAP $\xrightarrow{OH^-}$ PO_4^{-3} + Dihydroxyacetone

4) Derivatization with diazomethane.

PO_4^{-3} $\xrightarrow{H^+}$ $\xrightarrow{CH_2N_2}$ $(CH_3)_3PO_4$ (M_r = 140)

Scheme 2. Analysis of exchange.

CHEMICAL SYNTHESES

The following procedures were adapted from the methods of Reynolds et al.and Marschner et al (Reynolds, et al., 1983; Marschner,et al., 1983). The synthetic pathways are provided in Scheme1 and Scheme 2 of this chapter. Details are presented to enhance their reproducibility.

^{18}O Diethylphosphite: To an oven dried 500 ml round bottom flask equipped with a 25 mL addition funnel was added 250 mL tetrahydrofuran (THF) previously dried over Na / benzophenone. To this solution was added 10.2 g (100.8 mmole, 1.01 equiv) triethylamine (Aldrich) freshly distilled from BaO and 2.0 g (100.2 mmole, 1.00 equiv.) $H_2{}^{18}O$ (98.1 atom % ^{18}O, MSD Isotopes). To this stirring solution, cooled to 0° C, was added in a dropwise fashion 15.7 g (100.2 mmole, 1.00 equiv) freshly distilled diethyl chlorophosphite (Aldrich, bp. 154 - 156°C). Addition was over 30 min. The mixture was then allowed to stir overnight. The flocculent white precipitate (triethylammonium chloride) was then filtered through a fine scintered glass funnel and the THF removed. The remaining oily yellow residue was then vacuum distilled (bp. 84 - 86° C, 13 torr) to yield 12.2 g (87.1 mmole, 86.9 %) of clear liquid. The infrared spectrum compared to authentic sample (Aldrich) were identical. Of special significance is the P - H stretch of the phosphonate tautomer at 2425 cm^{-1}.

^{18}O-Bridge-Labeled Tetraethylthiopyrophosphate: (Arbuzov et al., 1954) To a 500 mL round bottom flask equipped with an addition funnel was added 12.2 g (87.1 mmole, 1.0 equiv) of previously synthesized ^{18}O diethyl phosphite and 8.82 g (87.1 mmole, 1.0 equiv) of triethylamine freshly distilled from BaO (Aldrich). The reaction mixture was warmed carefully to 45° C with an oil bath.

To this solution was added in small increments 2.79 g (87.1 mmole, 1.0 equiv) finely powdered sulfur (Aldrich) previously dried in vacuo over P_2O_5. The now yellow syrup was then diluted with 250 mL of dry, distilled THF. Fifteen grams (87.1 mmole, 1.0 equiv) of distilled diethyl chlorophosphate (Aldrich, bp. 84 - 86° C, 12 torr) was then added dropwise over 30 min. As the addition proceeded, the solution became cloudy as the insoluble triethylammonium chloride was formed. Stirring was continued overnight at room temperature. The reaction mixture was then filtered through a fine scintered glass funnel and the THF removed in vacuo. The yellow oil was then concentrated in vacuo and the vessel cooled to 0° C until fine needles appeared (residual triethylammonium chloride). The yellow oil was then re-equilibrated to room temperature and the needles filtered through glass wool in a disposable pipet. This yielded 25.5 g (82.7 mmole, 94.9%) of yellow oil. NMR analysis by ^{31}P at 97.57 MHz showed the product to be greater than 95% pure ($CDCl_3$, ^1H broadband decoupled) with d = 51.0 ppm (d,$^{1-3}J_{pp}$ = 22.0 Hz), in good agreement with literature value (Harris et al., 1967).

^{18}O-Bridge-Labeled Pyrophosphate, Tetrasodium Salt: (Chojnowski et al., 1982) In a 100 ml round-bottom flask equipped with a magnetic stirrer and an addition funnel was added 2.05 g (6.66 mmole, 1.0 equiv) of tetraethylthiopyrophosphate . The stirred solution was then cooled to approx. -40° C with the aid of a dry-ice acetone bath. To this solution was added 5.0 mL of dry methylene chloride (Mallinckrodt, distilled over P_2O_5) to decrease the viscosity at low temperature. To the stirred solution was added 4.17 mL (5.86 g, 29.3 mmole, 4.4 equiv) of iodotrimethylsilane (Aldrich). Addition was in a dropwise fashion over 30 min. Upon completion, the solution, which by now had darkened to an amber brown, was allowed to stir an additional 1 hr at -40°

C. The rapidly stirring solution was then allowed to equilibrate to room temperature, and stirring was continued for an additional three days followed by final reflux for two more hours. The dark brown solution was then quenched in an 80 mL solution of 1 M TAPS (tris(hydroxymethyl) methylaminopropane sulfonic acid) buffer, pH = 8.4. To this now clear solution was added 1 - 2 mL portions of bromine until a brick red color appeared (Lowe, et al., 1982). The solution was continually adjusted to a pH between 7 and 8 with 50% NaOH solution. Periodic addition of bromine with constant pH adjustment was continued until the brick red color persisted. The red solution was then allowed to stand an additional 5 min (note: red solution was not allowed to stand more than 15 min total from the initial addition of bromine). The red solution was then quenched with sodium bisulfite until the solution cleared. The excess methylene chloride was extracted with ether and the aqueous portion was diluted to1 L with distilled, deionized H_2O. The pH was then adjusted to pH = 8.5, and the solution was loaded onto a QAE Sephadex A-25 anion exchange column (2.5 cm x 25 cm) generated in the HCO_3^- form. The column was then eluted with a linear gradient consisting of TEA-HCO_3^- (triethylammonium bicarbonate - generated by bubbling CO_2 into an aqueous solution of triethylamine), pH = 8.5, 0.07 M - 0.7 M, containing 1.6 L in both the mixing and reservoir chambers. Fractions (30 mL) were collected. Aliquots (50 µL) were then taken from each fraction and assayed for inorganic phosphate according to the procedure of Ames (Ames, 1966). Inorganic orthophosphate typically eluted at an ionic strength of 0.15 M and pyrophosphate was found to elute at an ionic strength of 0.4 - 0.5 M. The pyrophosphate fractions were then consolidated and concentrated in vacuo until a dry, white precipitate remained. The precipitate was then dissolved repeatedly in absolute methanol and reconcentrated in vacuo until only a hazy film remained on the sides of the flask.

The film was then dissolved with three washings of absolute methanol (2 mL each) and the pyrophosphate precipitated as the sodium salt with addition of 1 M sodium iodide in dry acetone. The flocculent white precipitate was then centrifuged in 30 mL Corex tubes and the recovered precipitate resuspended and centrifuged at least three additional times with dry acetone. The precipitate was then collected and vacuum dried. The precipitate was then redissolved with 20 mL of distilled, deionized H_2O and the pH adjusted to approximately pH = 11.5 with 1N NaOH. Absolute ethanol was then added in a dropwise fashion until fine white needles appeared. The solution was then allowed to stand overnight under refrigeration. The product was collected via suction filtration and washed with absolute ethanol to yield 1.07 g (2.38 mmole, 35.8%) of fine white needles as the tetrasodium decahydrate salt.

A ^{31}P nmr spectrum generated at 202.5 MHz revealed the unlabeled resonance at 0.00 ppm and two peaks at -0.010 and -0.012 ppm upfield (see Figure 1). This is consistent with the ^{18}O non-bridge labeled pyrophosphate which imparts a small but discernible non-equivalence to the two phosphorus nuclei. Thus, these resonances represent a secondary coupling, the peaks being the inner peaks of an AB pattern. The resonance of -0.020 ppm represents the bridge-labeled pyrophosphate. The chemical shifts were consistent with those reported by Marshner et al (Marschner et al., 1983).

<u>β,γ-^{18}O-Bridge-Labeled ATP:</u> (Reynolds et al., 1983; Hoard and Ott, 1965) <u>A) Preparation of Tetra (n-butyl)-ammonium Pyrophosphate:</u> In 20 mL of distilled, deionized H_2O was dissolved 0.944 g (2.11 mmole, 2.0 equiv) of previously prepared ^{18}O pyrophosphate, tetrasodium salt. The solution was passed through a column of Dowex AG-50W X-8 cation-exchange resin generated in the pyridinium form (resin is swirled in 1 N HCl,

washed with H_2O, then swirled in pyridine and washed exhaustively with H_2O until little or no detectable pyridine odor was evident). The column was then eluted with approximately 250 mL of distilled, deionized H_2O. To this solution was added 2.07 mL (1.61 g, 8.44 mmole, 4 equiv.) of tributylamine (Aldrich, 97%). The solution was then concentrated in vacuo to yield a viscous yellow syrup The syrup was then dissolved in methylene chloride and reconcentrated three times to remove residual water. The syrup was then dissolved and reconcentrated three additional times with dimethylformamide (Mallinckrodt, distilled over CaH). The syrup was then left under vacuum overnight and maintained under vacuum until just prior to reaction.

B) Preparation of Activated AMP: To a 100 mL round-bottom flask was added 0.39 g (1.06 mmole, 1 equiv) of adenosine monophosphoric acid monohydrate (Sigma). To this was added 2.25 mL distilled, deionized water and 2.25 mL pyridine. This solution was swirled until completely dissolved. To this dissolved mixture was added 0.95 mL (0.77 g, 2.12 mmole, 2 equiv) of tri n-octylamine (Aldrich, 97%). The solution was swirled briefly and then concentrated in vacuo to yield a viscous syrup. The yellow tinted syrup was then dissolved in dry DMF and taken to dryness in vacuo a total of three times. The syrup was then dissolved in 9 mL of freshly distilled DMF and to this was added 0.51 g (3.16 mmole, 3 equiv) of crystalline 1, 1' carbonyldiimidazole (Sigma). The solution was then allowed to stir for 2 hr. Excess carbonyldiimidazole was then quenched with 214 µL (5.28 mmole, 5 equiv) of absolute methanol . This was then allowed to stir an additional 5 min.

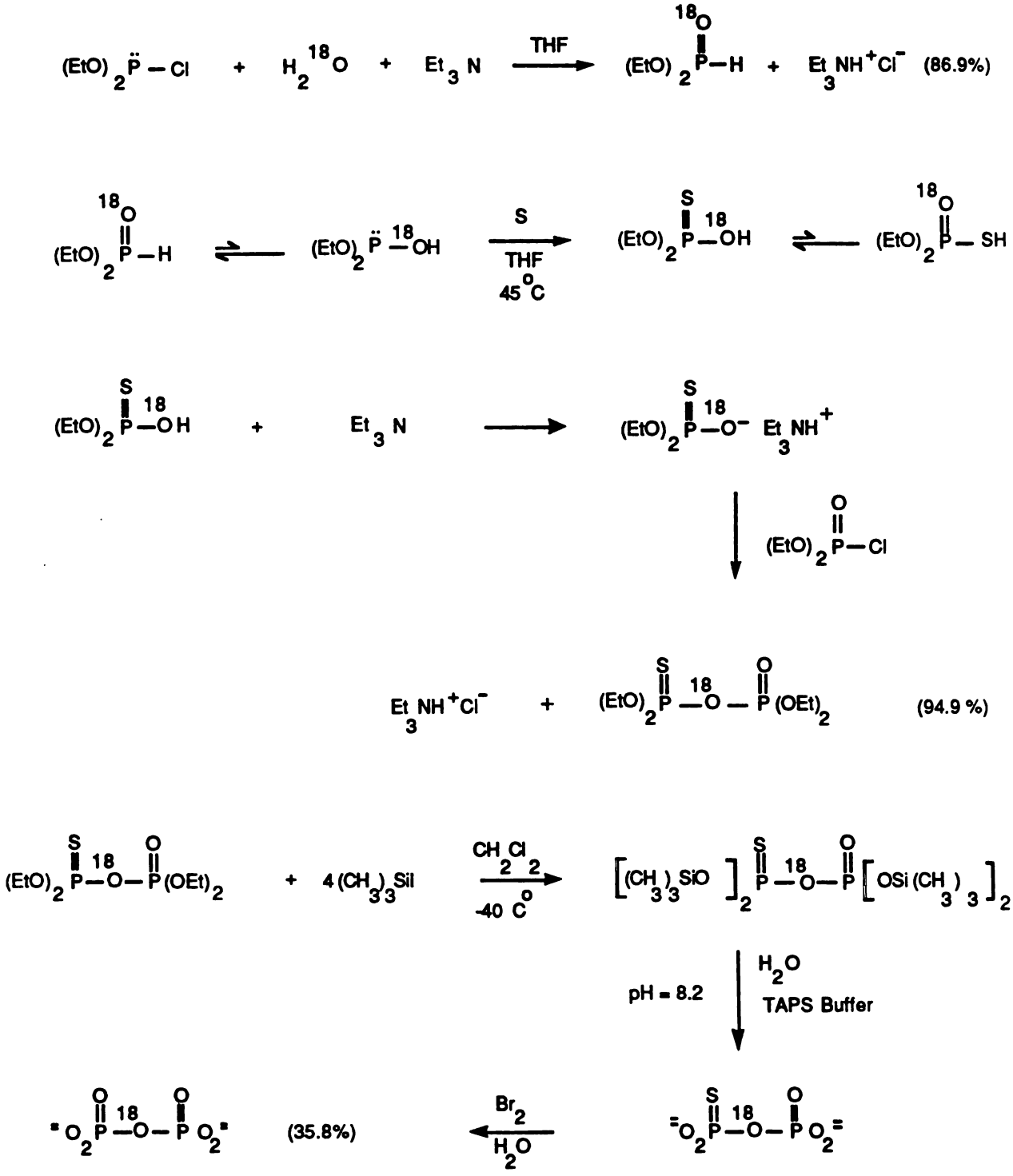
C) Synthesis: Dry tetrabutylammonium pyrophosphate prepared previously was then dissolved one last time in 8 mL of dry DMF and added dropwise to the stirring solution of activated AMP. Addition was over 30 min at room temperature. The flocculent mixture was then allowed to stir an additional 5 hr.

The mixture was then centrifuged at 5000 rpm in a Sorvall SS-34 rotor using 30 mL Corex tubes and the precipitate (imidazolium pyrophosphate) saved. The supernatant was then recovered and concentrated in vacuo to yield a viscous yellow oil. The yellow oil was then suspended into 150 mL of distilled, deionized water and the pH adjusted to 7.8 with 1 N NaOH. The solution was then loaded onto a DEAE Sephadex A-25 anion exchange column generated in the HCO_3^- form. The column was developed with a linear gradient of TEA-HCO_3^-, pH = 7.8, 0.07 M - 0.8 M, 1.5 L in both the mixing and reservoir chambers. Fractions were collected in 30 mL aliquots and analyzed by UV at 259 nm. Fractions corresponding to ATP were then pooled and the volume recorded. The UV A_{259} was recorded for purposes of determining the yield. The pooled fractions were then concentrated in vacuo to yield a white precipitate. The product was redisssolved in absolute methanol and dried in vacuo until only a white film remained in the flask. The product was then recovered by dissolution in small aliquots of methanol and conversion to the sodium salt by addition of 1 M NaI in acetone . The flocculent precipitate was then recovered by centrifugation. The recovered product was then resuspended in dry acetone and recentrifuged at least three times to remove any traces of NaI and triethylammonium iodide. The white precipitate was then dried overnight in vacuo to yield 786 mg (1.31 mmole, 57.5% by UV analysis, ε = 15,400) of base or 822 mg of the salt (corresponding to 1.5 equiv of Na^+).

A ^{31}P nmr spectrum generated at 202.5 MHz revealed three sets of triplets for the β-phosphorus (see Figure 2). The triplets are actually a doublet of doublets ($J_{P\alpha\text{-}P\beta}$ and $J_{P\beta\text{-}P\gamma}$) with fortuitously identical coupling constants rendering apparent triplets. The furthest downfield triplet (center peak set at 0.00 ppm) correspond to the unlabeled ATP. The most intense, shifted -0.016 ppm upfield, is the selectively ^{18}O-β, γ bridge-labeled ATP while the furthest

upfield peak represents the ^{18}O-β non-bridge labeled ATP. Enrichments typically ranged from 60%-78% ^{18}O bridge labeled, 8% - 20% ^{18}O non-bridge and 14% - 20% unlabeled ATP. The chemical shifts were in excellent agreement with those reported by Cohn & Hu (Cohn and Hu, 1980).

Quantitation of bridge vs non-bridge enrichments were performed by using the GEMCAP routine from the GE Nicolet 1280 software package. The middle peaks of each triplet were simulated and difference spectra were performed to generate the similated lines in line B. (see Figure 3). The three lines were then drawn as the independent components of the three lines and the areas quantitated (see Table 1). All other quantitations were conducted in the same manner.



Scheme 1. Synthesis of β,γ-^{18}O-ATP.

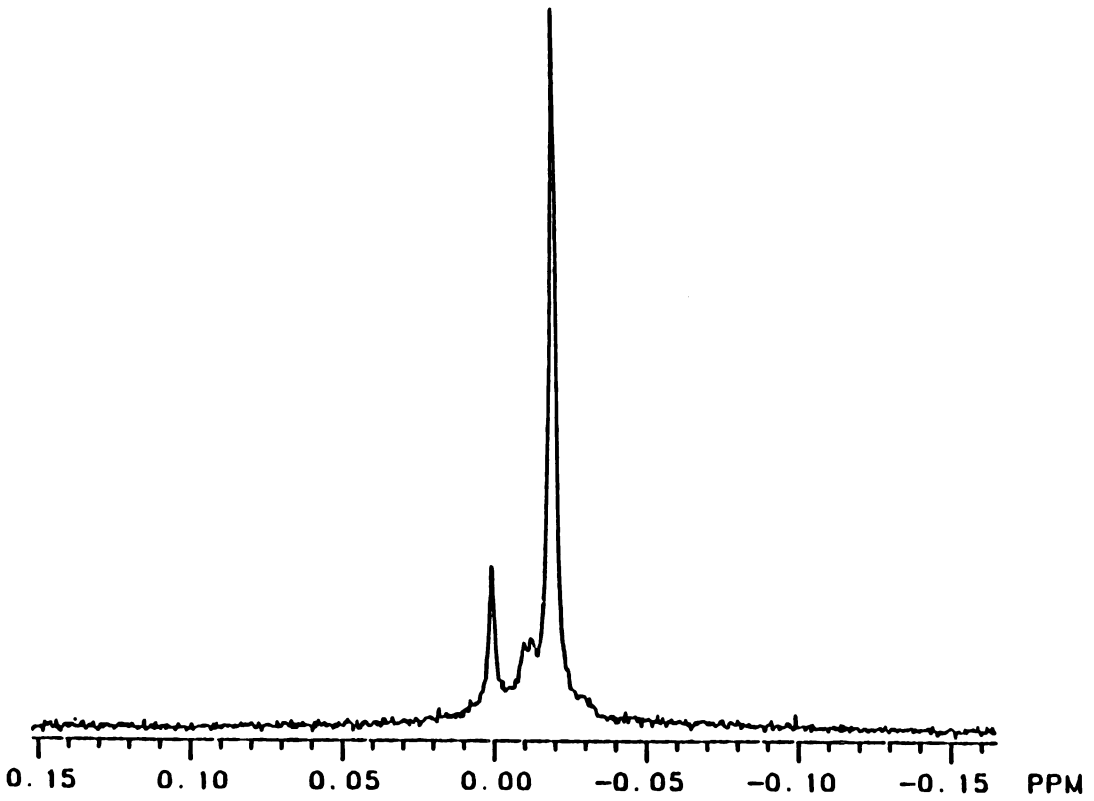
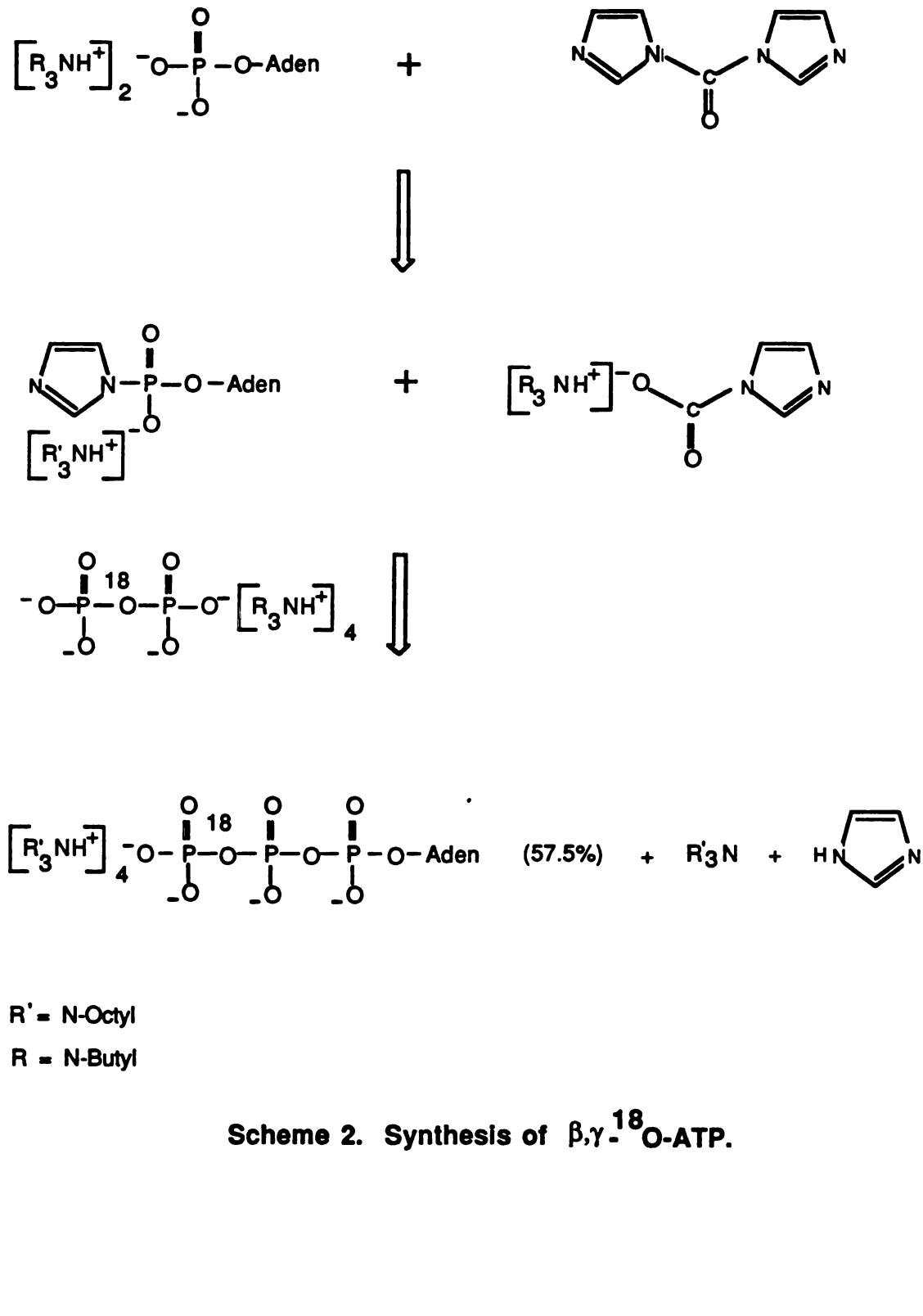


Figure 1. ^{31}P NMR at 202.5 MHz. of ^{18}O - pyrophosphate.



Scheme 2. Synthesis of β,γ-^{18}O-ATP.

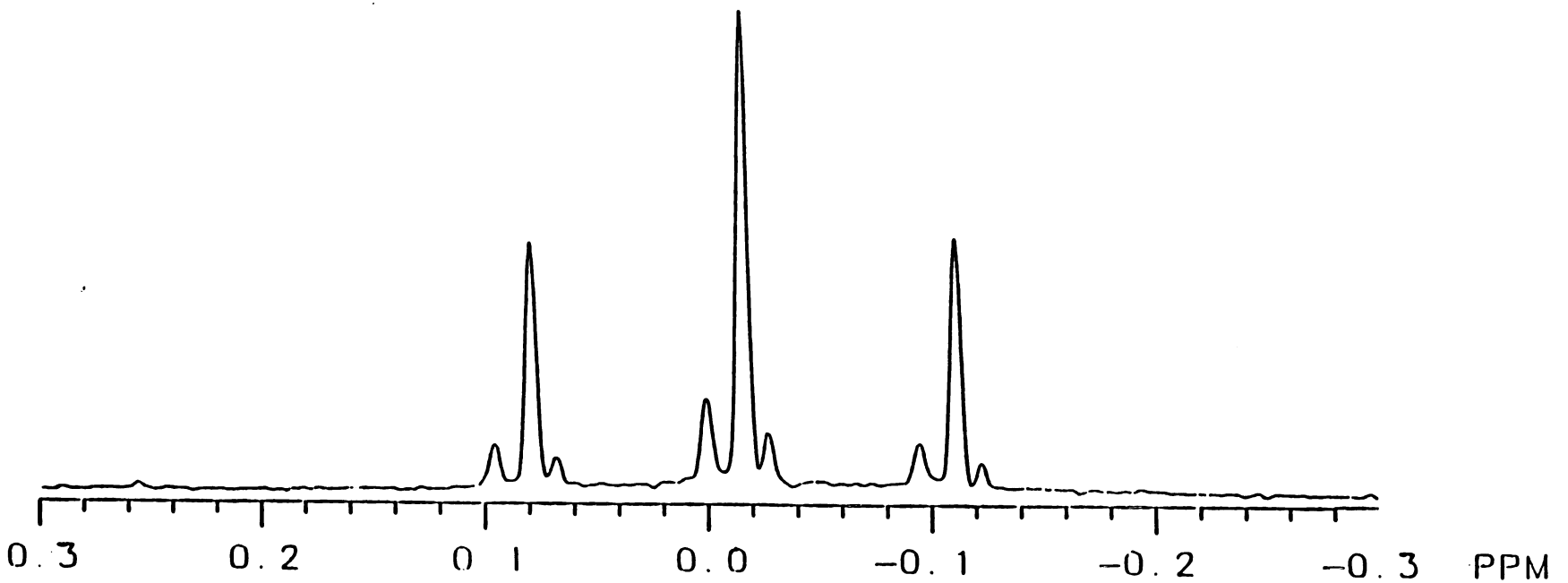


Figure 2. ^{31}P NMR at 202.5 MHz. of the β- phosphoryl resonances of ^{18}O - ATP.

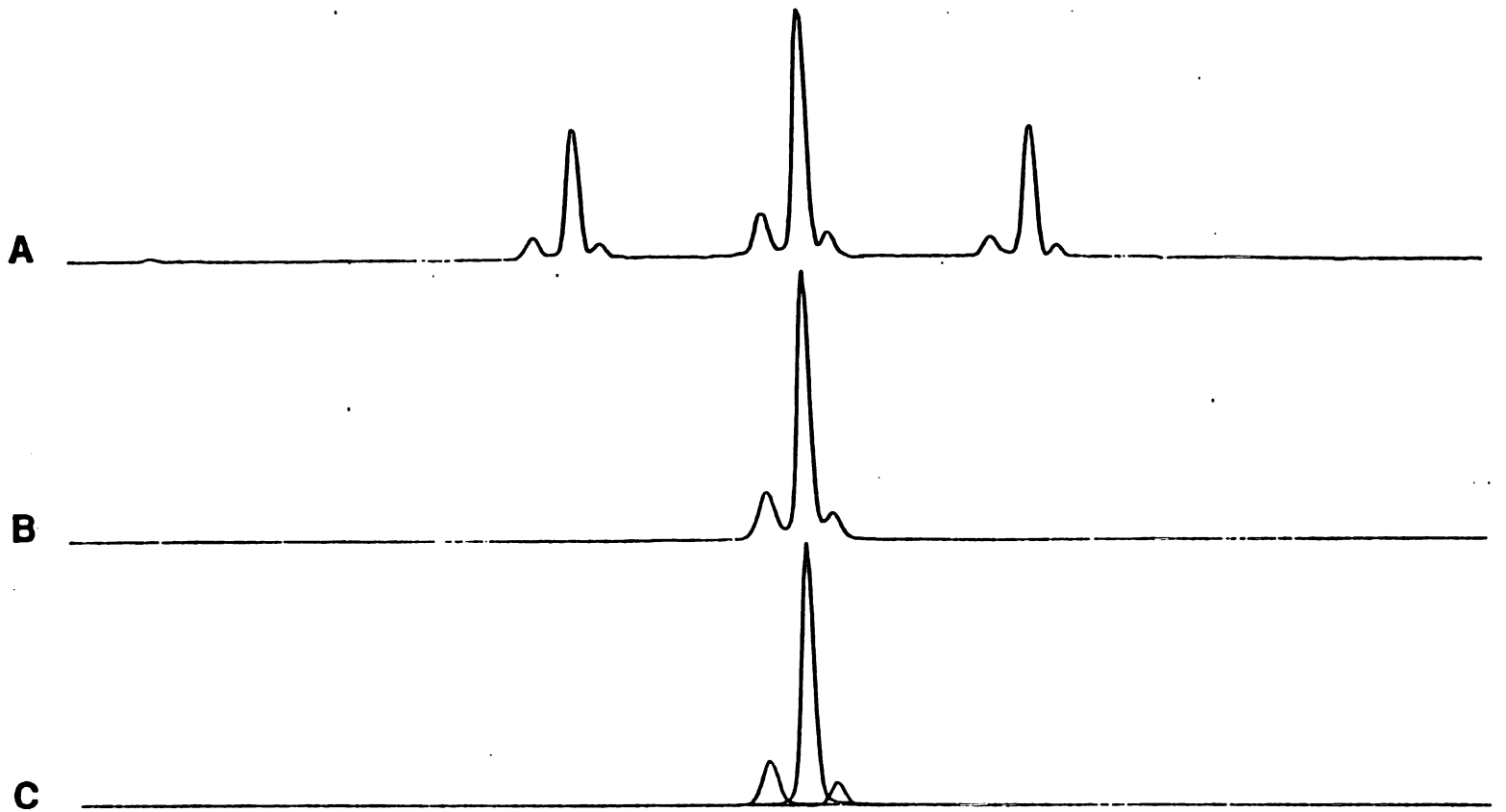


Figure 3. GEMCAP simulation of the β - phosphoryl resonances of β,γ -^{18}O-ATP.

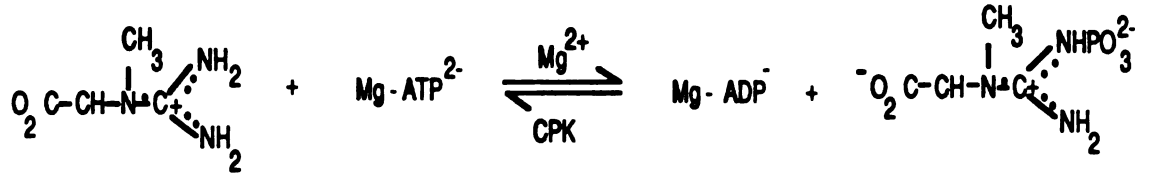
A.) Experimental ^{31}P NMR spectrum at 202.5 MHz. B.) GEMCAP simulation of the central peaks of NMR spectrum. C.) GEMCAP simulations of the individual resonances.

Table 1. Quantitation of GEMCAP routine for β,γ-^{18}O - ATP.

Chemical Shift (ppm)	Width	Intensity	Frequency (Hz)	Area	Area %
0.00	1.49	68	0.00	52	16.61
-0.016	1.49	387	-3.47	235	75.07
-0.027	1.40	37	-5.98	26	8.30

Creatine Kinase

Creatine kinase (adenosine 5'-triphosphate: creatine phosphotransferase, CPK, EC 2.7.3.2) catalyzes the reversible phosphoryl transfer from Mg^{2+}-ATP to creatine to form phosphocreatine and Mg^{2+}-ADP (Kenyon and Reed, 1983).



Phosphocreatine serves as a storage form of "high-energy phosphate" in muscle, heart, and brain tissue of all vertebrates. During periods of peak energy demand, the enzyme favors the formation of MgATP from phosphocreatine, which is the form used during metabolic need (Kenyon and Reed 1983).

The enzyme is an 82,600 MW unit consisting of identical 41,300 MW dimers. Under conditions of 0.25 M ionic strength, 38°C, and pH = 7.0, $K_{obs} = K_{eq} \cdot (H+) = 166$ where

$$K_{obs} = \frac{(MgATP)(Creatine)}{(MgADP)(Phosphocreatine)}$$

ATP BINDING SITE

The nucleotide binding site has been investigated by both chemical and NMR analysis which have provided information about the binding specificity for MgATP. Chemical modification studies with butanedione led Borders and Riordan to suggest the presence of an arginyl residue in the nucleotide binding site (Borders and Riordan, 1975). James found that the H-2 proton Nuclear Overhauser Enhancement (NOE) for ADP in its complex with creatine kinase was due to the β and γ methylene protons of an arginyl residue (James, 1976).

James and Cohn, using internuclear double resonance (INDOR) spectroscopy, inferred the presence of a lysyl residue near the transferable phosphoryl group (James and Cohn, 1974). It was the authors contention that the lysyl residue allowed the proper orientation for the phosphoryl transfer. In addition, they proposed that the electron-withdrawing properties of the lysyl ε-NH_3^+ group helped to facilitate attack by the nucleophilic creatine guanidino group upon the phosphoryl moiety. Rosevear and coworkers found the presence of a histidine residue in the vicinity of the nucleotide (Rosevear et al., 1985).

Steady-state kinetic studies by Morrison and James at pH = 8.0 indicated a rapid equilibrium, random bimolecular - bimolecular mechanism with the rate equation depicted in Scheme 1 (Morrison and James, 1965). Of notable exception to this is the interconversion of the central complexes [substrate complex = (PCr • MgADP•enzyme), product complex = (Cr • MgATP • enzyme)] which is slower than the dissociation rates of substates and products. Thus, the interconversion which involves phosphoryl transfer is the rate-limiting step. Engelborghs and coworkers, after extrapolating their kinetic data to zero, revealed that neither a transient phase nor a lag phase existed in either direction (Engleborghs, et al., 1975). They concluded that any possible isomerizations occurring before or after the chemical (catalytic) step must be much more rapid, thus confirming that the phosphoryl transfer is rate-limiting. Isotopic exchange work by Morrison and Cleland as well as Morrison and White were consistent with the proposed rapid equilibrium mechanism (Morrison and White, 1967; Morrison and Cleland, 1966). In addition, Morrison and Cleland found that an [enzyme • MgADP • creatine] dead-end complex occurred but found no evidence for an [enzyme • MgATP • phosphocreatine] complex (Morrison and Cleland, 1966).

The stereochemical course of the phosphoryl transfer in creatine kinase was studied by Hansen and Knowles (Hansen and Knowles, 1981). Using the adenosine S-(γ ^{16}O, ^{17}O, ^{18}O) triphosphate first synthesized by Blattler and Knowles (Blattler and Knowles, 1979) and determining the configuration at the Pγ by the method of Buchwald and Knowles (Buchwald and Knowles, 1982), they found the reaction to proceed with inversion of configuration, consistent with a single, associative, in-line transfer of the phosphoryl group between bound substrates.

Lowe and Sproat sought indirect evidence for an associative vs. dissociative mechanism by searching for the presence of PIX by ^{31}P - NMR spectroscopy (Lowe and Sproat, 1980). The authors conducted experiments using the inhibitor N-aminoiminomethyl-N-propylglycine and Mg-$\gamma(^{18}O_4)$-ATP. No evidence for PIX was found by ^{31}P NMR at 136 MHz. Lowe and Sproat concluded that since the creatine analog does not catalyze the turnover of MgATP to MgADP and the corresponding phosphorylated product, this was indirect evidence for an associative mechanism ($Sn2_P$) in the phosphoryl transfer step for creatine kinase.

Reed, Barton and Burns, while observing the planar ions SCN^-, N_3^-, and NO_3^- forming dead-end complexes with MgADP, creatine and creatine kinase, found evidence through IR data that the complex promoted a binding of these planar ions directly to the divalent cation (metal) despite intrinsically weak affinities of these anions for metal ions free in solution (Reed et al., 1978). The authors speculated that the coordination of a metaphosphate to the metal ion would not be expected to stabilize an already electron deficient intermediate. Hence, binding of the metal ion to the transferable phosphoryl group would favor an in-line or SN_2 process.

Reed and coworkers, using EPR spectroscopy to study the enzyme bound complex, were later able to identify six ligands about the metal ion (Reed and Leyh, 1980). They found that the Mn^{+2} is ligated to three non-exchangeable water molecules in the complex as well as to the three phosphate groups of ATP in the complex (see Figure 1).

Leyh and coworkers were further able to study the substrate : product complex by using sufficient enzyme to make the system substrate limiting. They found that on the enzyme, the K_{eq} for the substrate : product complex was approximately 1 (Leyh et al., 1985). In a series of experiments using pH-rate profiles and isotope trapping methods, Cook, Kenyon, and Cleland studied the partitioning of the enzyme • MgADP • phosphocreatine complex of creatine kinase (Cook et al., 1981). Using (^{14}C) phosphocreatine as a tracer, the authors found that the dissociation of phosphocreatine from the binary enzyme-phosphocreatine complex is 70-80 times faster than the net rate constant for catalysis and product release from the ternary complex. ($78 > k_2/k_5 > 67$). More importantly, however, was the observation that once MgADP was added to the assay, the partitioning of the [enzyme • phosphocreatine • MgADP] complex is about six times more in the direction of product-formation (enzyme • Mg-ATP • creatine) than back to substrates (enzyme: Mg-ADP: phosphocreatine) ($k_5/k_6 = 6$). This led to the conclusion that at pH = 7, phosphocreatine in the ternary complex (under conditions where the enzyme does <u>not</u> undergo a rapid random bimolecular-bimolecular mechanism) in the ternary complex is "sticky". Once bound, phosphocreatine became <u>commited</u> to catalysis and partitioned rapidly to products. At a pH above 8, the "stickiness" of the phosphocreatine disappears since catalysis is now slowed to the point where it is now rate-limiting. Reactant and product release, on the other hand, remain unaltered. This was consistent with the proposal for the existence of an active site histidine

which, during catalysis of phosphocreatine and MgADP to creatine and MgATP, acts as a general acid catalyst to provide a proton for the guanidinium nitrogen after the phosphoramidate bond of phosphocreatine is broken.

More recently, Reddick and Kenyon addressed the question of reversibility of the conversion of phosphocreatine and MgADP at pH = 8 when the mechanism is in the rapid random bimolecular-bimolecular form (Reddick, 1986). In this case, Reddick and Kenyon argue, one might expect to be better able to detect the existence of the reverse reaction since the "stickiness" of phosphocreatine becomes diminished. Since the reactant and product dissociation rates still remain constant under these conditions, catalysis now becomes the rate-limiting step. In this case, thus, we would now be investigating the possible interconversion of the substrate : product central complex. Reddick and Kenyon synthesized ^{15}N phosphocreatine selectively labeled in the phosphoramidate nitrogen. The authors used ^{31}P NMR (97.571 MHz) to investigate the rate of scrambling of the ^{15}N label from the phosphoramidate nitrogen into the guanidinium nitrogen when assaying the enzyme: ^{15}N-phosphocreatine: MgADP complex in the direction of creatine and MgATP formation. Since the J_{P-N} coupling is 18 Hz, one can see scrambling of the ^{15}N label as the intensity of the coupling should decrease and the ^{14}N-P bond (no J_{P-N} coupling) will exhibit a resonance increasing in intensity at a chemical shift between the two coupled resonances. Reddick and Kenyon found no evidence for exchange by NMR to a confidence level of 0.15 exchange/turnover ratio. Reddick and Kenyon concluded that the exchange rate is either not existent or below the level of detectability under their conditions. Alternatively, the detection of PIX might be masked since the guanidinium bond may either have a high barrier to rotation or be unusually restricted in the active site. Previous work by Kenyon et al. had concluded that

the rotational barrier about the guanidinium C-(N-Me) bond is not high enough in free solution to restrict rotation (G_{rot} = 14 kcal mole^{-1}, k_{rot} = 2.0 x 10^4 sec^{-1} (25°C)) (Kenyon, et al., 1976). Thus, Reddick and Kenyon addressed the likelihood of very restricted rotation about the guanidinium bond in the active site. This is a very plausible hypothesis as the k_{rev} value as well as the enzyme • MgATP • creatine complex concentration, Reddick and Kenyon argue, <u>are neccessarily non-zero values</u>. This would imply the back reaction must exist, but cannot be detected. Restricted rotation of the guanidinium in the active site would be consistent with an undetectable PIX. Should this be the case, the lack of PIX would imply a regiospecificity in the back reaction. In other words, during catalysis, the two nitrogens on the guanidinium group <u>are distinguishable</u> once bound to the enzyme. The question of regiospecificity had been indirectly addressed earlier by Struve and coworkers (Struve et al., 1977). Struve and coworkers, synthesizing the two possible phosphorylated products of the creatine analog 1-carboxymethyl-2-iminoimdiazolidine, compared the NMR spectrum of the isolated phosphorylated product after incubation with enzyme to the synthesized product. Their results showed that the enzyme produced only one phosphorylated product, that being the 1-carboxymethyl-3-phosphono-2-iminoimidazolidine product.

Indeed, it would be very interesting to study catalysis in the reverse direction by utilizing ^{18}O–β,γ-ATP ; however, in order to eliminate the possibility of PIX being a result of the product β - ($^{18}O_1$)-ADP acting in the reverse direction by becoming unbound and rebound, one <u>must</u> use a coupling system to remove all product ADP. Unfortunately, this experiment is not as yet feasible since an adequate coupling system has not yet been developed.

In a recent EPR study by Leyh and coworkers, the coordination of the MnATP complex was determined (Leyh, et al., 1985). Using the creatine

inhibitor 1-(carboxymethyl)-2-iminoimidazolidin-4-one (carboxycreatinine) , the specifically labeled substrates (α-^{17}O)-ATP, (β-^{17}O)-ATP, and (γ-^{17}O)-ATP, and Mn^{+2} ion, Leyh and coworkers found simultaneous coordination of the metal ligand to all three phosphate oxygens. The finding of this α,β,γ tridentate complex of Mn(II)-ATP thus predicts a plausible picture for the mechanism of catalysis, depicting a coordination that would require little conformational change to induce an in-line associative mechanism. Of recent interest was the finding by Reed and coworkers (unpublished results) that upon prolonged incubation of the β (^{17}O)-ATP with Mn^{2+} and carboxycreatinine, a slow but discernible disappearance of the Mn^{2+} - β-phosphate induced EPR relaxation occured (Reed, 1986). At the same time very little ATP hydrolysis was evident. A possible explanation of this result could be the breaking of the P_β - O bond with subsequent scrambling (torsional rotation) of the ^{17}O label. However, another explanation is that the equilibrium could lie very far to the side of the substrates such that catalysis may occur but without any discernible turnover to products.

We have investigated this possibility by seeking evidence for positional isotope exchange of β,γ-^{18}O-ATP. If the P_β - O bond is cleaving and randomizing the label in the back reaction, this should be evident by ^{31}P NMR as described in the introduction of this thesis.

Materials and Methods

Creatine kinase was initialy purified from rabbit muscle according to Method B of Kuby et al (Kuby et al., 1954). The FPLC method for subsequent repurification was developed by Smithers and Reed (Smithers and Reed, 1986). The purified protein was stored as a 30 - 50 mg/ml solution at 4°C prior to final purification by FPLC. Sodium azide (0.05%) was added to inhibit bacterial growth. This stock solution was then either dialyzed or gel filtered on Sephadex G - 25 into 10 mM Tris/HCl, pH = 8.0 and passed through a 0.22 μ membrane filter to remove particulate matter. A 25 - 50 mg aliquot was then injected onto an HR 10/10 Mono Q column (8 ml bed volume)(Pharmacia) previously equilibrated with the same Tris/HCl buffer. The column was then developed with the following:

Eluent A	10 mM Tris/HCl pH = 8.0
Eluent B	10 mM Tris/HCl 0.3 M NaCl pH = 8.0
Flow rate	4.0 ml/min.

The following column elution profile was utilized to isolate repurified creatine kinase:

	Total volume
1) 0 % B	64 mL
2) 0 % B - 4 % B	32 mL
3) 4 % B	32 mL
4) 4 % B - 25 % B	40 mL
5) 25 % B - 100 % B	8 mL
6) 100 % B	40 mL
7) 100 % B - 0 % B	8 mL
8) 0 % B	16 mL

Detection of proteins were monitored by UV analysis at 280 nm. The fraction corresponding to creatine kinase was then collected and pooled for NMR experiments. Figure 2 displays the elution profile, clearly displaying the elution of creatine kinase followed by other unidentified protein fractions.

Creatine kinase was assayed for activity by coupling ADP production to pyruvate kinase(Sigma) and lactate dehydrogenase(Sigma). Saturating amounts of creatine, ATP, and phosphoenolpyruvate were used. The activity was monitored by measuring the oxidation of NADH at 340 nm. Protein assays were performed according to the method of Bradford (Bradford, 1976). Specific activity for experiments varied from 50 - 60 units/mg.

Three 10 mL cocktails of ^{18}O-β,γ-ATP, 10 mM, and $Mg(OAc)_2$, 8mM, were prepared in 50 mM HEPES buffer (pH = 8). To one aliquot was added 20 mM carboxycreatinine, and to two were added 10 mg creatine kinase. The mixtures were incubated at 37°C in a shaker incubator. Hydrolysis of ATP was followed periodically by FPLC (Pharmacia) using an anion exchange column. Aliquots (20 µL) were removed at regular intervals and quenched immediately with 180 µL of 10% trichloroacetic acid (Pierce- HPLC spectrograde). The quenched mixture was then centrifuged for one minute with a Brinkmann 5414 centrifuge and 1.5 mL Eppendorf vials. The supernatant was eluted with a 0.5M - 1.0 M gradient of NH_4^+-HCO_3^-. Cocktails were incubated a total of 38 hours at which time they were quickly frozen. The solutions were then diluted ten-fold with distilled-deionized H_2O, the pH adjusted to pH = 7.6, and loaded onto a DEAE Sephadex A-25 anion exchange column and eluted with a TEA-HCO_3^- gradient, pH = 7.6, 0.07M - 0.7M. Elution was similar to that described for ATP isolation with nitrogenase. The ATP was isolated and prepared for nmr as previously described.

Denaturing polyacrylamide gel electrophoresis (PAGE SDS GEL) consisted of 20% polyacrylamide. Slabs were of 0.75 mm thickness. Gels were developed at either 7 amperes for a duration of 8 hours or 15 amperes for a duration of 1.5 hours. Standards consisting of five protein markers with molecular weights of 14.4 K, 20.1 K, 43 K, 67 K, and 94 K were obtained from Pharmacia. Molecular weights were determined by using a calibration curve of the log of the molecular weight standards vs. the distance migrated from the origin.

RESULTS

As can be seen in Figure 3, we see an obvious change in the bridge and non-bridge intensities; the unlabeled intensity remaining constant. The increase in the non-bridge intensity correlates very well to the relative decrease in the bridge peak, as would be expected since no washout of the ^{18}O label occurs. Unexpectedly, however, the control, lacking the inhibitor, also exchanged relative to the absolute control (see Figure 4). Comparing the amount of PIX, quantitation shows that the control (- inhibitor) underwent PIX more than the dead-end complex. One possibility comes immediately to mind. Should there be traces of contaminant in the enzyme, ie. adenylate kinase, one would expect scrambling of both the dead-end complex and control. Another possibility could be the role of acetate or bicarbonate acting as a substrate. Bicarbonate has been well documented as a substrate in a variety of ATP-utilizing enzymes (Raushel and Villafranca, 1980; Midelfort and Rose, 1976; Coon, 1959). In addition, Wimmer et al. have identified acetate as a substrate in the ATPase reaction of the enzyme (Wimmer et al., 1979). Should either of these act as a substrate, formation of a mixed anhydride intermediate is possible, allowing reversal of the reaction and hence, PIX. This would be consistent with finding PIX in both the control and the dead-end complex. Acetate could be implicated because it is the counterion of the Mg^{+2} salt in the assay mixture.(other anions are inhibitors). Lowe and coworkers, using a different inhibitor, found no evidence for PIX with creatine kinase; however, under their conditions, the incubation was over a significantly shorter period of time. The current incubation period was 38 hours and much larger quantities of enzyme were used compared to the Lowe experiments. Conceivably, if exchange were slow, we could find evidence for PIX whereas Lowe and

coworkers would not. A final possibility would be creatine kinase having an inherent ATPase activity.

At this point, the preparation of creatine kinase used was subjected to SDS gel electrophoresis. As can be noted in Figure 5, one can clearly see four prominent bands migrating to a molecular weight of approximately <14 K, 28 K, 38 K, and 42 K. However, this gel appeared clearly overloaded. A second gel using 1/200th the protein than previously shows two prominent bands corresponding to a molecular weight of approximately 42 K and 38 K (see Figure 6).

We have addressed the issue of the cause of the exchange reaction by further purifying creatine kinase by FPLC. The procedure, modified from the isolation procedure of Kuby, adds an additional purification step by passing the enzyme through a Mono - Q column (Kuby, 1954; Reed, 1986). Repeating the ESR experiments performed previously, Reed and coworkers found that the signal in the spectrum did <u>not</u> dissipate significantly with time over the period of 21 hours. Repeating the NMR experiments previously reported reveals the result shown in Figure 7. As one can see, the control sample is now devoid of any exchange after repeating the conditions as described for the previous sample. Of significance, however is the fact that the experimental sample still exchanged to a significant extent (approx 25.4 % ± 4.1%) (see Figure 8). Hence, because of the fact that the control did not exchange, we can rule-out that exchange is due to acetate or HCO_3^-. We can also rule-out reversibility secondary to an intrinsic ATPase reaction of the enzyme to the extent of the hydrolysis seen after prolonged incubation; however, we cannot rule-out the fact that an ATPase reaction exists under the present conditions because the amount of hydrolysis of ATP in the presence of enzyme without

carboxycreatinine was still significant compared to the rate of hydrolysis in the absence of enzyme.

As stated in the Materials and Methods section, Figure 2 shows the elution profile of the stock creatine kinase during purification by FPLC on the Mono Q column. Clearly, the peak corresponding to creatine kinase was the most prominent protein; however, one can see that the fraction previously utilized contained significant amounts of other proteins. A PAGE GEL of the rechromatographed creatine kinase now reveals only a single prominent band at a molecular weight of 42 K. (see Figure 9) Although not visible after photographing the gel, there was evidence of a very slight band at molecular weights of 38 K and 34 K. (Currently, I am not clear as to the origin of the 34 K band since it was not evident in the previous protein lot. Considering that the protein source was the same, I am inclined to believe it to be artifactual.) Clearly, however, the protein is significantly more homogeneous than the same preparation prior to the FPLC treatment. An isoelectric focusing gel run on the same protein preparation revealed two distinct bands and a third very faint band. It is not yet clear as to the identity of the three components. The pI's ranged from 6.5 to 6.8 with the two prominent bands being approximately 6.65 and 6.8. Despite the existence of two prominent bands, one can conclude by the NMR results that, prior to purification, a contaminating ATPase protein was co-isolated in this preparation. Clearly, it can be concluded that the addition of carboxycreatinine to the incubation mixture was responsible for a slow exchange over 38 hours. The actual exchange: turnover ratio is difficult to determine because of a significant amount of hydrolysis in the control sample. Subtracting out the amount of hydrolysis in the control sample relative to the experimental, we arrive at an additional hydrolysis of 6.9%. This would imply an estimated exchange : turnover = 3.7.

DISCUSSION and CONCLUSION

The results and spectra discussed previously argue for the fact that carboxycreatine in the presence of ^{18}O-β,γ-ATP and creatine kinase induces a very slow yet discernible scrambling of the ^{18}O label from the bridge to the non-bridge position. Interestingly, in the absence of carboxycreatinine, NMR analysis reveals no scrambling.

The results generated point to two possible conclusions. First, the carboxycreatinine may not be an inhibitor but actually may be a substrate with an extremely slow rate of turnover. One would not otherwise be able to detect the carboxycreatinine acting as a substrate by means of conventional enzymatic coupling methods because, as shown above, 40 hours were required to elicit the results. Unfortunately, attempts to isolate a phosphorylated product have been unsuccessful. Alternatively, however, the inability to isolate a phosphorylated product is not surprising since the equilibrium constant, $K_{obs} = K [H^+]$, lies far to the side of MgATP and creatine. Consequently, the finding of PIX may be a reflection of a small and slow, but reversible, product formation. In addition, if the phosphorylated product is unstable, it is very likely that after 38 hours incubation, all but an undetectable amount of product would be susceptible to hydrolysis. This is consistent with turnover data which show 23.5% hydrolysis for the experimental sample (+ carboxycreatinine) vs. 16.6% for the control sample (- carboxycreatinine) vs. 8.1% for the ^{18}O-β,γ-ATP in the absence of any enzyme. In addition, the possibility also exists that a very slow conversion of the central substrate complex to the central product complex may be occuring followed by slow or rapid conversion back to the central substrate

complex. In this case, products would not be detected since they would never be released.

Secondly, one could argue that carboxycreatinine is an inhibitor of the ATPase reaction of creatine kinase but is inducing β,γ bridge to β non-bridge scrambling via formation of a metaphosphate intermediate. Precedence for metaphosphate intermediates have been postulated for over 30 years (Butcher and Westheimer, 1955; Barnard et al., 1955). Recently, Buchwald and coworkers (Buchwald et al. , 1984) investigated the stereochemical course of methanolysis of phenyl [^{16}O, ^{17}O, ^{18}O] phosphate and 2,4 dinitrophenyl [^{16}O, ^{17}O, ^{18}O] phosphate in aqueous methanol and found complete inversion of stereochemistry. These findings, in conjunction with previous data concluding the existence of a metaphosphate intermediate, led Buchwald and coworkers to conclude that phospho-group transfer may occur via a pre-associative pathway in which the metaphosphate-like species is never free but already formed in the encounter complex, or by an "exploded" associative transition state (Jencks, 1981). Recently, Friedman and Knowles (Friedman and Knowles, 1985) and Cullis and Rous (Cullis and Rous, 1986) found evidence for randomization of chiral phosphate groups in an organic solvent (acetonitrile), attributing the mechanism possibly to solvent association and rapid interconversion of the putative metaphosphate intermediate with the aprotic solvent. To date, however, no evidence of metaphosphate has been detected in H_2O (Jencks, 1981). There have been reports of a dissociative mechanism speculating a metaphosphate intermediate; (Lowe and Sproat, 1978) however, the data is still equivocal.

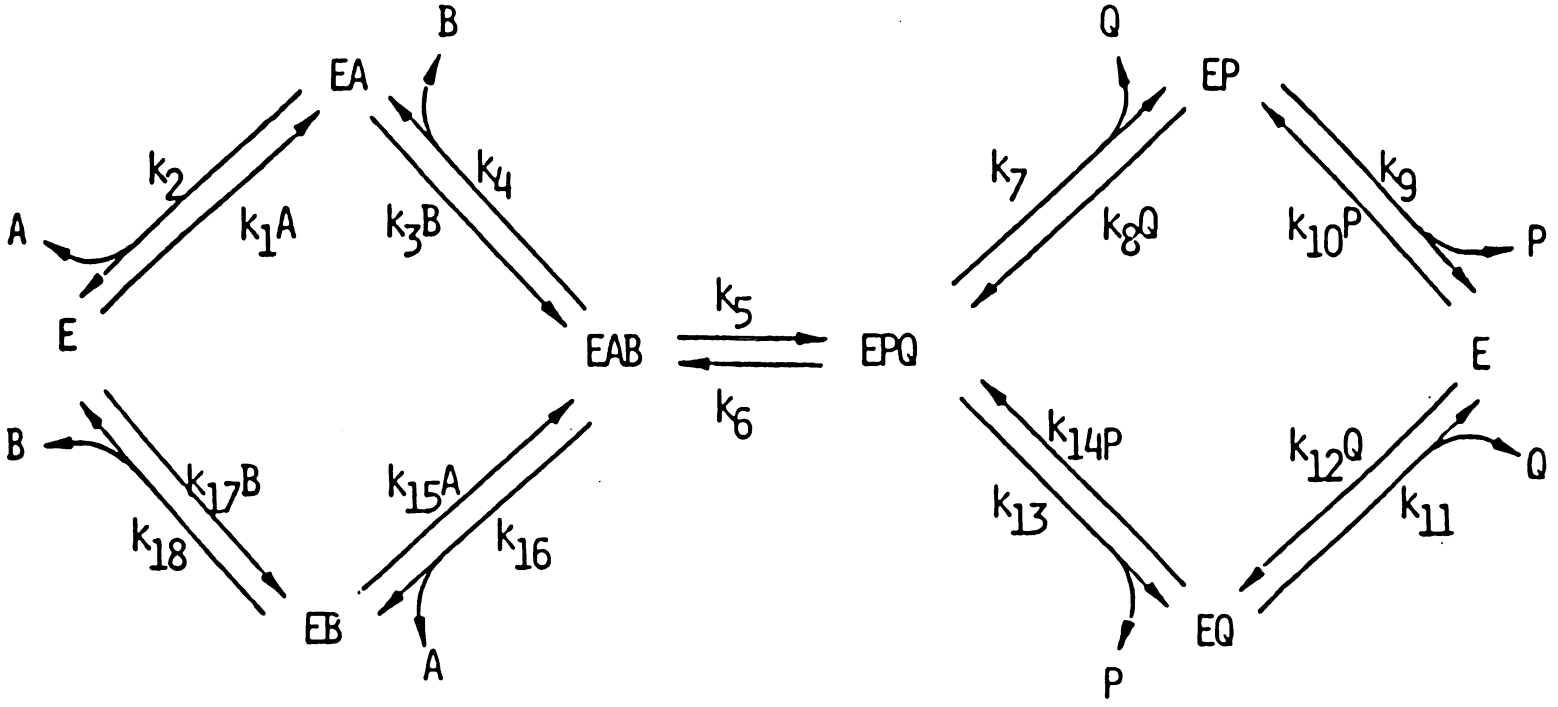
Pre-associative processes can be divided into concerted or stepwise. In a concerted mechanism, no intermediate, i.e., metaphosphate, is formed having a lifetime greater than one vibrational amplitude. This would require that bond

breaking be nearly complete while bond making has barely begun (Jencks, 1981). This has been termed by Jencks as an "exploded" or "loose" transition state. PIX in the presence of carboxycreatinine is inconsistent with this theory because even if carboxycreatinine were to form a bond, the lifetime of the transition state would be too short to allow any torsional rotation about the $P_\alpha O$-P_β bond. In addition, torsional rotation is not compatible with the existence of a transition state. Thus, one would expect a collapse back to the original substrate and hence, no PIX. If, however, the reaction is stepwise and involves metaphosphate, torsional rotation might be fast enough ($k_{rot} = 10^{12}$) (Midelfort and Rose, 1976) so that upon collapsing back to substrate, PIX may be allowed to occur.

Tuck and Lowe have studied this possibility by investigating the exchange of [β-^{18}O]-ADP in acetonitrile at 70°C (Tuck and Lowe, 1986). Although they found PIX, the authors questioned the conclusion of a stepwise pre-associative mechanism. It was the authors' contention that a [CH_3CN•PO_3^-•AMP] complex could allow the scrambling of the label in the α,β bridge [this is difficult to strictly rule-out]; however this was unlikely since control exchange experiments with ^{18}O-AMP and unlabeled ADP in acetonitrile at 70°C did not elicit any exchange. Thus, the authors felt the lifetime of the complex [CH_3CN^+-PO_3^{2-}•AMP] would be too short for scrambling and phosphoryl transfer of the phosphate group to occur. Alternatively, the authors suggested that if there is pre-association with a weak nucleophile, ie. acetonitrile, which cannot form a stable phosphorylated product, the extent to which the nucleophile can compete with the ultimate acceptor will determine the degree of racemization in stereochemical experiments. This can be considered consistent with our results if carboxycreatinine can form a transient phosphorylated product complex long enough for scrambling of the label to occur. This would be analogous to

formation of a transient [carboxycreatinine^{+}-PO_3^{2-} • ADP] complex whose lifetime is sufficiently long to allow scrambling of the ^{18}O label. Hence, collapse back to the ultimate acceptor (β-^{18}O-ADP) will lead to PIX. Clearly, all these phenomenon are slow processes (Tuck and Lowe required 40 hours and 70°C in acetonitrile to induce PIX. Our experiments required 55 IU/ml creatine kinase and 40 hours at 37°C to induce PIX).

The current data do not argue convincingly for either carboxycreatinine acting as a substrate acceptor to form phosphorylated product, or as an inhibitor allowing metaphosphate formation. Currently, we do not know of any experimental methods to strictly rule-out either of the two mechanisms. Hopefully, future work will resolve the mechanism in this case.



Scheme 1. The kinetic mechanism of creatine kinase where A = PCr; B = MgADP; C = Cr; Q = MgATP.

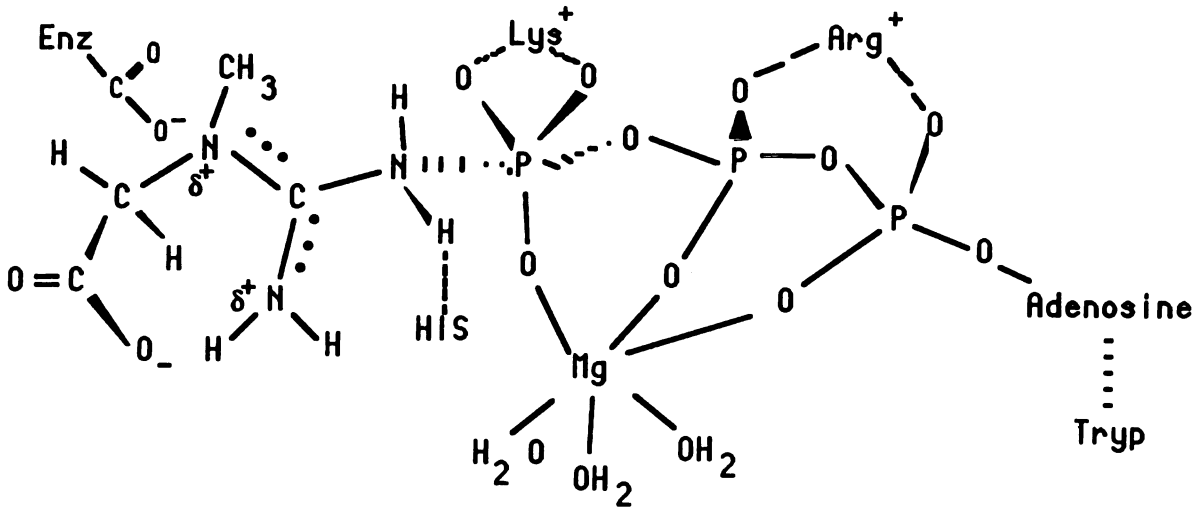


Figure 1.
Proposed active site of the central complex of creatine kinase.

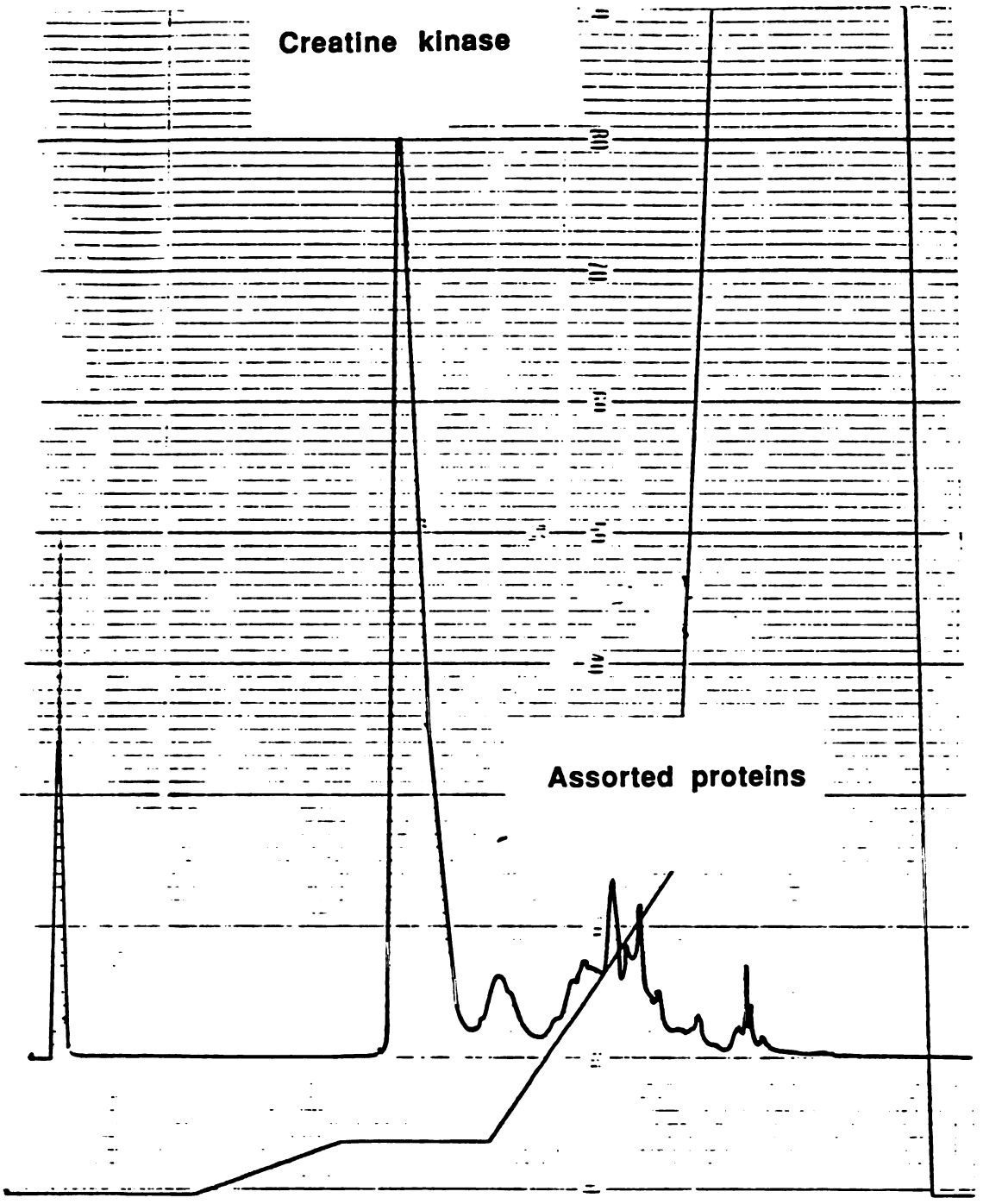


Figure 2. Elution profile of creatine kinase after repurification through an HR 10/10 Mono-Q anion exchange column. Detection was via UV spectrophotometry at 280 nm.

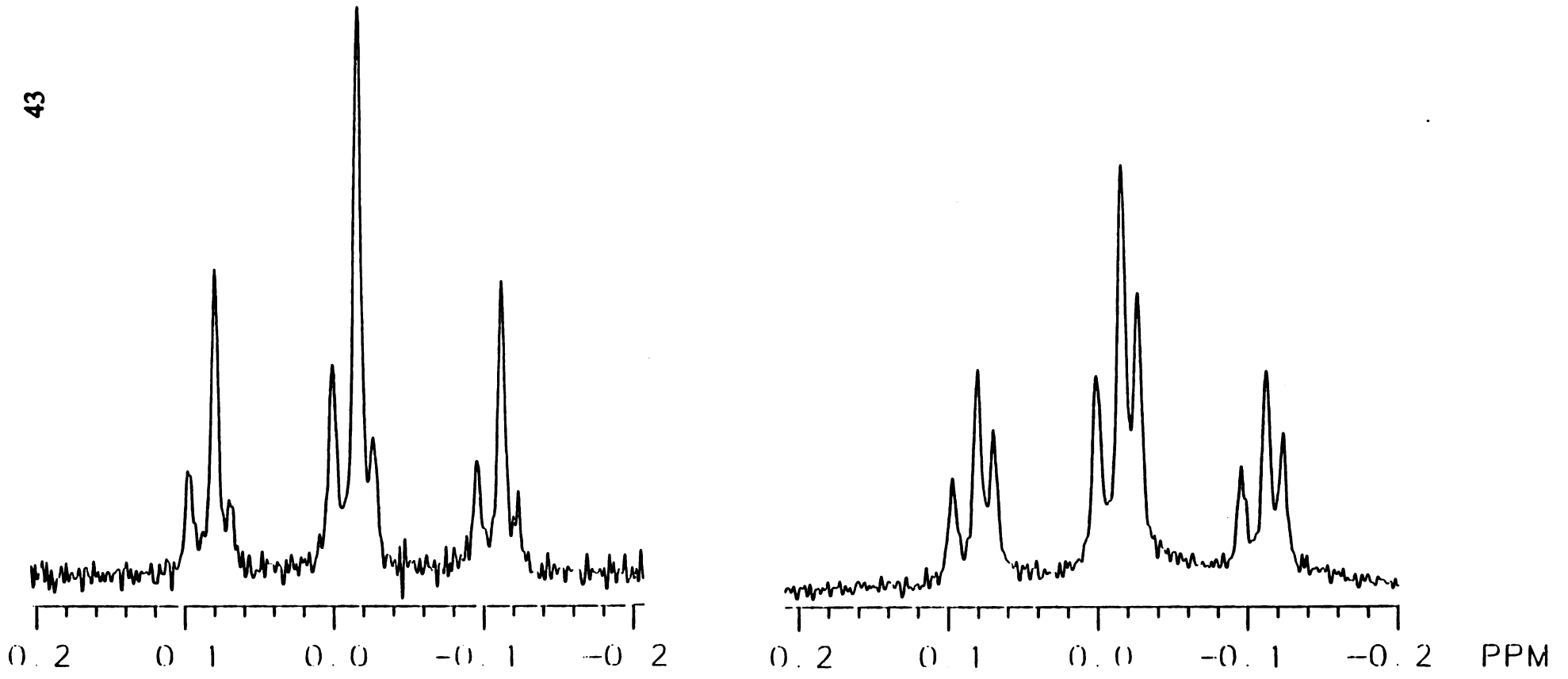


Figure 3. ^{31}P NMR spectra at 202.5 MHz of the β-phosphoryl group of β,γ-^{18}O-ATP. Incubation solution contained 8 mM magnesium acetate, 20 mM carboxycreatinine, and 10 mM labeled ATP at 37° C in 50 mM HEPES buffer at pH = 8.0. Spectrum on the left is after 38 hour incubation without enzyme. Spectrum on the right is with enzyme.

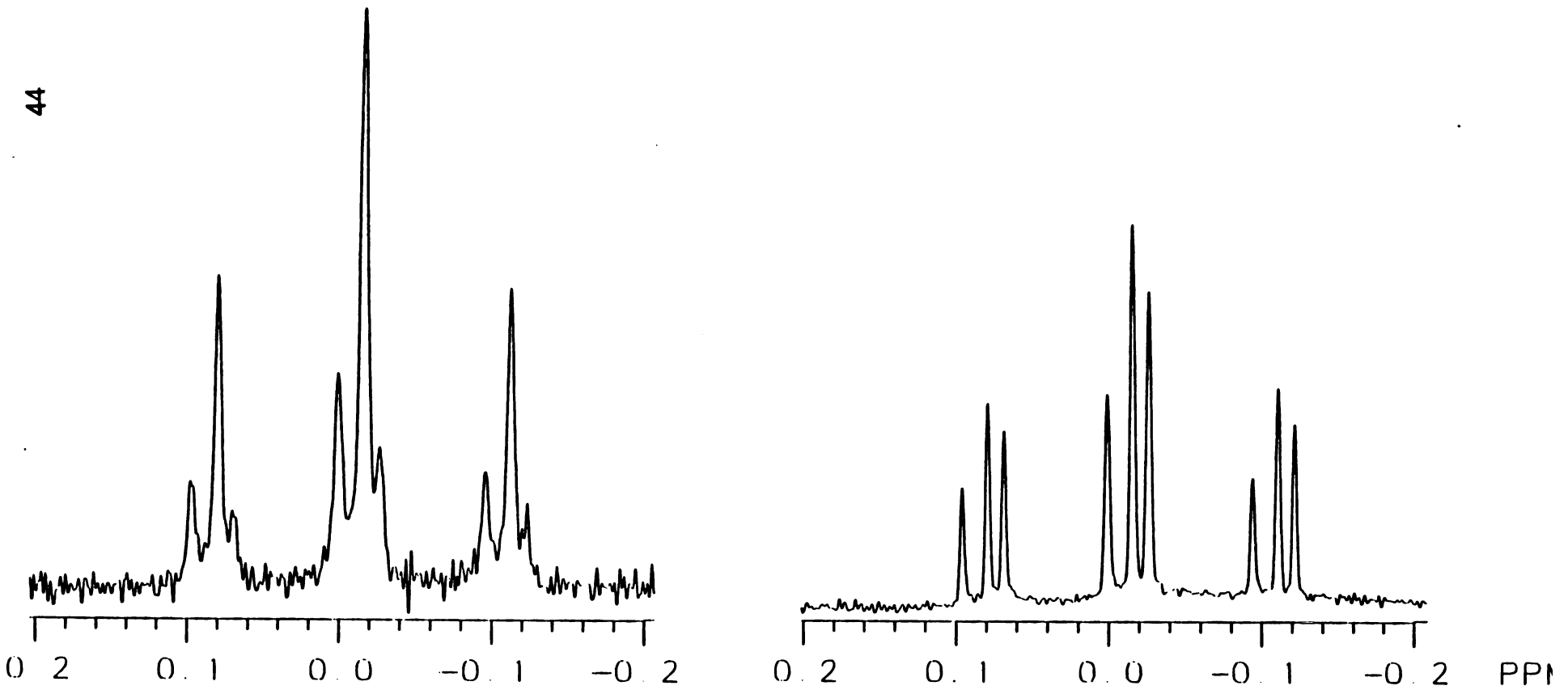


Figure 4. ^{31}P NMR spectra at 202.5 MHz of the β -phosphoryl group of β,γ-^{18}O-ATP. Incubation conditions were identical to those described in Figure 3 except carboxycreatinine was eliminated. Spectrum on the left is after 38 hour incubation at 37° C without enzyme. Spectrum on the right is with enzyme.

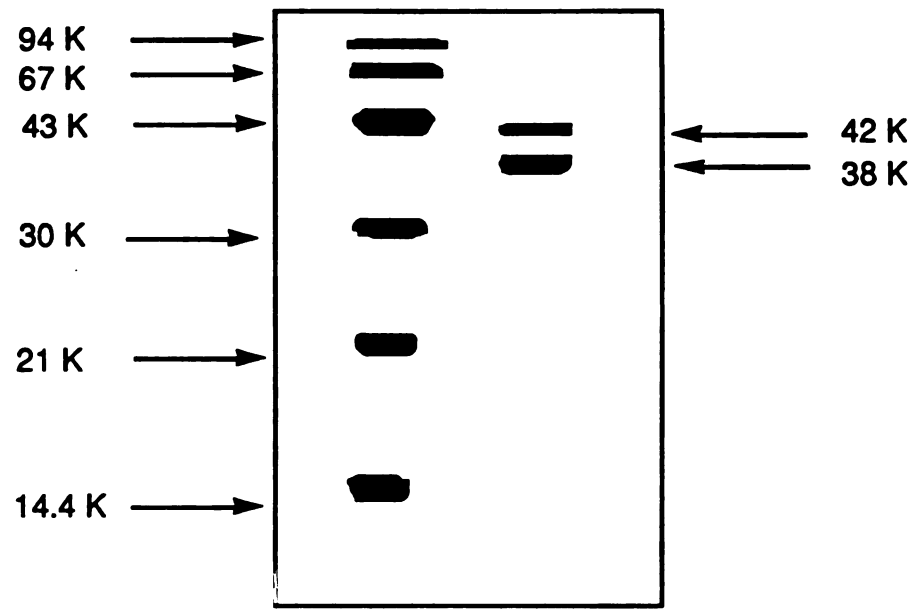


Figure 5. SDS polyacrylamide gel of purified creatine kinase prior to repurification by fast protein liquid chromatography. Gels are recopied from original by the "Thunderscan" program on an Apple Macintosh computer.

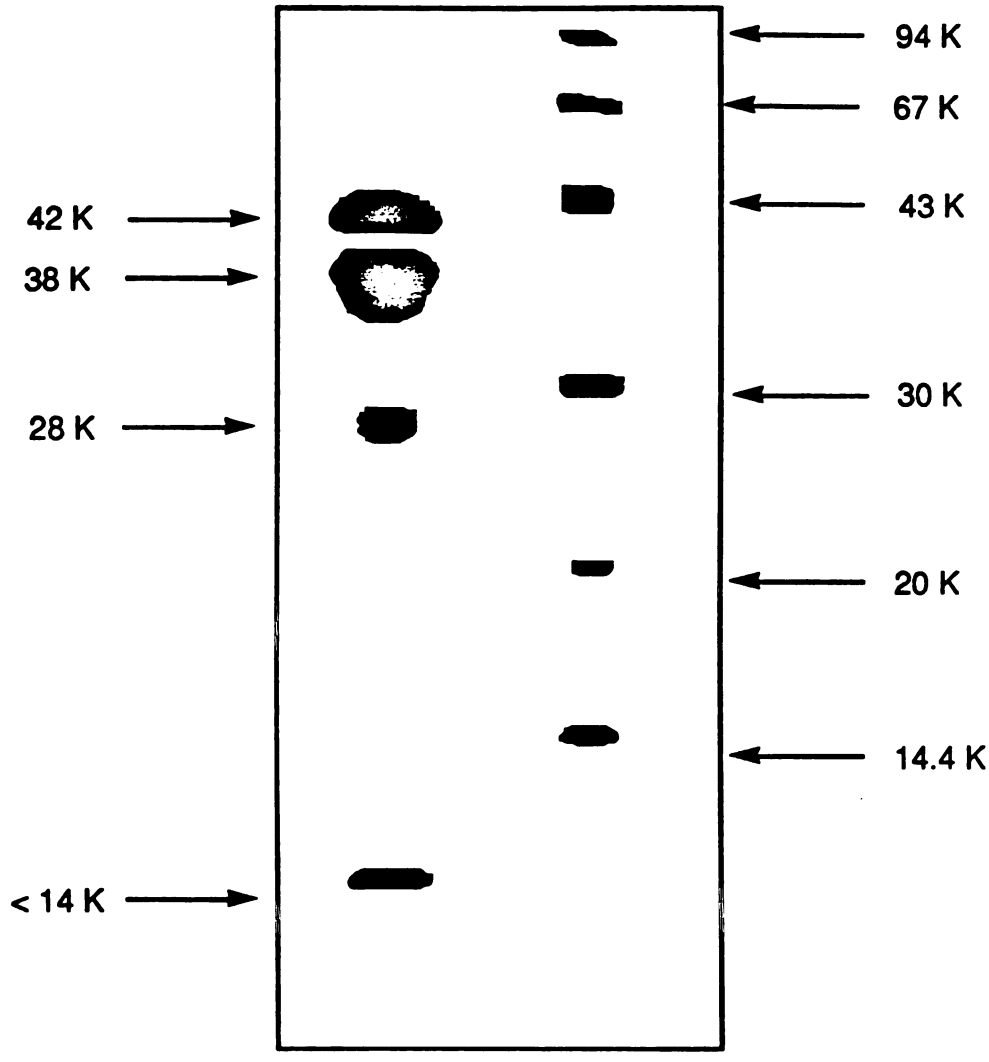


Figure 6. SDS polyacrylamide gel of purified creatine kinase prior to repurification by fast protein liquid chromatography on a Mono-Q anion exchange column. Note creatine kinase sample is from the same source as in Figure 5 but sample is overloaded. However, one can note two additional bands now visible.

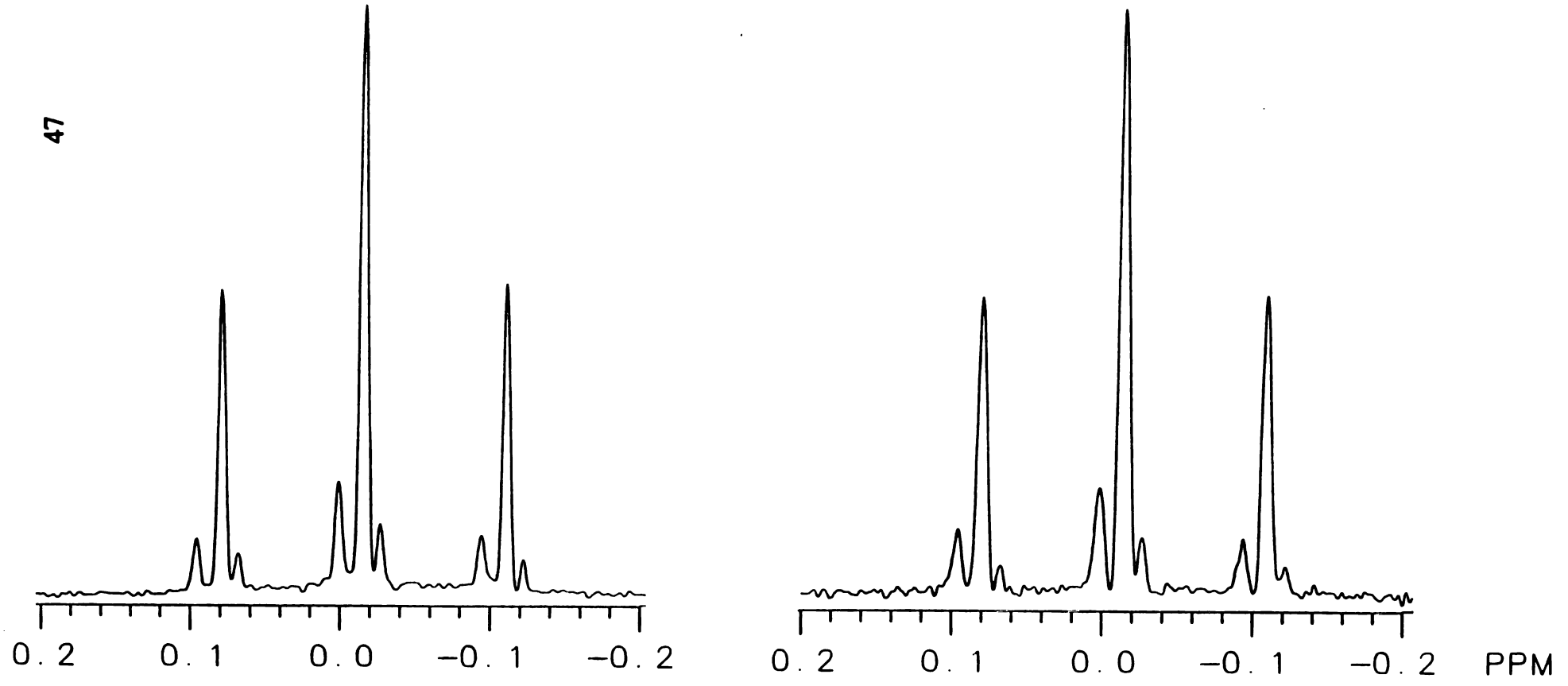


Figure 7. ^{31}P NMR spectra at 202.5 MHz of the β-phosphoryl group of β,γ-^{18}O-ATP. Incubation conditions were identical to those described in Figure 4 (without carboxycreatinine). Spectrum on the left is after 38 hour incubation without enzyme. Spectrum on the right is after incubation with enzyme.

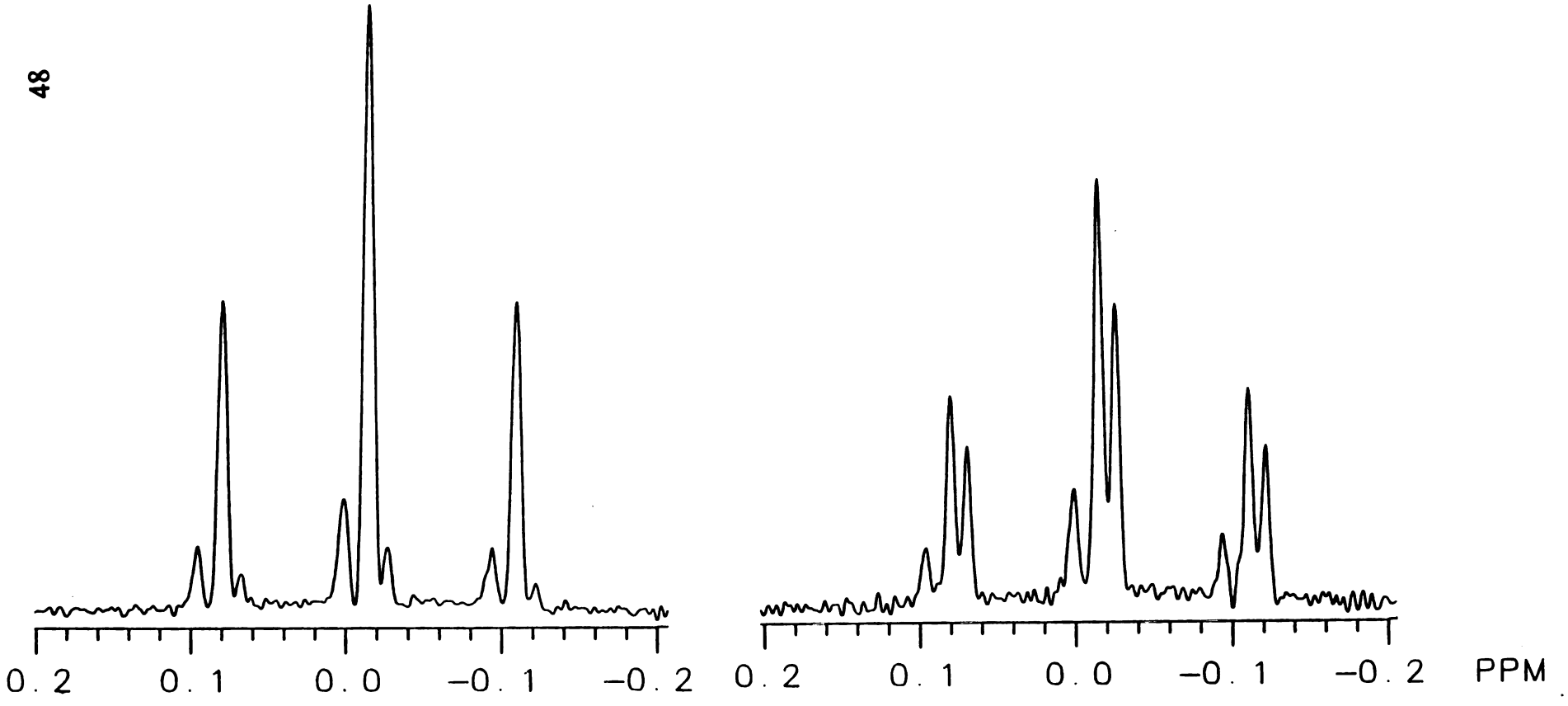


Figure 8. ^{31}P NMR spectra at 202.5 MHz of the β -phosphoryl group of β,γ-^{18}O-ATP. Incubation conditions are identical to those described in Figure 3 (with carboxy-creatinine). Spectrum on the left is after 38 hour incubation without enzyme. Spectrum on the right is with enzyme.

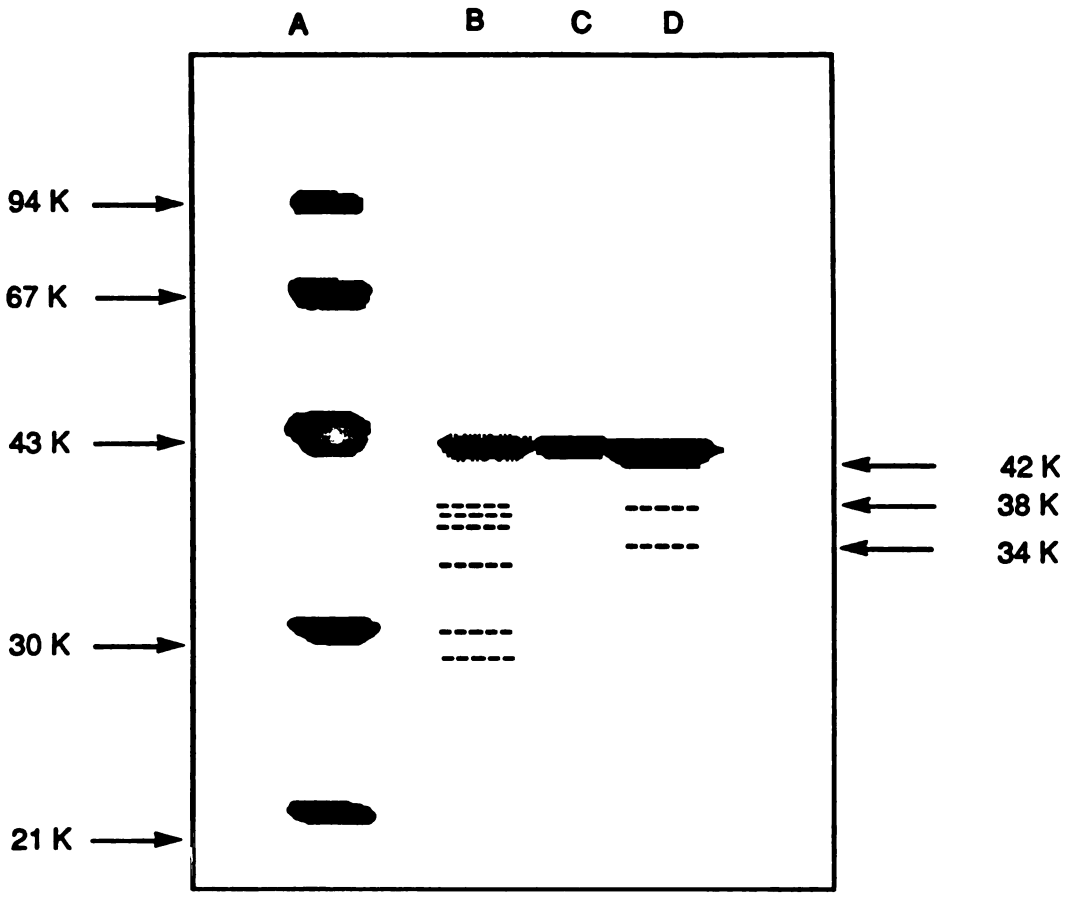
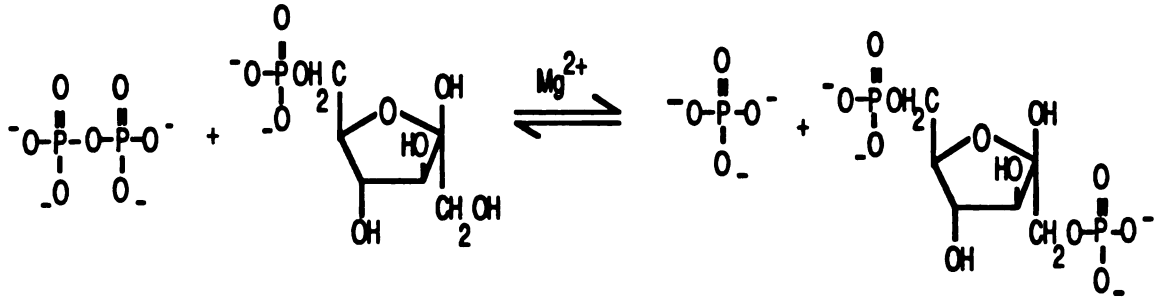


FIGURE 9. SDS polyacrylamide gel electrophoresis of creatine kinase after repurification by fast protein liquid chromatography using an HR 10/10 Mono - Q anion exchange column.
Lane A. Protein standards with molecular weights as labeled.
Lane B. Creatine kinase from Sigma.
Lanes C and D. Repurified creatine kinase with different amounts of protein. Broken lines denote faint bands of protein that were barely detectable.

Pyrophosphate: D-fructose 6-phosphate 1-phosphotransferase

Pyrophosphate: D-fructose 6-phosphate 1-phosphotransferase (PPi-PFK) (EC.2.7.2.90) is a 95,000 MW dimer that catalyzes the reaction;



The enzyme was first discovered by Reeves and coworkers in 1974, isolating the enzyme from *Entamoeba histolytica* (Reeves et al., 1974). O'Brien and coworkers isolated PP_i-PFK from the bacteria *Propionibacterium shermanii* (O'Brien et al., 1975). The enzyme has also been reported in aerobic marine bacteria (Sawyer et al., 1977), photosynthetic bacteria (Pfleiderer and Klemme, 1980), and both photosynthetic and non-photosynthetic plant tissues (Carnal and Black, 1979; Sabularse and Anderson, 1981; Van Schaftingen et al., 1982 ; Kruger et al., 1983), and the cytoplasm of spinach leaves (Cs'eke et al., 1982). To date, there is no evidence of this enzyme in animal tissue. Interestingly, the enzyme isolated from Mung bean, *Phaseolus aureus* , becomes 5 $x10^2$ times more active in the presence of the allosteric activator fructose-2,6-bisphosphate (F2,6dP) (Sabularse and Anderson, 1981a; Sabularse and Anderson, 1981b). The enzyme is specific for F6P and F1,6dP as no other mono or disubstituted sugar can by substituted(O'Brien, et al., 1975). As well, no nucleotide triphosphate or polyphosphate can replace PP_i in the forward reaction. Arsenate, however, can replace phosphate in the reverse reaction. In *E. histolytica* , Mn^{2+}, and Co^{2+} increase the forward rate in the

presence of Mg^{2+}; however, alternately, Ni^{2+}, Zn^{2+}, and Ca^{2+} inhibit the rate (Sawyer et al,1977).

Initial studies by Reeves and coworkers postulated the enzyme mechanism to undergo a random bimolecular-bimolecular mechanism (Reeves et al., 1976). In addition, F6P and P_i were non-competitive with respect to each other, suggesting the formation of a dead-end complex being formed. Work by Bertagnolli and Cook found that sulfate competitively inhibits P_i. In disagreement with Reeves and coworkers, Bertagnolli and Cook found that the reverse reaction revealed evidence for a terreactant type of mechanism. They found Mg^{2+} as the third substrate. In the forward reaction, the Mg^{2+} pyrophosphate chelate was the substrate. Their data was consistent with a rapid equilibrium, random bimolecular-termolecular mechanism (Bertagnolli and Cook, 1984). In addition, exchange against the flow experiments using ^{32}P phosphate while looking at the phosphoryl transfer of F6P, Bertagnolli & Cook found no evidence for exchange.

Despite the fact that exchange against the flow showed no incorporation of ^{32}P into the pyrophosphate starting material, this does not address the question of exchange possibly occurring on the enzyme during catalysis. In other words, during catalysis, is there a fast exchange between the substrate complex (enzyme • Mg-PP_i • F6P) and product complex (enzyme • Mg^{2+} • P_i • F1, 6 dP) or alternatively, once the substrate complex is formed, are they commited to turnover and release? Exchange against the flow studies address the question of released products binding again to enzyme and being catalyzed back to substrates but does not address the question of what is occurring to the enzyme-bound complex.

We have addressed this question by looking for positional isotope exchange (PIX) by ^{31}P nmr and the compound ^{18}O bridge labeled

pyrophosphate synthesized from the method of Reynolds et al (Reynolds et al, 1983). Experiments have been conducted for the enzyme isolated from *P. freudenreicheii* and from the *P. Aureus* both in the presence and absence of F2,6dP.

MATERIALS AND METHODS

Initial velocity measurements were made by coupling Fructose 1,6-bisphosphate to aldolase, triosephosphate isomerase , and glycerolphosphate-dehydrogenase while monitoring the oxidation of NADH by observing the disappearance of absorbtion at 340 nm. Alternatively, product inhibition studies were conducted by coupling inorganic phosphate production to glyceraldehyde-3-phosphate dehydrogenase and 3-phosphoglycerate kinase while monitoring reduction of NAD^+ by observing the increase in absorbtion at 340 nm. Assays were done in one mL aliquots and catalyzed by the addition of approximately 0.01 units (1 µL) of PPi-PFK.

Sodium pyrophosphate decahydrate, fructose 6-phosphate, fructose 1,6 bisphosphate, NAD^+, NADH, Fructose 2,6 bisphosphate, $MgCl_2 \cdot 6\ H_2O$, and D-glyceraldehyde 3-phosphate (diethyl acetal, dicyclohexylammonium salt), ADP, and TAPS [tris(hydroxymethyl)methylaminopropane sulfonic acid] were obtained from Sigma. Magnesium sulfate and magnesium chloride were obtained from Mallinckrodt.

^{18}O Bridge labeled pyrophosphate was synthesized according to the method adapted from Reynolds et al (Reynolds et al., 1983). Quantitation of bridged, nonbridged and unlabeled ^{18}O pyrophosphate were calculated by taking the relative peak areas from the ^{31}P nmr spectra taken at 97.57 MHz. and 202.45 MHz. as representative of the respective enrichments. These measurements were made using the GEMCAP routine from the Nicolet software package (see Appendix 1).

The D-glyceraldehyde 3-phosphate was prepared by dissolving 50 mg of the diethyl acetal with 2 mL of H_2O and approximately 2 ml of Dowex 50W-X8 in the H^+ form. The solution was submerged in boiling H_2O for approximately 30 seconds, quickly cooled, and the resin removed by filtration. The orange tinted

solution was either used immediately or quickly frozen and stored at -20°C for later use. Assays were run in 100 mM TAPS buffer, pH = 8.0. Fructose 1,6-diphosphate aldolase (E.C. 4.1.2.13) from rabbit muscle, triose-phosphate isomerase (EC.5.3.1.1) and glycerol phosphate dehydrogenase (EC.1.1.1.8) from rabbit muscle were obtained as the ammonium sulfate suspensions and the sulfate-free lyophilized powder from Sigma. If the ammonium sulfate suspension was used, the enzymes were rigorously dialyzed (3 x 1 : 1000 dilution) against PIPES buffer, pH = 7.3 (4°C) prior to use.

CORRECTION FOR METAL CHELATE COMPLEXES

The following dissociation constants were used. Mg-PP_i, 0.002 mM; Mg-FDP, 2 mM; Mg-F6P, 25.7 mM, (Martell & Smith, 1977), Mg-$NADP^+$, 19.1 mM; Mg-NADH, 18.6 mM (Apps, 1973).

The concentration of total ligand was calculated from

$$[L_t] = [L_f] + [L_f][Mg_f] / K_{MgLI}$$

Total (and free) Mg^{+2} concentrations were calculated according to the following;

$$[Mg_t] = [Mg_f] + S\ [(Mg_f)(L_{If})] / K_{MgLI}$$

where $[L_t]$, $[L_f]$, $[Mg_t]$, and $[Mg_f]$ are total and free concentrations of ligand and Mg^{+2} respectively. K_{MgLi} is the dissociation constant for the metal-ligand complex while K_{MgLi} is the dissociation constant for the ith component of the complex.

For product inhibition studies, it was noted by Dr. Paul Cook that a significant amount of dead-end complex forms (enzyme : FDP : $MgPP_i$) in the presence of the appropriate compounds (Cook, 1986). K_i values as communicated by Dr. Cook were as follows;

$K_{i(Mg\text{-}PPi)}$ = 23 mM

$K_{i(FDP)}$ = 0.65 mM

The following km values were used for calculations when appropriate.

	F6P	F1,6dP	$MgPP_i$
P. freudenreicheii	127 μM	30 μM	4.6 μM
Mung bean			
(+ 2,6 bis-phosphate)	200 μM	9 μM	
(- 2,6 bis-phosphate)	1 mM	65 μM	

The use of $SO_4^=$ salts were avoided because it acts as a competitive inhibitor vs $HPO_4^=$ and non-competitive vs FDP with a K_i = 20 mM.

Data for competitive inhibition was fitted to the following equation:

$$v = \frac{VA}{K_a(1 + I/K_i) + A}$$

where V is the maximum velocity and I is the concentration of F1,6dP. K_i is the inhibition constant for F1,6 dP in the dead-end complex.

From the ^{31}P spectrum generated at 97.3 MHz, the bridged, unbridged and unlabeled species corresponded to 73.1%, 10.0 %, and 16.9% respectively. Analysis was made by measuring peak intensities. At 202.5 MHz., the bridge, unbridged, and unlabeled species corresponded to 68.7 %, 15 %, and 16.3 % respectively. Analysis at 202.5 MHz. was determined by peak areas using the GEMCAP™ curve analysis and deconvolution on the Nicolet 1280 software package.

For product inhibition studies, concentrations of fructose 6 - phosphate concentrations were maintained at or no more than three times the km value

(0.127 mM). The amount of metal chelate complexes were corrected so as to keep the initial Mg^{2+} concentration at 1 mM free ligand in solution. ^{18}O-pyrophosphate concentrations were initially 0.5 mM. Fructose 1,6 bisphosphate concentrations were 0.65 mM (0.43 mM as the non-ligand substrate).

For turnover experiments using Ca^{2+}, $MgCl_2$ was replaced with 1 mM $CaCl_2$. Addition of higher concentrations were not feasible as the calcium pyrophosphate salt is insoluble (even at 1 mM $CaCl_2$, some precipitate was evident). Initial concentrations for remaining substrates consisted of the following:

	Concentration
^{18}O-pyrophosphate	10 mM
fructose 6-phosphate	10 mM
$CaCl_2$	1 mM

For turnover experiments with Ce^{3+}, incubation mixtures consisted of the following:

^{18}O-pyrophosphate	2 mM
$CeCl_3 \cdot 6\ H_2O$	2.02 mM
fructose 6-phosphate	2 mM
$MgCl_2$	0.5 mM

A 30 mL solution of 100 mM TAPS buffer (pH = 8.0) was prepared. To this was added magnesium chloride , 8 mM, fructose 6-phosphate (10 mM), and ^{18}O bridge labeled pyrophosphate (10 mM). A 3.0 mL aliquot was removed for assay purposes. To each 1 mL aliquot was added 1 µL of PP_i-PFK in 20% glycerol/PIPES buffer (pH = 7.2). Fructose 1,6 - bisphosphate aldolase, 13 units; glycerol-phosphate dehydrogenase, triosephosphate isomerase, 2.5 units and 25 units, respectively, were then added along with 0.2 mM NADPH. For the inorganic phosphate assay, 7 units of glyceraldehyde 3-phosphate

dehydrogenase and 3-phosphoglycerate phosphokinase were added to the 1 mL assay mixture along with NAD^+ (0.3mM) and ADP (0.3 mM). In both cases, the reaction was followed by observing the decrease (oxidation of NADPH) or increase (reduction of NAD^+) at 340 nm.

The remaining 27 mL of cocktail was then incubated in a water bath at 25°C and to this was added 27 µL of the PP_i-PFK enzyme. The incubation mixture was then allowed to react variable amounts of time depending upon the rate of turnover. Incubation times varied from as short as four hours to as long as 42 hours. Total turnover was determined by taking a 5 µL - 20 µL sample of the quenched assay mixture and performing endpoint analysis using the aldolase-glycerol phosphate dehydrogenase-triosephosphate isomerase assay. Turnover varied from as little as 10% (using Ca^{2+} and enzymne from *P. freudenreicheii*) to as much as 57%. All experiments involving product inhibition of the substitution of metal ligands were performed only with enzyme from *P. freudenreicheii*. Samples were then quenched by quickly freezing and stored for later analysis. The remaining cocktail was then diluted with cold distilled deionized H_2O (200 mls), the pH readusted to pH = 8, and the entire contents loaded onto a DEAE Sephadex A-25 anion exchange column (Pharmacia) generated in the HCO_3^- form. The column was developed with a linear gradient of TEA-HCO_3^-, 0.07 M - 0.7 M, pH = 8, and 400 mL in both the reservoir and the mixing chambers. Fractions were collected in 30 mL aliquots with a Gilson FC-100 fraction collector. Fractions containing inorganic phosphate (and hydrolyzed pyrophosphate) were assayed according to the method of Ames (Ames, 1966). Fractions containing pyrophosphate (eluting between approximately 0.35 M - 0.4 M), were then consolidated and concentrated in vacuo to a dry powder. Absolute methanol (Mallinckrodt) was then repeatedly added and the dissolved mixture dried in vacuo to remove all

remaining traces of TEA-HCO_3^-. The remaining film lining the bottom of the round-bottom flask was then dissolved with 3 x 1 mL washes of absolute methanol and the washes consolidated into a 30 mL Corex centrifuge tube. The product was then converted to the Na+ salt by precipitation with the dropwise addition of 1 N NaI/acetone. The flocculent white precipitate was then centrifuged at 6000 RPM in a Sorvall SS-34 rotor centrifuge. The white pellet was then isolated, resuspended with absolute acetone, and recentrifuged at least four times until only a clean white pellet remained. The pellet was then isolated and dissolved in a minimal amount of distilled deionized H_2O. The pH was adjusted to pH = 11.5 - 12 with 1 N NaOH and then absolute ethanol was added slowly in a dropwise fashion until fine needles began to appear. The solution was then refrigerated approximately one to two hours. The crystalline needles were then suction filtered, washed with ethanol and stored.

NMR PREPARATION

NMR samples were prepared in 600 µL of 100 mM HEPES buffer, pH = 8.0 . The sample was then diluted to 10 mL with distillied-deionized H_2O, then passed twice through Chelex - 100 cation exchange resin (Sigma). Strict attention was paid to making all glassware metal-free by initial soaking in 50% (v:v) H_2SO_4 : HNO_3 followed by washing thoroughly with distilled deionized H_2O. After passage through Chelex, samples were lyophilized and the prepararation reconstituted with 33% HOD (Aldrich, low paramagnetic, T_1 = 48.1 sec.).

NMR Spectroscopy were run at 97.571 MHz. (5.6 Tesla), using a sweepwidth of ±500 Hz. and a 4.09 second acquisition time. Spectra were digitized by 8,192 data points producing digital resolution of 0.0026 ppm/data point. Zero - filling twice produced resolution to 0.00065 ppm/data point.

Typically, 200 - 1000 acquisitions were required to yield signal : noise ratios ≥90. Spectra were resolved to ± 3%. Appodization by double exponential multiplication (DM) was utilized to enhance resolution of spectra (see Appendix I). Hi - field NMR spectra were also generated at 202.5 MHz. (11.6 Tesla) utilizing a sweep width = ±1000 Hz and a spectral size of 16,384 data points. Acquisition time was 4.09 seconds with a 3 second delay. Digital resolution was identical as that at 97.57 MHz. both before and after zero-filling. Typcially, 200 - 1000 acquisitions were required to yield signal : noise ratios ≥ 127.4. Spectra were resolved to ± 1.6%.

RESULTS

Figure 1 and Figure 2 represent NMR spectra at 97.57 MHz for ^{18}O-pyrophosphate incubated with PPi-PFK from *P. Aureus* in both the presence and absence of fructose 2,6 bisphosphate. Figure 3 represents an NMR spectra at 202.5 MHz. for ^{18}O-pyrophosphate incubated with PPi-PFK from *P. freudenreicheii* compared to its control. As can be seen, in either case, there is clearly little or no difference in bridge or non-bridge intensities compared to the corresponding controls. It should be noted that in the hi-field spectrum, the non-bridge peak appears to be split into an apparent doublet. This is due to the fact that ^{18}O in the non-bridge position now imparts upon the molecule a small but discernible amount of non-equivalence to the two ^{31}P nuclei in the non-bridged pyrophosphate. Thus, what is actually seen are the inner peaks of an AB pattern. This was shown by Marschner et al. at higher field (202.5 MHz) where the inner peaks are resolved (Marschner et al., 1983) Integrating areas under the curves, one sees that there is little to no change in the enrichments of the pyrophosphate in the presence or in the absence of enzyme.

From the above acquired spectra and experiments, it seems reasonably clear that PIX is not occurring to any reasonable extent. Extrapolating from the confidence limits of the spectra generated (S/N ≈ 90 at 97.5 MHz. and S/N ≈ 127.4 at 202.5 MHz.), we can now place a lower limit on the rate of partitioning to products from the central complex vs. partitioning to substrates under saturating conditions. Table 1 lists the minimal product : substrate ratio for partitioning for *P. freudenreicheii* to be at least 33 and for *P. Aureus* to be at least 20. This corresponds to an upper limit to the exchange : turnover ratio equal to 0.03 and 0.05, respectively.

Attempts to induce PIX were undertaken by replacing the Mg^{2+} with Ca^{2+}. Precedence for using Ca^{2+} stems from work by von der Saal et al (von der Saal et al., 1985). who found that for guanosine monophosphate synthetase (GMP synthetase), the PIX rate was enhanced in the presence of Ca^{2+} vs. Mg^{2+}. This could be due to either a slower rate of dissociation of the product complex or, alternatively, a faster rate of dissociation of the substrate complex. If dissociation of the product complex is slow, then reversal of the catalytic step may now be fast enough to allow detection of exchange. Alternatively, if substrate complex dissociation is rapid, and if there is interconversion of the substrate: product central complex, exchange may be evident. In addition, von der Saal and coworkers noted that nothing is known about the rate of dissociation of the Mg • O - P bond relative to the Ca • O - P bond when bound to enzyme and that this may be important in determining the rate of rotation of the ligand complex in the PIX reaction (von der Saal and Villafranca, 1986).

Similar experiments were performed with Ce^{3+} in the presence of a trace amount of Mg^{2+}. Bertagnolli and Cook (Bertagnolli and Cook, 1986) found that when using Ce^{3+} as the metal ligand, the velocity is enhance by Mg^{2+}. Mg^{2+} in this case apparently acts as an allosteric activator.

Additional experiments were conducted at pH = 6.5 to determine if decreasing the pH will have any effect on exchange vs. turnover. Although the spectra are not presented in this text, pH did not have any effect on promoting any exchange. As well, the calcium ligand did not produce any exchange of the label; however; it must be noted that when using the calcium pyrophosphate ligand we were able to generate only ~ 10 % turnover to products after 42 hours. Thus, we might be able to generate PIX if we were to generate more turnover, but the amount of enzyme required to generate a sufficient amount of turnover was limited. We in addition were not able to determine the effects of the cerium ligand as the NMR of the labeled pyrophosphate resonance resulted in only a very broad singlet (> 5 Hz.). Despite repeated attempts to re-chelate away the cerium, we were unsuccessful in increasing the resolution of pyrophosphate resonance. Hence, no conclusions could be drawn for exchange under these conditions.

Attempts were subsequently made to slow the reaction by the use of product inhibition. Garrard and Raushel (Garrard and Raushel, 1984) and Reddick and Kenyon (Reddick, 1986) used the concept of slowing down the initial velocity by adding the non-isotopic (non PIXing) product and coupling away the PIXing product during the incubation. It is imperative that the PIXing product be coupled away completely as we must rule-out the possibility of exchange due to the product coming off the enzyme and the reaction being catalyzed in the reverse direction.

Taking into account the K_i for the F1,6dP for forming the Enzyme : F1,6dP : Mg-PP_i dead-end complex, and with F6P at saturating concentrations (to eliminate competitive inhibition by F1,6dP against F6P), we analyzed the initial veloctiy. The table below lists the kinetic experiments compiled:

	Velocity	Velocity after F1,6dP	Predicted
F1,6 dP (0.43 mM)			
Expt. 1	2.09×10^{-2} IU/μL	1.29×10^{-2} IU/μL	1.38×10^{-2} IU/μL
Expt. 2	1.64×10^{-2} IU/μL	1.12×10^{-2} IU/μL	1.09×10^{-2} IU/μL

Hence, it appears likely that there can be a (F1,6dP • MgPPi•enzyme) dead-end complex being formed. Regardless of the reason, however, experiments at such low substrate concentrations make these experiments impractical because 2 - 3 liter volumes would be required to generate enough turnover as well as provide enough pyrophosphate for analysis. The amount of enzyme neccessary would be prohibitive.

DISCUSSION AND CONCLUSIONS

The above results can be interpreted in the following manner:

1) Once the catalysis takes place, the reverse reaction is no longer possible and the dissociation of the products is appreciably faster than either catalysis or the k_{on} rate in the reverse direction. This fast partitioning to products has been noted previously by Cook and coworkers when investigating the kinetics of creatine kinase (Cook et al., 1981). Using ^{32}P labeled phosphocreatine, Cook and coworkers found that the partitioning of PCr and Mg-ADP to Cr and Mg-ATP occurred faster than the partitioning backwards off the enzyme. They attributed this fast partitioning to the "stickiness" of phosphocreatine. In other words, once the rate-limiting chemical step occurs, the products complex is "commited" to dissociation off of the enzyme. The authors attributed this phenomenon to an active site histidinium that acts as a general acid catalyst . Using a similar rationale, it can be proposed that in PP_i-PFK, an amino acid residue in the vicinity of the C-1 OH group may be acting as a general base catalyst, removing the proton from the hydroxyl moiety and commiting the substrates to turnover to products (see Figure 4). Should the dissociation of the product complex be much faster than the interconversion of the central complex (Mg^{+2}-PP_i • F6P• enzyme ===P_i•F1,6dP•Mg^{+2}• enzyme) as can be the case in a rapid random mechanism, one would expect little exchange to occur. Thus, the absence of (or very little) PIX in this case would be expected. Supplementing these results with the data previously generated by Bertagnolli and Cook showing no evidence for any exchange against the flow, one can conclude that , within the limits of sensitivity by NMR, there is no evidence for either reversibility or exchange of the central complex in direction of F6P phosphorylation (Bertagnolli and Cook, 1986). In a rapid-random

mechanism, substrates can bind in any order with equal probability; however; more importantly, all steps are in rapid equilibrium except that concerned with the interconversion of the central complexes. Thus, it is not surprising that if catalysis is a slow step in the process, conversion from the substrate complex to the product complex leads to a rapid dissociation (partition to products) before there is an opportunity to revert back to the substrate complex with subsequent release.This data is consistent with their postulatating a rapid random bi-ter mechanism for the enzyme. In addition, the experiments were conducted at pH = 6.5 with the rationale that if phosphorylation occurs by a general base type of catalysis abstracting a proton from the C-1' hydroxyl, decreasing the pH would eliminate the general base mechanism allowing a different mechanism to prevail, allowing the occurrence of PIX (see Figure 4). Unfortunately, pH - rate profile data are not available at this time so we do not know which amino acid may be responsible for a general base mechanism should one exist (Cook, 1986). Regardless, this experiment did not result in any evidence for PIX.

2) Catalysis may occur, but the enzyme may not provide enough freedom to allow a free rotation about the O -P bond so the oxygens in the intermediate are not torsionally symmetric. This is rather unlikely because quantum mechanical calculations have shown this barrier to rotation to be very low (ΔG = 1 kcal, $k_{rot} = 10^{12}$). Nevertheless, this possibility cannot be strictly ruled-out either. This is a case whereby the back reaction would occur, but PIX would not be observed.

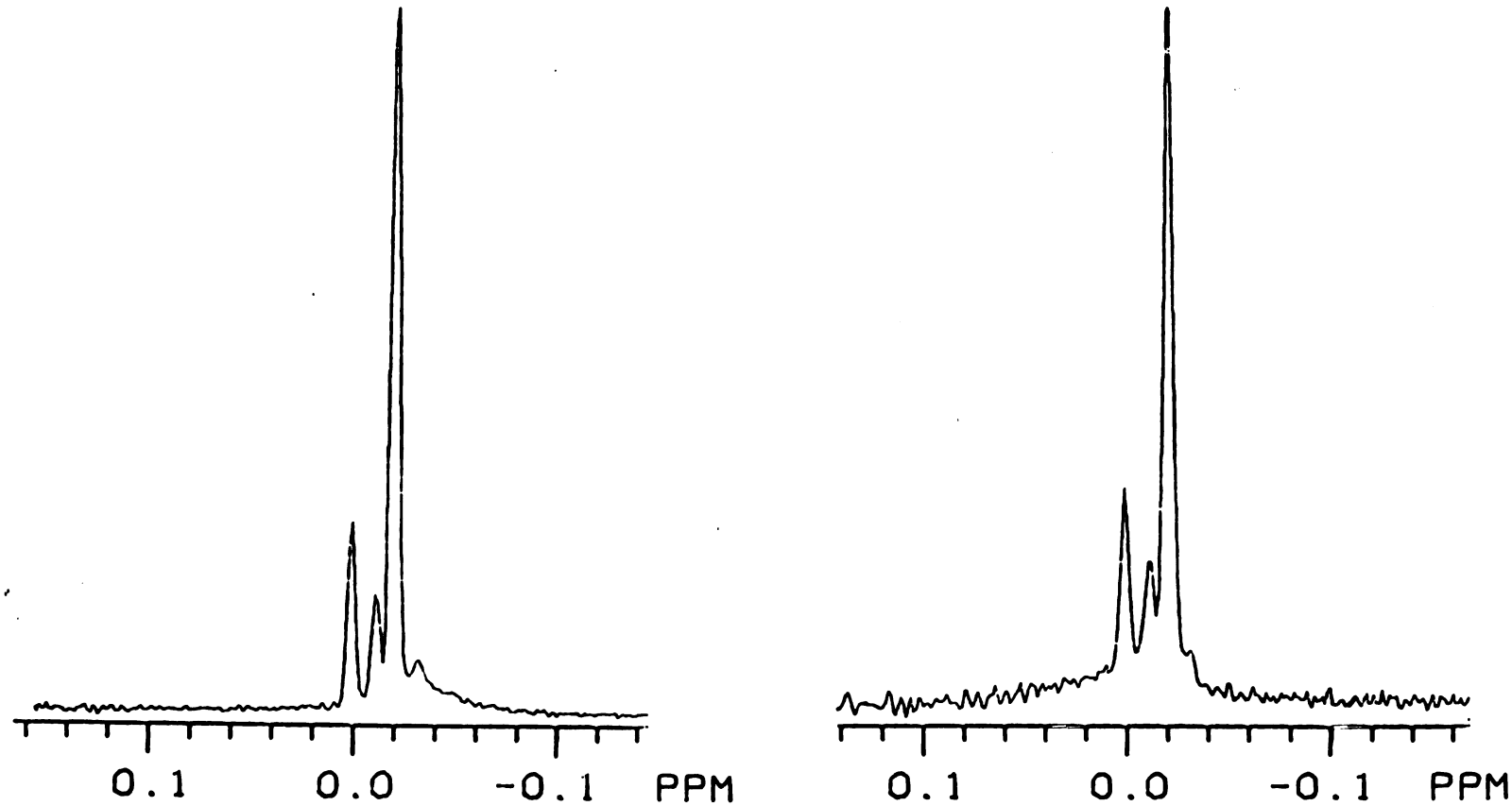


Figure 1. ^{31}P NMR at 97.57 MHz of the phosphoryl resonances of ^{18}O-pyrophosphate. Incubation solution contained 10 mM fructose 6-phosphate, 8 mM magnesium chloride, and 10 mM of the labeled pyrophosphate at 25° C in 100 mM TAPS buffer at pH = 8.0. Spectrum on the left is after a 24 hour incubation without enzyme. Spectrum on the right is with enzyme. Pyrophosphate-dependent phosphofructokinase is from P. aureus.

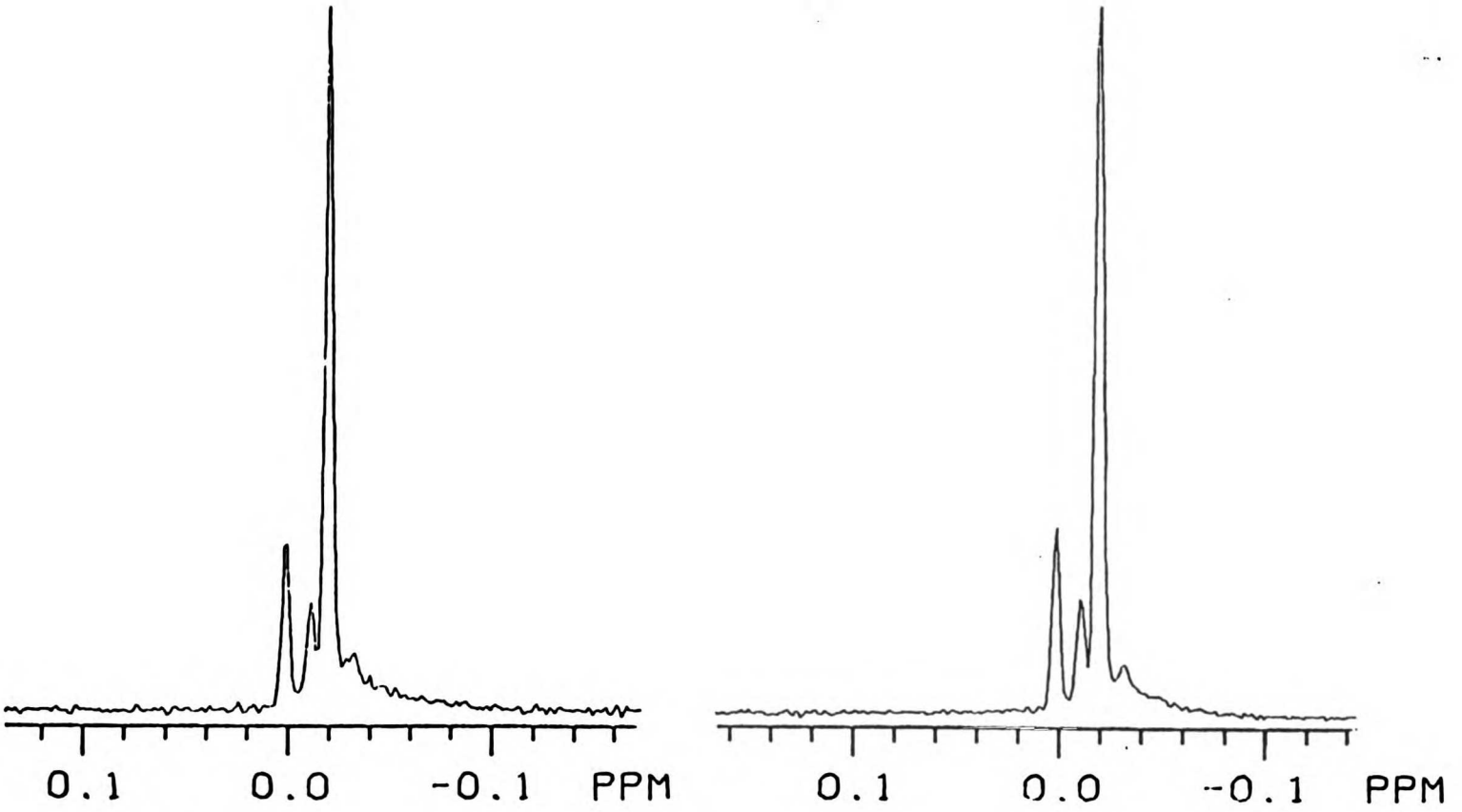


Figure 2. ^{31}P NMR at 97.57 MHz of the phosphoryl resonances of ^{18}O-pyrophosphate. Incubation conditions were identical to those described in Figure 1 except 1 μM fructose 2,6-bisphosphate was added. Spectrum on the left is after a six hour incubation without enzyme. Spectrum on the right is with enzyme. Pyrophosphate-dependent phosphofructokinase is from P. aureus.

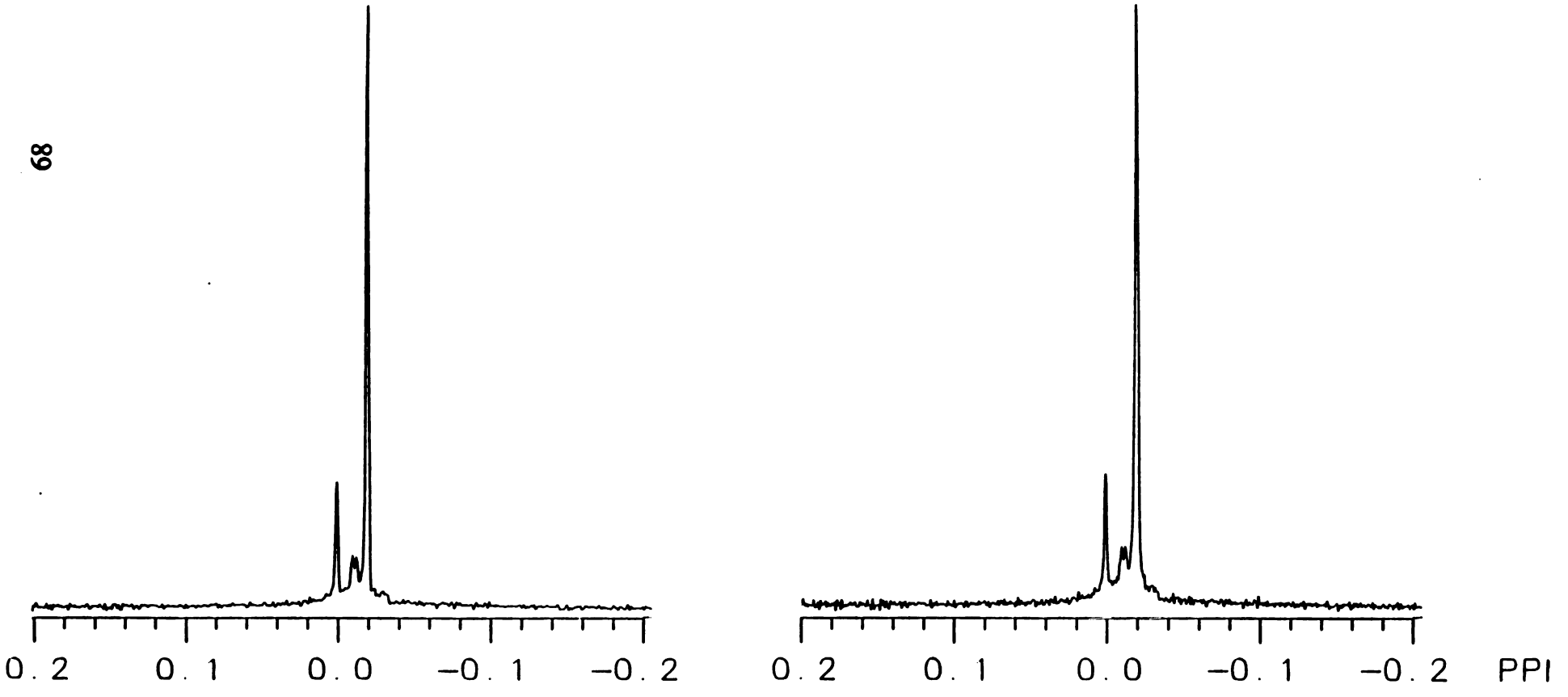


Figure 3. ^{31}P NMR at 202.5 MHz of the phosphoryl resonances of ^{18}O-pyrophosphate. Incubation conditions were identical to those described in Figure 1. Spectrum on the left is after a four hour incubation without enzyme. Spectrum on the right is with enzyme. Enzyme source is from P. freudenreicheii.

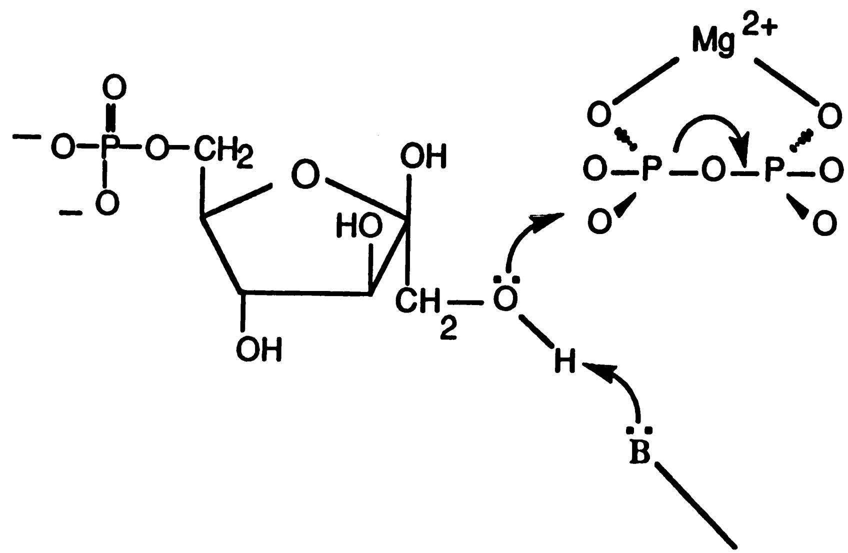


Figure 4. Proposed general base catalytic mechanism for pyrophosphate-dependent phosphofructokinase.

Table 1. Table of Results

Results of positional isotope exchange experiments using selexstively bridge-labeled ^{18}O-pyrophosphate.

	Fraction of chemical Rxn.	Fraction of exchange Rxn.	Maximal undetectable exch./turn-over ratio.
P. Freudenreicheii (202.5 MHz.)	0.57	0 ± .03	.03
P. Aureus (97.5 MHz.)			
(+ 2,6 bisphosphate)	0.29	0 ± .04	.05
(- 2.6 bisphosphate)	0.02	0 ± .04	-----*

^{18}O-pyrophosphate exchanged = $(X / (\ln (1-x)) (PPi_{initial})(\ln 1 - F)$

X = fraction of pyrophosphate lost.

$F = (P_t - P_0) / P_\infty - P_0)$ where P refers to percentage of ^{18}O non-bridge label at time t, zero and at equilibrium. Since the bridge label can scramble into any one of three non-bridge positions (assuming torsional symmetry), then at equilibrium, the $P_\infty \approx 63.1\%$,

$P_0 \approx 10.0\%$, and $P_t \approx$ % non-bridged at time t at 97.5 MHz. At 202.5 MHz., the $P_\infty \approx$

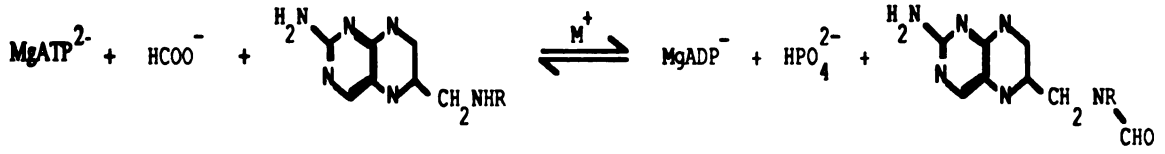
62.4 %, $P_0 \approx 14.5$ %, and $P_t \approx$ % non-bridged at time t.

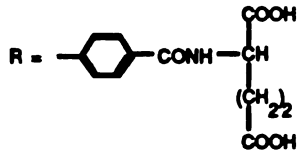
S/N measured by computation of GE-Nicolet 1280 software program. Actual measurement yields maximal signal to maximal noise ≈ 45. This was used to determine maximal undetectable exchange/turnover ratio.

* Amount of turnover was too small to determine maximal exchange/turnover ratio.

Formyltetrahydrofolate Synthetase

Formyltetrahydrofolate synthetase (E.C. 6.3.4.3) catalyzes the ATP-dependent formylation of tetrahydrofolate at the N-10 position (Himes and Harmony, 1973).





The enzyme is responsible for incorporating formate into the one-carbon metabolic pool since the product, N^{10}-formyltetrahydrofolate (N^{10}-formyl-H_4 folate), is used directly in the biosynthesis of purines and f-Met tRNA. N^{10}-formyl-H_4 folate is reduced and used in the biosynthesis of pyrimidines, serine, and methionine. The enzyme is found in plants, animal tissue and bacteria. Clinically, the enzyme is found at increased levels in acute leukemia and infectious mononucleosis (Bertino et al., 1962). Bacteria (i.e., *M. aerogenes* , *C. cylindrosporum* , *C. acidi urici* , and *C. thermoaceticum*) unquestionably possess the highest concentrations of the enzyme, possibly because of the fact that these bacteria utilize purines as sources of carbon and nitrogen (Rabinowitz and Pricer, 1956).

PHYSICAL PROPERTIES

The active enzyme from Clostridial species is a 240,000 MW tetramer of four identical subunits (MacKenzie and Rabinowitz, 1962). For maximum

catalytic activity, the enzyme requires monovalent cations (i.e., K^+, Rb^+, NH_4^+). The purpose of the cation appears to be to prevent the catalytically active tetramer from dissociating into the monomeric components as well as increasing the catalytic activity of the tetrameric unit (MacKenzie and Rabinowitz, 1967; Scott and Rabinowitz, 1967; Welch et al., 1968) Apparently, the addition of the monovalent cations has no effect on the velocity, but, instead, acts to decrease the k_m of the substrate formate from 50 mM to 5 mM in the presence of saturating concentrations of cation (Welch et al., 1968). However, the mechanism by which this increased activity occurs is still unknown. Anionic counterions also induce differential activity, sulfate being one of the more active anions in promoting tetramer formation.

Earlier studies have determined that the equilibrium lies far in the direction of N^{10}-formyl-H_4 folate. The equilibrium constant ranges between 41 and 115 (Himes and Rabinowitz, 1962). Curthoys and Rabinowitz determined the equilibrium constant from the Haldane relationship to be 76, in good agreement with previous values (Curthoys and Rabinowitz, 1972) The enzyme is sensitive to pH-induced dissociation of the tetrameric unit at pH values below 7 and above 9.5 (Himes and Wilder, 1968). pH - rate profiles suggest that there are 2 groups with pKa values of approximately 6 and 9.2 that are responsible for catalysis. MacKenzie et al. provided evidence for the presence of a histidine moiety in the active site. Pseudo-first order rate constants for inactivation by photooxidation in the presence of methylene blue correlated with the oxidation of a histidine. pH - rate profiles for activation revealed an inflection point of pH = 6.3, also consistent with histidine group involvement (MacKenzie et al., 1972).

ATP BINDING SITE

ATP and deoxy ATP appear to be the only substrates for the enzyme, as GTP, ITP, CTP, and UTP are all inactive. A K_m value for MgATP (the actual substrate) has been measured to be 1.4×10^{-4} M (Himes and Wilder, 1965). Four independent binding sites are present on the enzyme. Binding still occurs when the enzyme is in the monomeric form; however, catalytic activity occurs only when the tetrameric form is present.

Mejillano et al. recently investigated the stereochemistry of the metal - nucleotide chelate complex of ATP phosphorothioates (Mejillano, et al., 1986). The authors found that with both Mg^{2+} or Cd^{2+} no stereoselectivity was observed when the ATPαS isomer was used. This also suggested that the enzyme did not utilize metal ions coordinated to the α - phosphate. In contrast, the enzyme showed a preference for the S_p isomer of Mg^{+2} - ATPβS and the R_p isomer of Cd^{+2} - ATPβS (see Figure 1). Interestingly, the S_p isomer of Mg^{+2} - ATPβS and R_p isomer of Cd^{+2} - ATPβS both assume the D or right - handed screw sense configuration of the metal - chelate ring. Additionally, the corresponding L or left - handed screw sense isomers exhibited competitive inhibition with the lowest K_i values. Also, unpublished EPR studies of enzyme - Mn^{2+} - nucleotide complexes using [α - ^{17}O] ADP, [β - ^{17}O] ADP, and [β - ^{17}O] ATP demonstrate Mn^{+2} coordination to the β - phosphate but not the α - phosphate (Smithers et al., 1986). These results suggest that the reactive metal - nucleotide substrate is a β - γ - bidentate complex. However, the disclaimer is made that the β - monodentate complex cannot be ruled out.

REACTION MECHANISM

A variety of mechanisms have been proposed for this enzyme. Whiteley and Huennekens proposed a mechanism whereby the tetrahydrofolate substrate is activated by phosphorylation at the N - 10 position, followed by displacement of P_i by formate; however, results of exchange studies showed this mechanism to be highly unlikely (Whiteley and Huennekens, 1962). Other work by Jaenicke and Brode proposed other phosphorylated tetrahydrofolate intermediates; however, their work appeared to be flawed by the presence of considerable amounts of contaminating enzymes (ie. myokinase, nucleoside diphosphate kinase, and ATPase activities) (Jaenicke and Brode, 1961a; Jaenicke and Brode, 1961b; Jaenicke and Brode, 1961c).

A concerted mechanism has been proposed for the reaction catalyzed by the enzyme from *C. cylindrosporum* . The following data argues favorably for such a mechanism: 1) Exchange of $^{32}P_i$ and ATP requires both tetrahydrofolate and formate. 2) Exchange of ^{14}C - formate and N-10 formyltetrahydrofolate requires P_i and ADP. 3) Arsenolysis of N-10 formyltetrahydrofolate requires ADP. 4) Formation of ADP and ATP requires both formate and tetrahydrofolate. 5) Di - ^{18}O labeled formate transfers <u>one</u> ^{18}O label to P_i. and 6) Maximum exchange of ADP and ATP is dependent upon formate and tetrahydrofolate (Himes and Rabinowitz, 1962; Uyeda and Rabinowitz, 1964). Additionally, as previously stated, a histidine has been proposed as the general base that catalyzes the reaction.

Of significance is the fact that the mechanism does <u>not</u> involve the formation of freely dissociable intermediates, which is consistent with kinetic and equilibrium exchange studies by Joyce and Himes (Joyce and Himes, 1966a; Joyce and Himes, 1966b).

However, despite the fact that the data are consistent with a concerted type of mechanism, the possibility of tight-binding intermediates must also be considered.

Formyl phosphate has been proposed as a possible intermediate in the catalytic mechanism (see Figure 2). Original work by Sly and Stadtman did not exhibit any evidence for formyl phosphate acting as a substrate (Sly and Stadtman, 1963). However, the failure to observe the formyl phosphate may be due to the requirement of tetrahydrofolate to induce the correct conformational change to elicit substrate binding. NMR work by Himes and Cohn showed that tetrahydrofolate caused a de-enhancement of the Mn^{2+} induced relaxation rate of the water protons. This was suggestive of tetrahydrofolate inducing the formation of enzyme Mg-ATP • H_4-folate • enzyme quarternary complex (Himes and Cohn, 1967). Buttlaire and coworkers provided evidence supporting a formyl phosphate intermediate when they catalyzed ATP formation from ADP and carbamyl phosphate, a structural analog to the formyl phosphate intermediate (see Figure 2) (Buttlaire et al., 1976; Buttlaire et al., 1979) In a recent reinvestigation of the formyl phosphate intermediate, Smithers and coworkers were able to demonstrate the ability of formyltetrahydrofolate synthetase to utilize formyl phosphate as a substrate, providing strong evidence for a sequential mechanism instead of a concerted mechanism (Smithers, et al., 1986). In addition, in the presence of pteroyltriglutamate (PGA), a competitive inhibitor of H_4 folate, the authors were able to generate MgATP from formyl phosphate and MgADP (see Figure 3). The authors were also able to catalyze the reverse reaction, hydrolyzing MgATP in the presence of formate.

We have set out to investigate the possibility of a formyl phosphate intermediate by looking at the reaction by PIX experiments. From the data cited above, we a priori would expect to see exchange of the ^{18}O bridge label in ^{18}O-

β,γ-ATP. Hence, we should expect to see PIX. A positive PIX result in the presence of formate and pteroyltriglutamate would be consistent with the proposal of a formyl phosphate intermediate.

MATERIALS AND METHODS

For all PIX experiments, 10 mL cocktails were made in 100 mM triethanolamine buffer, pH = 8.0. To the buffer was added in the following:

Magnesium Chloride	10 mM
Potassium Chloride	10 mM
NCOO- Na+	8 mM
^{18}O β-γ ATP (1.5 Na+)	8 mM
β-Mercaptoethanol (from Stock Soln. in Tris buffer, pH = 8.0)	100 mM

For initial turnover experiments with H_4 folate, the concentration was 2 mM. The concentration of PGA was 1 mM when the inhibitor was substituted.

Two sets of controls were run in a side by side manner with the experiment under turnover conditions. The following cocktails were run:

Sample	Incubation Time	Initial ATP Conc.	Final ATP Conc.
1) Experimental	8 hours	8 mM	4.8 mM
2) Control (- H_4 folate)	8 hours	8 mM	6.8 mM
3) Control (- H4 folate) (- formate)	8 hours	8 mM	7.1 mM

Formyltetrahydrofolate synthetase, stabilized as the 50% $(NH_4)_2SO_4$ suspension, was placed in dialysis tubing and dialyzed against the following buffer.

Triethanolamine pH = 8.0	100 mM
KCl	50 mM
β-Mercaptoethanol	50 mM

The enzyme was dialyzed against a 1 : 100 (vol : vol) dialysate for three hours. This was repeated three times. The enzyme was removed and 6.5 mL (24.7 mG) was added to the incubation mixture. The cocktail was incubated at 37°C for eight hours in the cases where no H_4-folate was added. In the presence of H_4-folate, turnover was continued until 40% turnover occurred. Turnover was assayed by removing an aliquot from the cocktail and quenching with 0.36N HCl. Quenching with acid converts the 10-formyl H_4-folate to the 5-10 methenyl product which can be detected by UV at λ = 350 nm (ε = 24,500) (Rabinowitz and Pricer, 1962). Proteins were assayed by the method of Bradford (Bradford, 1976). Specific activity for all experiments was 445 units/mg. In the case of the experiments with pteroyltriglutamate, ATP hydrolysis was followed by HPLC using the method of Himes (Himes, 1986).

After incubation, the samples were diluted 10 - fold with distilled-deionized H_2O and the ATP isolated by DEAE-Sephadex A-25 anion exchange resin as described previously (see nitrogenase Materials and Methods Section). ATP samples were then prepared for NMR Spectroscopy as described previously in this thesis.

One additional experiment was conducted that increased the enzyme : substrate ratio so that, in the event of an extremely slow exchange rate, we may be able to detect PIX under higher enzyme concentration. The experiment was performed in the presence of pteroyltriglutamate. In this experiment, an enzyme active site : substrate ratio of 10^{-3} was used. Conditions of the experiment were as follows:

Triethanolamine • HCl	100 mM	pH = 8.0
2 - mercaptoethanol	100 mM	
$MgCl_2$	20 mM	
β,γ - ^{18}O-ATP	10 mM	
KCl	50 mM	
Enzyme active sites	10^{-5}	

The following table shows the amount of turnover after ten hours incubation at 37°C:

Sample	Incubation Time	Initial ATP Conc.	Final ATP Conc.
1) Experimental	10 hours	8 mM	5.3 mM
2) Control (-) pteroyltri-glutamate	10 hours	8 mM	6.5 mM
3) Control (-) pteroyltri-glutamate (-) formate	10 hours	8 mM	7 mM

RESULTS

An NMR spectrum at 97.57 MHz revealed no evidence for exchange under turnover conditions with saturating substrate concentrations. Signal : noise was equal to 45. Because we determined the total turnover for the above conditions, we can assign an upper limit to the exchange : turnover ratio to be 0.042.* Figure 4 shows NMR spectra at 202.5 MHz of the ^{18}O-β,γ bridge labeled ATP after incubation in the presence of formate, Mg^{2+}, enzyme and PGA. These spectra were generated at 202.5 MHz with a signal : noise for control and experimental samples equal to 200 : 1 and 95 : 1, respectively. As can be seen, the control and experimental spectra appear virtually identical. If we make the assumption that the difference in ATP concentrations between the incubation in the presence of PGA and enzyme *vs.* no enzyme (chemical hydrolysis), we can get an idea of the upper limit for exchange *vs.* turnover. Using the equation for exchange by Wimmer and Rose, we have calculated the exchange : turnover ratio as ≤ 0.01 (Wimmer and Rose, 1980). The experiment using PGA was repeated using more enzyme and incubating for a longer period. Although the spectrum is not presented, the result is virtually identical to that in Figure 4.

* Note: The upper limit of exchange *vs.* turnover is based upon the maximum possible exchange in the NMR spectrum that could go undetected because of interference in the noise. It is very likely that our upper limit of exchange : turnover numbers are much less; however, only limits are reported that can be reliably extrapolated from the NMR data.

CONCLUSION AND DISCUSSION

The absence of PIX under normal turnover conditions (saturating substrate concentrations) is not considered surprising to us as no other ATP-utilizing enzyme to date has exhibited any exchange under saturating substrate conditions. This appears consistent with the product being "committed" to turnover once catalysis occurs. This seems to imply that the k_{off} rate is significantly faster than the catalytic rate in the reverse direction. The "commitment" is assisted by the general base catalytic mechanism which aids in abstracting the proton from the N-10 nitrogen, making it more nucleophilic and hence, able to catalyze the reaction. Similar results were obtained with pyrophosphate-dependent phosphofructokinase discussed elsewhere in this thesis.

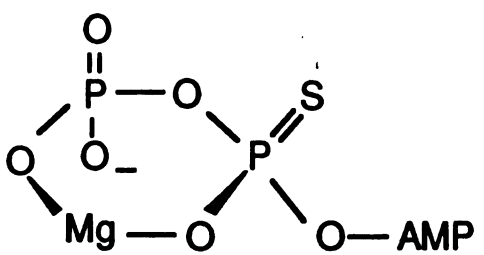
Surprising to us, however, was the absence of PIX when we replaced the H_4 folate with the inhibitor pteroyltriglutamate (PGA). From unpublished results of Smithers et al., we know that formyl phosphate in the presence of PGA can promote the synthesis of ATP when ADP is present (Smithers et al., 1986). In addition, it was proposed that in the presence of THF, formyl phosphate promotes a slow synthesis of both ATP and N-10 formyl THF, primarily because of a slow-binding and slow-release of the putative formyl phosphate intermediate. The catalytic rate for formation of ATP in the presence of formyl phosphate, MgADP and PGA is approximately 20% that of ATP formation when H_4 folate is present. Hence, we originally assumed that a similar slow-release phenomenon would exist in the case of the ^{18}O-β,γ - ATP •formate•PGA•enzyme incubation. Consequently, if slow release of the intermediate formyl phosphate were to exist, one might expect the enzyme to allow ample opportunity for exchange of the bridge label. This was not the case.

The above results do not argue convincingly for either the presence or absence of a formyl phosphate intermediate. Arguments for a concerted mechanism have been reviewed previously (Himes and Rabinowitz, 1962; Uyeda and Rabinowitz, 1964). The results of our PIX experiments with PGA are consistent with a concerted mechanism since the absence of the nucleophilic substrate, H_4 folate, would not lead either to catalysis or reversal of catalysis. However, if the mechanism were concerted, one would expect PGA to protect against hydrolysis of MgATP because a dead-end complex would form, discouraging hydrolysis. In addition, the ability of the enzyme to utilize formyl phosphate, albeit slowly, argues against a concerted mechanism.

Our results in the presence of PGA does not neccessarily argue against a formyl phosphate intermediate, but rather, may reflect the release of the putative intermedate. A lack of PIX can still be consistent with a formyl phosphate intermediate if the rate of release of the intermediate is slow but still much faster than the reversal of catalysis from the central product complex. Recall that the forward rate in the presence of PGA was 20% of that observed with H_4 folate. Apparently the slow dissociation of formyl phosphate (if indeed formyl phosphate is the intermediate in this case) from the central complex is faster than the reverse reaction and dissociation from the substrate central complex.

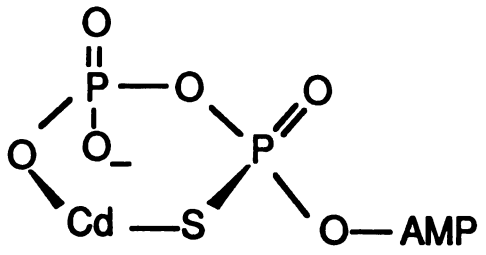
Screw Sense

Δ



MgATP βS

S_p



CdATP βS

R_p

FIGURE 1. Structure and screw sense configuration of β, γ - bidentate metal - ATP substrate analogs of formyltetrahydrofolate synthetase.

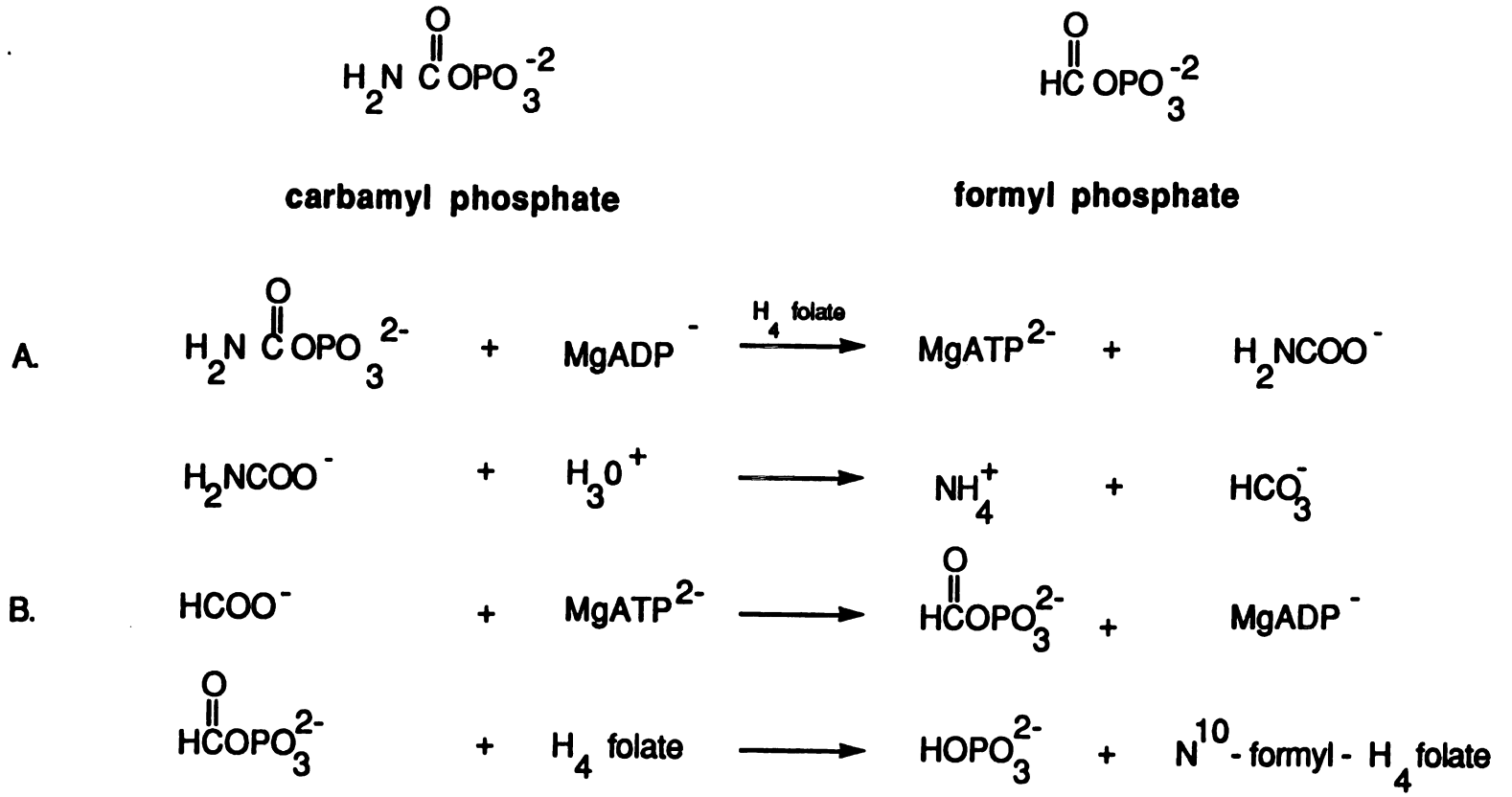
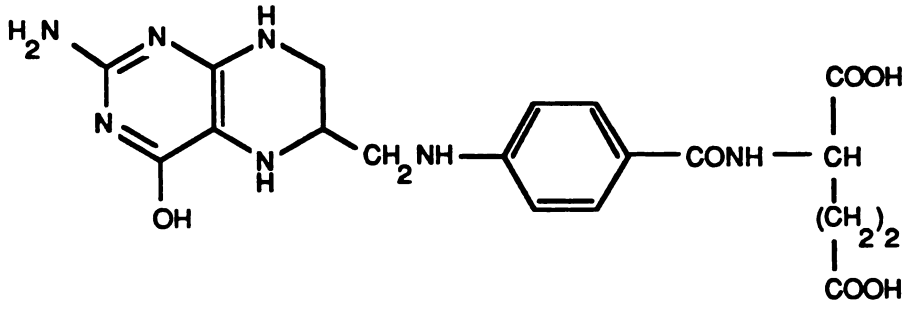
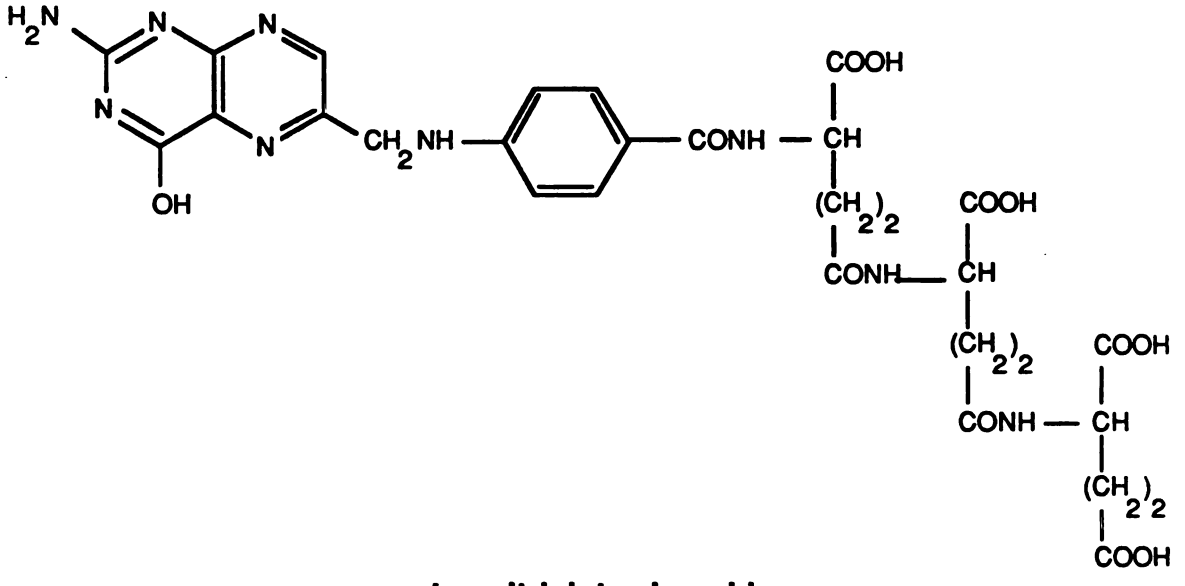


FIGURE 2. Proposed mechanisms for formyltetrahydrofolate synthetase.

A) Proposed mechanism of catalysis with the intermediate analog carbamyl phosphate.
B) Proposed mechanism of catalysis with the substrate formate.



tetrahydrofolic acid (H_4 folate)



pteroyltriglutamic acid

Figure 3. Structures of tetrahydrofolic acid and pteroyltriglutamic acid.

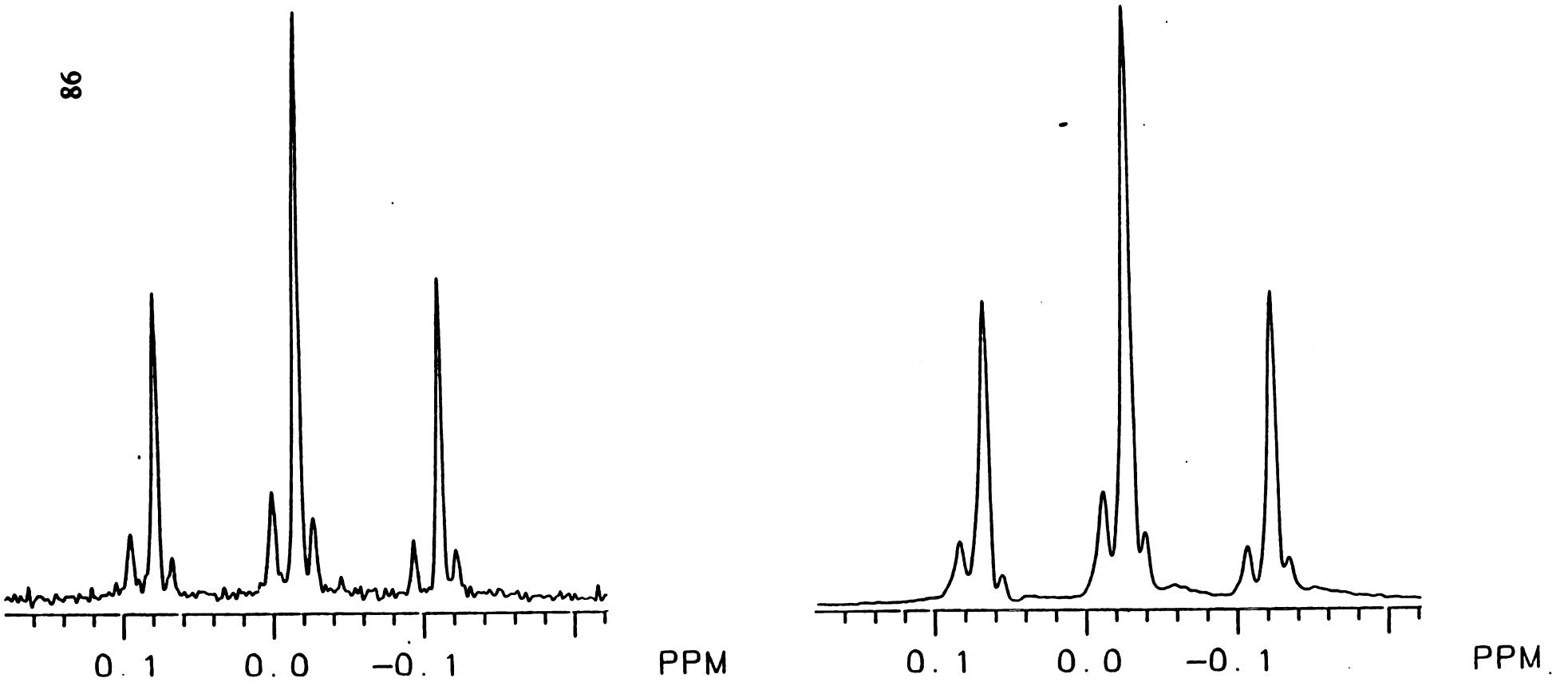


Figure 4. ^{31}P NMR at 202.5 MHz of the β-phosphoryl resonances of β,γ-^{18}O-ATP. Incubation solution contained 50 mM potassium chloride, 8 mM sodium formate, 20 mM magnesium chloride, 100 mM 2-mercaptoethanol, and 10 mM of the labeled ATP at 37° C in 100 mM triethanolamine buffer at pH = 8.0. Spectrum on the left is after a ten hour incubation with 10^{-3} active site equivalents of formyltetrahydrofolate synthetase per labeled ATP. Spectrum on the right had undergone identical conditions but in addition contained 1 mM pteroyltriglutamate.

NITROGENASE

The nitrogenase system in bacteria is responsible for the sequential reduction of dinitrogen (N_2) to ammonia. It is referred to commonly as a nitrogen fixing system as it mobilizes atmospheric N_2 and converts it to ammonia for use in various de novo synthetic pathways (i.e., amino acid, purine and pyrimidine biosynthesis). Bacteria containing such systems are important in agriculture as they provide a source of nitrogen for plants that otherwise may be unable to mobilize their own N_2. This is commonly referred to as *Symbiotic Nitrogen Fixation*. (Lehninger, 1972) This system, commonly found in leguminous plants (i.e., peas, beans, clover, alfalfa, and soybeans), consists of these bacteria permeating the root system via the cortical parenchyma, giving rise to nodules. Here, the bacteria are in association (physical and biological) with the vascular system of the plant. It has been concluded that the bacteria "*fixes*" the N_2 and funnels the ammonia through the plant via the plant root system while, in turn, the plant provides essential components lacking in the bacteria (Newton and Burgess, 1983).

A net reaction for nitrogenase has been proposed:

$$N_2 + 4\,S_2O_4^{2-} + 16\,MgATP^{2-} + 24\,H_2O = 2\,NH_3 + H_2 + 16\,MgADP^{-} + 8\,SO_3^{2-} + 16\,P_i^{2-} + 24\,H^+$$

An additional aspect of nitrogenase action, however, is the relationship between H_2 evolution and N_2 reduction. In the absence of N_2, nitrogenase catalyzes the reduction of protons to H_2:

$$2\,H^+ + 2e^- = H_2$$

In the presence of N_2, H_2 evolution is partially suppressed, such that the limiting stoichiometry is observed: (Rivera-Ortiz and Burris, 1975; Burgess et al., 1981; Guth and Burris, 1983)

$$8\,H^+ + N_2 + 8\,e^- = 2NH_3 + H_2$$

Nitrogenase contains two specific functional protein subunits as well as a cofactor. In *Azotobacter vinelandii,* the enzyme we currently are studying, there is a Fe-Mo component (Av_1 or dinitrogenase component) that is an $\alpha_2\beta_2$ tetramer of MW = 240,000 and that contains Mo, Fe, and S in an approximate ratio of 1 : 15 : 15 consisting of four Fe_4S_4 clusters and probably two Fe-Mo cofactors (Newton and Burgess, 1983). The Fe-Mo-cofactor has been isolated containing a Mo : Fe : S ratio of 1 : 8 : 6 (Shah and Brill, 1977). The second component, the Fe protein (Av_2 or dinitrogenase reductase), contains a single Fe_4S_4 cluster and consists of identical subunits with a total MW = 60,000 (Newton and Burgess,1983)(Gillum and Mortenson, 1977).

MECHANISM OF REDUCTION

Although the enzyme was first isolated by Carnahan and coworkers in 1960, the mechanism of enzyme action has not been elucidated (Carnahan, et al., 1960). However, some general information has been agreed upon: 1) The enzyme requires an electron source to generate reduction. In vivo, an electron transport system, i.e., ferredoxin, is associated with the enzyme. In vitro, dithionite ($Na_2S_2O_4$) has been used as the electron source. 2) MgATP is required for catalysis. Other nucleotides, i.e., GTP, CTP, TTP, and ITP, cannot replace ATP. The stoichiometry of reaction appears to be 4 ATP: 2 e^- transferred; however, this ratio varies substantially with subunit ratio, enzyme concentration, and amount of product formation. 3) N_2 is the natural substrate, in the absence of which protons are reduced and H_2 is evolved. 4) Mg^{2+} is required for activity. Other divalent cations, i.e., Mn^{2+}, Co^{2+}, Fe^{2+}, and Ni^{2+} may replace Mg^{2+}, but with reduced activity. 5) Nitrogenase has been found to reduce alternate substrates, generally those with existing parallel π bonds, i.e., acetylene, propyne, azide anion, and hydrazoic acid (HN_3), to name a few. Doubly bonded molecules, on the other hand, are neither substrates nor are they significant inhibitors (McKenna, 1980). Carbon monoxide is a commonly used non-competitive inhibitor that seems to act preferentially to inhibit substrates except for protons (Burris, 1971). Recently, N_2O has been identified as a competitive inhibitor, the only one known to date (Jensen and Burris, 1986).

BINDING OF ATP

The site of ATP binding was initially shown by Bui and Mortensen who found that ATP binds to the Fe subunit but not to the Fe-Mo subunit. Bui and Mortensen used gel equilibration to demonstrate ATP binding to two independent sites of the Fe subunit of *C. pasteurianum* (Cp_2). ADP, the hydrolysis product, appeared to be competitive at one site, but at the same time increasing the affinity of ATP at the second site (Bui and Mortensen, 1968). Tso and Burris, using EPR and gel equilibration, reported similar results to those of Bui and Mortensen, finding a stoichiometry of 2 ATP's per active Fe protein subunit (Tso and Burris, 1973). Ljones and Burris supported this result by finding evidence that ATP induced the interaction between bathophenanthrolene disulfonate and the Fe_4S_4 cluster of the Fe protein subunit (Ljones and Burris, 1978). Walker and Mortensen found a similar ATP-induced interaction between the Fe : S cluster and the iron chelator α,α'-dipyridyl. Of additional importance was the fact that the Fe - Mo protein did not exhibit any such effect (Walker and Mortensen, 1974).

Other authors, using a variety of other techniques, have reported contrasting results with regard to the number of nucleotide binding sites. McKenna et al., using circular dichroism studies, found that oxidized Av_2 and Kp_2 have a high affinity for two molecules of ATP and probably ADP as well (McKenna, et al., 1984). Mortensen and Thorneley reinvestigated the ATP binding to Cp_2 with gel equilibration and reported 5 ± 2 binding sites per Fe subunit (Mortensen and Thorneley, 1977). Miller et al. reported binding of four ATP's to the Fe-Mo subunit of purified *K. pneumoniae* (Kp_1) and *Azotobacter chrococcum* (Ac_1) (Miller et al., 1980). Zumft et al., using EPR, concluded that Cp_2 binds two molecules of ATP (Zumft et al., 1973; Zumft et al., 1974).

Florescence probe data by You and coworkers indicated that the Av_2 subunit separately binds two ATP molecules and at least two ADP molecules per subunit (You et al., 1984). In summary, the literature has reports of a variety of combinations of ATP and ADP bound to both Fe and Fe-Mo subunits of nitrogenases. To date, however, it appears that no one has addressed the possible differences between nitrogenases from different sources.

Recently, in a reinvestigation of nucleotide binding to the Fe subunit of *A. vinelandii*, Cordewener et al. studied the number of ATP and ADP binding sites. They found that the number of binding sites, as determined by radiochemical labeling, varied markedly with specific activity. Nitrogenase with a specific activity > 2000 nm C_2H_4 min^{-1} mg^{-1} Av_2 showed less variation in the number of binding sites. Their Scatchard plot analysis was consistent with two binding sites. Of significance was their result that ATP exhibited a negative cooperativity for the second binding site whereas ADP showed a positive cooperativity. In addition, their kinetic data, when computer fit to previously reported K_d values, matched the binding equations for two nucleotide binding sites. Thus, in the reduced species, Cordewener et al. supported the contention that there are only two nucleotide binding sites with competitive inhibition being significant in only one of those sites (Cordewener, et al., 1985).

CATALYTIC ROLE OF ATP

The question of the specific role of ATP in catalysis has also been studied. Work sited earlier by Walker and Mortensen demonstrated the ability of ATP to induce an interaction between the Fe_4S_4 cluster and α,α' dipyridyl. This provided evidence that ATP induced a conformational change, binding to the Fe protein and exposing the Fe_4S_4 cluster to the surface of the protein (or at least an accessible area of the protein) , allowing the cluster to transfer electrons to the Fe-Mo component (Walker and Mortensen, 1974). This

conclusion was supported by the work of Zumft, Palmer, and Mortenson whose EPR spectra of the Fe subunit in the presence of ATP revealed virtually identical shifts to those found with 5 M urea and the Fe subunit alone. The authors concluded that urea, a denaturant of the protein at low urea concentrations (≤0.5 M), acts at high concentrations to release the Fe_4S_4 cluster from the Fe subunit in a fashion similar to ATP (Zumft, et al., 1973). In addition, the direction of the shift in the redox potential to a lower potential was found to be consistent with what would be expected for the cluster to be moving from the inside to the outside of the protein, as compared to synthetic iron : sulfur model compounds (Holm and Ibers, 1977).

Zumft and coworkers, working with *C. pasteurianum*, measured the change in redox potential for the Fe-protein and found a value of appoximately -110 mV in the presence of ATP. The potential shift was found to be consistent with a proposed conformational change in the protein caused by binding of <u>two</u> ATP molecules. Ljones and Burris, when studying the ATP-induced chelation of bathophenanthrolenedisulfonate with the Fe_4S_4 cluster of the Fe protein, also found that the chelation kinetics were consistent with a model whereby both ATP sites must be occupied to allow the conformational change leading to the reactivity of the Fe_4S_4 cluster (Ljones and Burris, 1978). They concluded that the kinetics were consistent with the necessity of both nucleotide binding sites being occupied before the Fe protein subunit is allowed to undergo a conformational change.

Kinetic studies by Tso and Burris found that the presence of ADP blocked one of the sites and resulted in a sigmoidal curve when measuring the velocity of product formation. In other words, as the amount of ADP increased, the enzyme became progressively inhibited. This was consistent with the hypothesis that inhibition of ATP binding at one of the sites will prevent the ATP-

induced conformational change neccessary to initiate catalysis (Tso and Burris, 1973). It was felt that an ATP-induced conformational change of the Fe protein could alter the local environment of the Fe_4S_4 cluster complex, making electron transfer to the Fe-Mo complex favorable. In addition, hydrolysis of MgATP did not occur if the Fe-Mo protein was not present. This was evidence that both subunits had to be present to initiate the ATP-induced catalysis.

Studies to determine the sequence of events prior to, during, and after ATP hydrolysis are inconclusive. Initial studies from Bulen and LeComte using *A. vinelandii* established the absolute requirement of ATP for catalysis and H_2 evolution (Bulen and LeComte, 1968) EPR work by Smith, Lowe, and Bray showed that a dithionite-reduced Kp_2 transfers electrons to Kp_1 in an ATP-dependent reaction (Smith et al., 1973) Ljones and Burris reported that the transfer of electrons between subunits occurred with only one electron transferred per event (Ljones and Burris, 1978). Hence the enzyme undergoes a series of one-electron transfers during catalysis.

Kinetic studies by Watt and Burns found that a lag time existed between the initial incubation of the enzyme : ATP cocktail and detectable H_2 formation, leading to the conclusion that ATP hydrolysis and presumably electron transfer occur prior to proton reduction and H_2 evolution (Watt and Burns, 1977). Hageman and Burris, observing changes in the EPR signal during this lag period prior to H_2 evolution, found that the first electron transfer occurred rapidly with no H_2 evolution and that reduction and H_2 evolution occurred concomitantly with the second electron transfer (Hageman and Burris, 1978)

The ATP /2e- ratio determination has produced a variety of results. Watt et al. found that in *A. vinelandii* nitrogenase, the optimal ATP/$2e^-$ ratio is approximately 4, being optimal at 20°C; however, deviations from the optimal 4 ATP/ $2e^-$ ratio for reduction have been reported (Watt et al., 1975). Variations

with temperature were reported by Watt and Burns. At low temperature (10°C), they found that the enzyme completely dissociated electron transfer and ATPase activities. The authors postulated a temperature-induced alteration in the protein structure of either the Fe and/or Fe-Mo protein. At less than 20°C, the protein(s) may be "frozen" into a conformation incapable of inducing electron transfer but still capable of ATP hydrolysis. They postulated that only when sufficient energy has been supplied does the "frozen" form "melt" to allow productive ATP hydrolysis associated electron transfer (Watt and Burns, 1977). This is consistent with a higher activation energy for product formation (being higher below 20°C). This work confirmed previous results by Burns who initially reported a biphasic Arrhenius plot with a point of intersection of 21°C. Activation energies *for electron transfer* of 14.6 and 39 kcal $mole^{-1}$ were found above and below this temperature.(Burns, 1969; Shilov and Likhtenshtein, 1971) Watt and Burns confirmed this result, measuring activation energies of 12.3 and 35 kcal $mole^{-1}$. In addition, only a single slope occurs for ATP hydrolysis with an activation energy of 17 kcal $mole^{-1}$.

Simply, Watt and Burns attempt to explain the question of temperature-dependent ATP : $2e^-$ ratios in terms of the determined activation energies. Above 20°C, ATP hydrolysis has a higher activation energy than electron transfer; thus, an increase in temperature will produce a correspondingly greater increase in the rate of ATP hydrolysis than electron transfer. Below 20°C, a similar scenario occurs except that now the activation energy for electron transfer is higher than that for ATP hydrolysis. Consequently, at less than 20°C, ATP hydrolysis will proceed but the activation barrier for electron transfer is now too high and the enzyme becomes essentially an ATPase.

Combining the above results together may lead one to conclude that the Fe protein probably contains two ATP binding sites that must be occupied for

the subunit to undergo the correct conformationas change ideal for electron transfer from the Fe subunit to the Fe-Mo subunit; the electron transfers occur one at a time, and the optimal ratio is probably 4 MgATP : 2 e^- transferred.

Hageman, Orme-Johnson, and Burris looked at the sequence of events during electron transfer using *A. vinelandii*. (Hageman, et al., 1980). By utilizing pre-steady state kinetics, they observed a rapid burst of MgATP hydrolysis occurring on the same time-scale as electron transfer. This burst corresponded to two ATP's hydrolyzed for each electron transferred between proteins. Their result is consistent with the Hageman and Burris data regarding the initial rapid first electron transfer (Ljones and Burris, 1978). In addition, the k_m's for MgATP were measured during these pre-steady state kinetics resulting in values of 200 μM and 970 μM respectively. These k_m values were similar to the k_d values for MgATP from the Fe protein, indicating a much slower electron transfer rate relative to the binding and dissociation of MgATP from Av_2. Comparing the above data to the steady-state H_2 evolution, k_m values for ATP were 390 μM and 30 μM, respectively. Because of the large decrease in k_m of the steady-state H_2 evolution relative to the pre-steady state condition, the authors concluded that a very slow, essentially irreversible step occurred after electron transfer from Av_2 to Av_1. Hageman and coworkers proposed that binding and hydrolysis of two ATP molecules on Av_2 is sufficient to account for all of the ATP hydrolysis occurring in the steady state reaction. Combined with previous results arguing for two binding sites, they proposed that ATP is used solely for the purpose of facilitating electron transfer. All other ATP hydrolysis could be explained by non-productive or futile hydrolysis.

Recently, in a series of papers by Lowe and Thorneley, the sequence of events determining the mechanism and order of ATP hydrolysis was addressed (Lowe and Thorneley, 1984a; Thorneley and Lowe, 1984a; Lowe and

Thorneley, 1984b; Thorneley and Lowe, 1984b). Using a computer simulation method applied to the pre-steady state (stop-flow) kinetics of *K. pneumonia* ($K_{p)}$ nitrogenase, Lowe and Thorneley propose a detailed mechanism for the enzyme. Scheme 1 depicts the basic unit of the Fe-Mo protein cycle. In order to generate the proposed products in the limiting stoichiometry (Equation 2), eight electrons are required (Rivera-Ortiz and Burris, 1975; Burgess et al., 1981; Guth and Burris, 1983). This is acheived by eight sequential cycles of the Fe protein cycle, (Scheme 1), which, when coupled together, comprise one Fe-Mo protein cycle whereby one N_2 molecule is reduced to two ammonia molecules. Scheme 2 depicts the kinetic sequence of the proposed Fe-Mo protein cycle. In addition, the Lowe and Thorneley mechanism shows a single rate by which ATP hydrolysis and electron transfer are coupled. Thus, once electrons are passed from Fe protein to Fe-Mo protein (k_{+2}, Scheme 1), the hydrolysis of ATP is essentially irreversible. Thorneley and Lowe also depict the dissociation of oxidized Fe protein from reduced Fe-Mo protein (k_{+3}, Scheme 1) as rate-limiting. This rate-limiting process of bound Fe protein to Fe-Mo protein presumably suppresses H_2 evolution by essentially "sandwiching" the H_2 product in between the Fe and Fe-Mo complex. Thus, according to the authors, the Fe protein can be said to have a second role, that being to stabilize the metal-hydrogen interaction in the Fe : Fe-Mo complex to quenching and H_2 evolution. It is only at this time that N_2 can bind by displacing an H_2 present in the Fe_{ox} - $Fe\text{-}Mo_{red}$ complex (k_{+10}, k_{+11}, Scheme 2). Once bound, N_2 can then be sequentially reduced to ammonia.

The mechanism proposed by Thorneley and Lowe has become disturbing to us because should protein dissociation between oxidized Fe protein and reduced Fe-Mo protein be rate-determining, then the activation energy for dissociation should be the highest in the scheme. This would imply

that the distribution of intermediate steps prior to the rate-limiting one should accumulate until a steady state is acheived. This would neccessitate some reversibility of the ATPase reaction. This seems to raise the question as to the irreversibility of the ATP hydrolysis and the concomitant electron transfer step.

The emphasis of our work has been directed towards the molecular mechanism of ATP hydrolysis. Using the specifically labeled ^{18}O-β,γ-ATP previously synthesized in our laboratories, we set out to investigate the direction of bond-cleavage during ATP hydrolysis (Reynolds et al., 1983). By taking advantage of the ^{18}O selectively labeled between the P_β and P_γ, we can, by the use of high-resolution mass spectrometry, determine whether the P_β - O or the P_γ - O bond is broken. Conventional ATPases catalyze cleavage of the P_β - O - P_γ linkage at the P_γ - O position and speculation about the role of ATP in nitrogenase has assumed similar behavior. Breaking of the P_β - O bond has never been reported as it would imply attack at the β-phosphorus. Breakage of the Pγ bond would imply attack by a nucleophile on the γ-phosphorus, leading to the more conventional product.

We in addition have addressed the question of the reversibility of ATP hydrolysis by looking for evidence of positional isotope exchange in the nitrogenase system from *A vinelandii.* Taking advantage of the selective synthesis of ^{18}O-β, γ-ATP, we have set out to determine if the enzyme will induce scrambling of the bridge-label and hence to determine if ATP hydrolysis and electron transfer are separable components of the mechanism. Evidence of PIX would be significant because then we may be able to explore either the reversibility or irreversibility of the reduction mechanism (i.e., PIX could still be associated with futile hydrolysis and not productive electron transfer). A lack of PIX would imply a result consistent with that of Hageman et al and Thorneley and Lowe (Hageman et al., 1980; Thorneley and Lowe, 1984). Should this be

the case, we may be able to set an upper limit on the exchange rate of ATP hydrolysis based upon the sensitivity (signal : noise) of the NMR spectra generated.

MATERIALS & METHODS

Stock solutions were prepared by initial degassing and Argon purge to insure an inert atmosphere. This included base for pH titration of stock. Argon gas (99.9%), used as the inert atmosphere, was initially deoxygenated by bubbling through a chromium, K_2SO_4 solution over amalgamated zinc. High purity acetylene (99.5%) was passed thru a CO_2 / isopropanol trap to remove any residual acetone. High purity hydrogen was used for gas chromatographic standards (Shilov and Likhtenshtein, 1971). ^{18}O-β, γ-ATP (prepared according to the method of Reynolds et al.) was dissolved in HEPES buffer (25 mM), and the pH adjusted to 7.4. $MgCl_2 \cdot 6\ H_2O$ was also dissolved in 25 mM HEPES buffer, pH = 7.4, as a 40 mM stock solution. Sodium dithionite was obtained from Alpha (86 - 90%) and purified by recrystallization from aqueous methanol. Stock solutions of dithionite (0.08 M, pH = 7.2 - 7.8) were prepared by addition of 0.564 g of the salt under Ar to 40 mL of degassed 0.02 M NaOH. Solutions were maintained on ice and discarded after four hours. Nucleotide concentrations for stock solutions were determined by UV_{A259} using an extinction coefficient of ε = 15,400. HPLC analysis of the ATP stock solution determined a purity of 97%. (3% ADP).

Proteins were prepared according to the procedure of McKenna (McKenna, 1979). Calculations for the Fe protein were performed assuming a MW = 64,000. Protein concentrations were determined by the method of Bradford (Bradford, 1976). Stock Fe protein concentrations varied between 8.3 -15.0 mg mL^{-1} with specific activities varying between 1650 - 1950 nmoles mg^{-1} min^{-1} based on reduction of acetylene. Fe-Mo protein calculations were performed assuming a MW = 220,000 with a concentration = 20.4 mg mL^{-1} and specific activity equal to 2000 nmoles mg^{-1} min^{-1}.

For each 1 mL assay, final concentrations were as follows,

$Na_2S_2O_4$ -	5 mM
^{18}O-β,γ-ATP	7.5 mM
$MgCl_2$ -	6.5 mM
C_2H_2	1 mL

For positional isotope exchange experiments, 250 mL incubation bottles were used. Cocktail volumes were changed to 11.6 mL in these bottles with the concentrations and relative volumes kept constant. Cocktails were run in a constant temperature bath, T = 25° C.

Assays were performed under saturating conditions of dithionite, C_2H_2, and Mg-ATP. Assays were prepared with all reagents <u>except</u> Fe protein and then initiated with that subunit. ATP hydrolysis was initially followed at 15 minute intervals by HPLC analysis. Upon completion, reaction mixtures were quenched with an equal volume of absolute methanol and cooled to -20°C.

Gas chromatographic analysis was used to determine the C_2H_4 / C_2H_2 product / substrate ratio by injection of 150 µL of the assay gas mixture to a Pora-pak N column. The mode was flame ionization. Data recording was by interfacing to a Hewlett-Packard 3390A integrator. Relative sensitivity of the GC for the two gasses were prepared in a 21.5 mL assay bottle with 1.0 mL H_2O, 50 µL C_2H_4, and 1.0 mL C_2H_2. The standard was based to simulate the approximate level of C_2H_4 to be produced in the assay mixture. H_2 was analyzed by GC analysis on a molecular sieve column using thermal conductivity detection. Standard assay was identical to the one for acetylene except 10 µL, 20 µL, 40 µL, and 50 µL aliquots of H_2 were utilized. In large scale reactions, the standards were scaled accordingly in a similar manner.

Product formation was determined using the following calculations:

$$N_e = V_{C2H2} \times P/760 \times 1/RT \times (V_f - V_{rm})/[V_{C2H2} + (V_c - V_{rm})] \times C_{C2H4}/(GCR \times C_{C2H2} + C_{C2H4}) \times 10^9 \text{ nmoles/ mole}$$

N_e = nmoles ethylene produced

V_{C2H2} = initial volume acetylene in assay bottle in liters

P = room pressure = 755 mm

R = ideal gas constant = 0.08205 L atm. mole $^{-1}$ $^{o}K^{-1}$

V_f = flash volume in liters

V_{rm} = reaction mixture volume in liters

C_{C2H4} = counts ethylene from gas chromatograph

C_{C2H2} = counts acetylene from gas chromatograph

GCR = gas chromatograph ratio of ethylene vs acetylene detection from standards

ATP HYDROLYSIS

Based upon $2e^-$ requirement for reduction of acetylene and 2 ATP's hydrolyzed per $1e^-$ transferred.

ATP hydrolyzed = 4 x (nmoles C_2H_4 + nmoles H_2 produced.)

Additional ATP hydrolysis quantitations were determined by analytical HPLC using a C-18 reverse phase column and eluting with an isocratic system consisting of the following:

74 % 10 mM tetra n-butyl ammonium bromide
30 mM KH_2PO_4

26% acetonitrile

pH = 3.0

ISOLATION OF PRODUCT

The methanol quenched solutions were then quickly frozen and stored at -20°C until ready to be analyzed. Just prior to isolation, the solution was thawed, dried in vacuo, and dissolved to 10-fold the original assay volume with distilled - deionized H_2O. The pH was then adjusted to pH = 7.6 with 0.25 N NaOH. The entire solution was then loaded onto a DEAE Sephadex A-25 anion exchange column (Pharmacia) generated in the HCO_3^- form. The column was developed with a linear gradient of TEA - HCO_3^-, 0.07M - 0.8M. Column size and reservoir volumes were scaled according to the amount of nucleotide to be isolated.

For mass spectrometry analysis, fractions were collected in 10 mL aliquots and assayed for inorganic phosphate according to the method of Ames (Ames, 1966). Fractions corresponding to the inorganic orthophosphate were combined, solvent removed in vacuo and washed with absolute methanol which was also removed in vacuo. This procedure was repeated until all of the TEA - HCO_3^- was removed. The recovered P_i was then converted to the permethylated product according to the method of Midlefort and Rose (Midelfort and Rose, 1976). The process involved passing the isolated triethylammonium salt of P_i through a Dowex AG-50W X-8 (H^+ form) column, concentration in vacuo followed by treatment with 50 µL of 10% aqueous methanol and dropwise addition of freshly prepared diazomethane etherate until a yellow tint persisted. Stirring was maintained for three hours. The mixture was then evaporated under a stream of N_2 gas to yield a yellow oil. This oil was then reserved for analysis by high resolution mass spectrometry. ADP and ATP fractions were isolated in a similar manner. Fractions were detected by UV spectrophotometry at 259 nm. Fractions were consolidated and isolated in the same manner and were converted to Na^+ salt by exchanging the methanol-

soluble triethylammonium salt of the nucleotide with saturated NaI in acetone. The precipitate was isolated by centrifugation at 5000 rpm on a Sorvall SS-34 rotor using 30-mL Corex centrifuge tubes. The supernatant was decanted away and the pellet resuspended with absolute acetone. This procedure was always repeated at least four times. The product was then dried under vacuum desiccation and stored at -10°C until prepared for nmr spectroscopy. The precipitation procedure was eliminated in later experiments.

PREPARATION OF NMR SAMPLES

Nmr spectra were obtained using 5 mm Wilmad 528PP thin-walled tubes. All glassware, pipettes, filters and tubes were pre-washed with a concentrated H_2SO_4 : HNO_3 ,(1 : 1, v : v), soaked for 24 hours and exhaustively washed with distilled, deionized H_2O. All samples were dissolved in 600 µL of 100 mM HEPES buffer, pH = 8.0, and passed through a Chelex column generated in the Na^+ form, using Whatman 934AH glass-fibre filters as resin filler plugs. This procedure was always repeated at least twice. To this effluent was added 30 µL of 30 mM sodium (bis-ethyleneglycol)tetracetic acid (EGTA) (pH = 8.0) and the entire sample readjusted to pH = 8.0. The sample was then frozen and lyophilized. The lyophilized sample was then reconstituted to 600 µL using distilled - deionized H_2O that was previously Argon purged for 15 minutes and 100% D_2O (low paramagnetic, Aldrich, T_1 = 48 Sec.)

Mass Spectrometry Data

Mass spectra were determined on the Kratos MS-50 high resolution spectrometer at the University of California, Berkeley. The mode was electron impact. Three scans were collected (scan #14, #15, and #16) and the peaks corresponding only to the unlabeled (140.0232 mass units) and the ^{18}O-labeled (142.0281 mass units) trimethylphosphate (the permethylated product) were quantitated. It should be noted that we had to resort to high resolution mass spectrometry because of too much interference by common molecular weight side products and fragments when quantitated by conventional low resolution mass spectrometry. The 140 : 142 mass ratio was then calculated and compared to expected calculated value.

MASS SPECTROMETRY RESULTS

ADP and ATP Recovery Data

(based on % of total ATP = 40 umoles)

	Fe + Fe-Mo	Fe only	Fe-Mo only	No protein
ATP	8.18 µmoles (20.5%)	26.9 µmoles (67.3%)	22.9 µmoles (57.2%)	34.8 µmoles (87.0%)
ADP	14.8 µmoles (37.0%)	1.16 µmoles (2.9%)	1.06 µmoles (2.65%)	1.45 µmoles (3.6%)

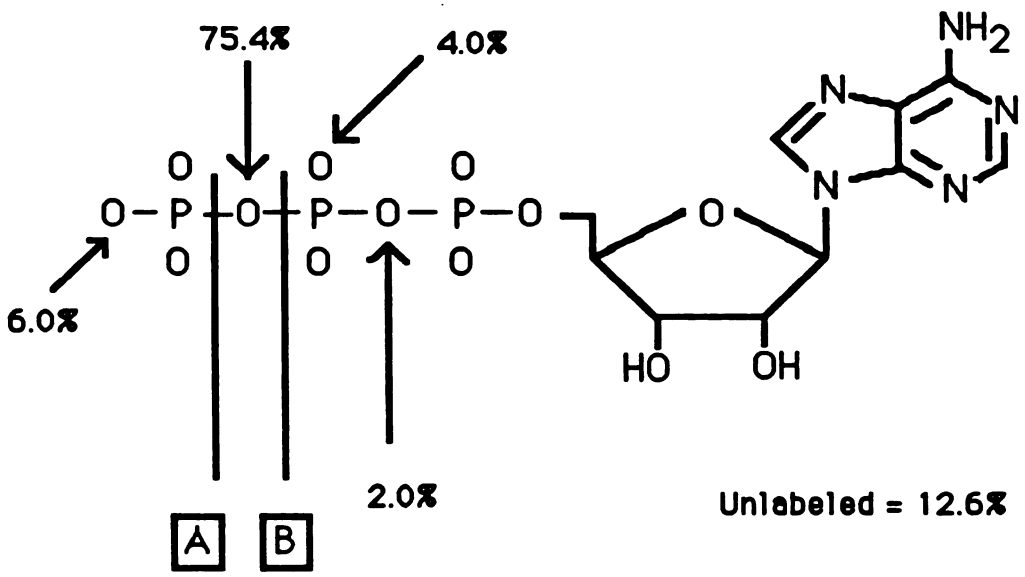
$\varepsilon = 15{,}400$

Cocktails Run

The following assays and controls were run

	Fe-Mo Protein	Fe- Protein	Cocktail
A) Cocktail plus both subunits.	(+)	(+)	(+)
B) Cocktail with only Fe-Mo subunit*	(+)	(-)	(+)
C) Cocktail with only Fe subunit*	(-)	(+)	(+)
D) Cocktail with no enzyme*	(-)	(-)	(+)

* Note: No detectable inorganic phosphate was isolated from these assay cocktails.



Assuming a cleavage of the P_β - O bond, one would expect from the permethylated derivative cleaved at B:

142.0281 (^{18}O) = 81.4%

140.0232 (^{16}O) = 18.6% Relative Ratio 140.0232 : 142.0281 = 0.229

Assuming a cleavage of P_γ - O bond, one would expect from the permethylated derivative cleaved at A;

142.0281 (^{18}O) = 6.0%

140.0232 (^{16}O) = 94.0% Relative Ratio 140.0232 : 142.0281 = 15.6

RESULTS

N = 3

PRODUCT	$PO(OMe)_3$	$Me^{18}O\ PO(OMe)_2$
MASS	140.0232	142.0281
ENRICHMENT X	100%	7.25%
RATIO 140.0232 : 142.0281	13.8 (average of 3 scans)	
STANDARD DEVIATION	2.79	
PREDICTED 140.0232 : 142.0281	A = 15.6	B = 0.229

Thus, within a standard deviation, our results conclude hydrolysis by cleavage of the P_γ - O bond.

POSITIONAL ISOTOPE EXCHANGE EXPERIMENTS

Initial spectra were acquired on a hybrid spectrometer at a field of 5.6 Tesla from Cryomagnets Inc. The wide-bore magnet was fitted with a 10 mm probe tuned to 97.571 MHz and temperature controlled to 20°C. The spectrometer was interfaced to a Nicolet Technologies 1180 computer and pulse programmer. Subsequent spectra were acquired at 202.45 MHz on a GE - Nicolet 500 MHz spectrometer programmed with the Nicolet 1280 software package. A 10-mm heteronuclear probe was used.

Spectral parameters on the hybrid spectrometer consisted of 8192 data points with a sweep width of ±400 Hz., 5.12 second acquisition time, one second delay, and 49° pulse angle. Typically, 2000 pulses were accumulated, generating a signal : noise of approx. 45. Line broadening of 0 Hz. and 8192 data points of zero filling was applied. Spectra were apodized by double exponential multiplication (DM) to enhance resolution. (see Appendix 1) T_1 for

P_β was equal to 2.71 seconds. Final spectra were defined without zero-filling by 0.002 ppm / data point and 0.001 ppm / data point with zero-filling.

Spectral parameters at 202.45 MHz. consisted of 8192 data points with a sweep width of ± 250 Hz., 90^0 pulse angle, 8.1 second acquisition, and 500 msec pulse delay. Typically, 100 - 2000 FID's were accumulated generating signal : noise > 50. A maximum of 0 - 0.3 hz. line broadening and the spectra were zero-filled twice. The "DM" routine was applied for resolution enhancement; however, under no circumstance were the relative intensities changed by greater than 1.5% when using resolution enhancement routines (see Appendix 1). Final spectra without zero-filling were defined by 0.0006 ppm/datapoint and with zero filling was 0.00015 ppm/data point. T_1 for the P_β was equal to 0.71 seconds.

To insure that spectral intensities were not influenced by foldover peaks, the carrier frequency, pulse angle and spectral width (and data points) were alternately varied to insure peak intensities remained constant. When foldover peaks were detected, the carrier frequency was moved until they were no longer detected.

^{31}P NMR RESULTS

A list of the NMR results from nitrogenase experiments is shown in Table 1. Three different Fe subunit lots (Fe G29, Ge F33, and Fe G36) and two FeMo subunit lots (FeMo S29 and FeMo S30) were used. Incubation times varied from 55 minutes to as much as five hours and turnover of ATP for fixing systems generally ranged between 70% - 90%. Under normal fixing systems utilizing H^+ and C_2H_2 as the substrates, two of the three fixing systems did not exhibit any significant amount of PIX (0 - 3% ± 4%). Initial experiments with Fe G29 and FeMo S29 exhibited no evidence for PIX (0 ± 4% experimental ; 0± 8% for

control) In one system (Fe G33 + FeMo S30) a significant amount of PIX was seen (17 - 35% ± 4 - 10%); however, the control experiment with Fe G33 alone also exhibited a significant amount of exchange of the isotopic label (24% ± 5%). In addition, turnover experiments in the presence of carbon monoxide (CO), a non-competitive inhibitor of C_2H_2, also exhibited a significant amount of exchange (12 - 26% ± 4%). (It should be noted that one control experiment with Fe G33 did not exhibit any significant evidence for exchange (< 0.9% ± 4%) ; however, we were unsure of the validity of this result as the enzyme was not later checked for activity.) Under no circumstance did any exchange ever occur in the presence of the FeMo subunit alone. The third set of experiments (Fe G36 and FeMo S30) revealed a significant amount of exchange when the Fe G36 subunit control was incubated (6% ± 4%). For this reason, the resultant protein subunit was then further purified by column chromatography on a Sephacryl S-200 exclusion column (Pharmacia). Repeat experiments with the Fe G36 subunit (now designated as Fe "G36") exhibited no significant evidence for exchange (0.9% ± 4%). Repeating previous fixing experiments with the repurified Fe "G36" now exhibited only a small (≤ 3% ± 4%) amount of exchange. In addition, the amount of exchange did not vary when the Fe : FeMo ratio was varied from 0.8 to 1.3. The reason for using two different Fe : FeMo ratios was to test the observation that the experiments with Fe G29, performed with a Fe : FeMo ratio = 0.8, demonstrated no significant PIX whereas the experiments with Fe G33, performed with a Fe : FeMo ratio = 1.3, exhibited significant PIX. In addition, one can see that under similar turnover conditions, the amount of product formation (nmoles H_2 + nmoles C_2H_2) remained similar when the Fe subunit lot was changed, thus insuring little variation in activity of lots (3.38 - 4.9 x 10^4 nmoles for Fe G33 + FeMo S30 vs.

3.3 - 3.4 x 10^4 nmoles for Fe "G36" + FeMo S30). Total ATP turnover rates were also similar in both sets of experiments (74 - 90% total ATPase range).

Figure 1 show NMR spectra generated at 97.57 MHz of the initial fixing experiments with Fe G29 and FeMo S30 vs. FeMo S30 alone (control). As is evident, there is no evidence for PIX. Signal : noise was 48 for the experimental and 37.8 for the control. Although not presented, the control using Fe G29 exhibited similar results. The remaining figures are generated at 202.5 MHz. on the GN 500 spectrometer at UCSF. As is evident, all experiments using the Fe G33 subunit exhibited significant exchange. Subsequent spectra with Fe "G36" are also presented. The original control with Fe G36 is not presented; however, incubation experiments resulted in 6% ± 4% exchange as catalogued in the tables in this chapter.

From the above data, we can assign an upper limit based upon the signal : noise from the spectra generated by NMR. With an uncertainty of ± 4%, we can conclude that the ATPase reaction proceeds with an upper limit of exchange / turnover ≤ 0.029.

DISCUSSION AND CONCLUSION

The observations from experiments using three different lots of Fe subunit initially appear ambiguous; however, they can probably be explained by assuming that the Fe G33 subunit may be significantly contaminated with an enzyme capable of catalyzing PIX (possibly an ATPase). The justification for this is the fact that when Fe G33 is incubated in the absence of FeMo protein, a significant amount of PIX can still be observed. Alternatively, the original experiments with the Fe G29 and FeMo S29 subunits did not exhibit any evidence for exchange (± 5%) while the repurified Fe "G36" sample exhibited only an insignificant amount of exchange after 85% turnover (3% ± 4%). This

has led us to believe that the exchange or reversibility of ATP hydrolysis for the ATP-coupled reduction in nitrogenase is either very small or essentially irreversible. The following are the reasons for this assumption:

1) If exchange were to be associated with either product formation (C_2H_2 + H_2) or ATP hydrolysis, we should see a definite correlation between the two. We have not been able to observe any such correlation between either the substrates used or products formed. Thus , the amount of exchange appears to be random even when the exact same parameters are repeated. In addition, the question should be asked whether the insignificant (<3%) amount of exchange with the repurified protein subunits may be associated with turnover. We think not because, compared to control (Fe "G36" alone), the amount of exchange at best could have changed by little more than three-fold (<0.9% vs. <3%) yet the amount of turnover now has increased over four orders of magnitude (1.2 nmoles product formed for Fe "G36" control vs. 3.3×10^4 nmoles produced for Fe "G36" + FeMo S30, normal fixing conditions).

2) The two Fe subunits did not show any significant amounts of ATP exchange, and in addition, we did not see any significant amounts of ATP exchange under fixing conditions after large amounts of ATP hydrolysis (74 - 95% hydrolysis). The only Fe subunit that did induce bridge to non-bridge ATP exchange under large amonts of hydrolysis also exhibited exchange under non-fixing conditions with the Fe subunit alone.

3) The Fe subunit which originally showed significant amounts of exchange under non-fixing conditions later showed only insignificant amounts of exchange under fixing conditions after an additional purification step.

The other question that could be addressed is as follows: can the large amount of exchange be associated with a very large Fe : FeMo ratio? This must be addressed since the apparent Fe G33 control which exhibited a good deal of

exchange also exhibited some evidence of turnover (734 nmoles $C_2H_4 + H_2$ produced). This appears to imply that a small amount of FeMo subunit was present in this preparation. Hence, we cannot strictly rule out that a large Fe : FeMo ratio could be somehow inducing an exhange reaction. Tso and Burris reported earlier that fixing cannot occur with both subunits present (Tso and Burris, 1973).. In addition, the authors claimed there was no alteration in ATP : $2e^-$ ratio when the Fe : FeMo ratio was greater than two. This data appear to imply that this large Fe : FeMo ratio is not altering the mechanism to any significant extent and that exchange probably is due to a contaminant. We can, however, probably rule out the possibility that the exchange phenomenon being due to a futile hydrolysis because the ATP : $2e^-$ ratio was nearly maximal (3.69 vs. 4 for ideal conditions).

Although the use of CO as a non-competitive inhibitor did elicit PIX (12 - 26%, 85 - 90%ATP hydrolyzed), we cannot yet argue as to whether or not this could be attributed to a contaminant because: 1) the same Fe G33 subunit was utilized in this instance, and 2) CO could be inducing exchange independently of the possible contaminating ATPase.

The results generated from our PIX experiments now bring us back to the question proposed in our introduction. Is the absence of exchange consistent with protein dissociation being rate-limiting? Clearly, the results point to the conclusion that the ATP hydrolysis step in nitrogenase (k_2 in Scheme 1) is essentially irreversible. Of course, we can only conclude that our results are as accurate as the spectra generated on the NMR spectrometer (i.e. upper limits for exchange are based upon the signal : noise ratios of the experimental spectra presented). This is consistent with the results of Hageman et al. which states that a slow, irreversible process is occurring after electron transfer (Hageman et al., 1980). In addition, our results seem to indicate that the slow, irreversible

step may be associated with the relative irreversibility of the nitrogenase ATPase reaction (< 0.029 exchange / turnover). In addition, it may be proposed that this slow step may be the reason for an essentially irreversible ATPase in nitrogenase.

It is not clear exactly why the ATPase reaction is essentially irreversible. Mechanistically, one could propose a release of inorganic phosphate from the $[Fe_{ox} \cdot (MgADP + P_i)_2 \cdot FeMo_{red}]$ central complex as being consistent with an essentially irreversible process. Hageman et al. have experimental evidence that inorganic phosphate release parallels the burst phase and slow steady-state hydrolysis of MgATP (Hageman et al., 1980). Although kinetic data is lacking, a slow release in addition to very slow or non-existent rebinding of inorganic phosphate to the complex could retard the reversibility of the complex to render the ATPase reaction essentially irreversible. A possible experiment to verify this would be to add excess P_i as a product inhibitor. This may promote the reversal of the ATPase reaction.

Alternatively, one could propose a possible isomerization of the $[Fe_{ox} \cdot (MgADP + P_i)_2 \cdot FeMo_{red}]$ central complex such that now the compolex cannot undergo a reversal of the ATPase reaction. In other words, one could conceive of a situation whereby the redox potential of the complex is so unfavorable that the reverse reaction is not feasible.

But, can a rate-limiting protein dissociation be consistent with a prior irreversible step? In personal communications with Prof. Paul F. Cook, North Texas State University, it has become clear that a rate-limiting step can be consistent with an irreversible step occurring earlier in the scheme (see Scheme 1). However, it should be noted that we may not be able to interpret the scheme 1 of the Lowe and Thorneley mechanism in terms of a reasonable activation energy profile (Lowe and Thorneley, 1984a). Recall that if there is not

a reverse rate constant, k_{-2}, in Scheme 1, this would neccessarily imply the intermediate complex [Fe_{ox} • (MgADP + Pi) • $FeMo_{red}$] would be lying in an essentially infinitely deep well in the activation energy profile. Hence, we cannot reasonably interpret Scheme 1 in terms of a continuous activation evergy profile. At best, we can say only that the measured rate constant for protein dissociation is rate-limiting compared to all the kinetic parameters compiled. But clearly, an unknown component of the mechanism exists between catalysis and protein dissociation that makes the scheme discontinuous.

Thus, the actual mechanism of the ATPase reaction in nitrogenase remains unclear. However, we have provided evidence through exchange experiments that the reversal of the ATPase reaction coupled to electron transfer in nitrogenase is indeed small, not only under initial velocity conditions but also under prolonged turnover conditions (>85% ATP hydrolysis).

FUTURE EXPERIMENTS

Although the available results have led to the proposal that the ATPase reaction coupled to reduction in nitrogenase is essentially irreversible (and that exchange catalyzed by the Fe G33 subunit was probably due to a contaminant), clearly the results are equivocal. THe following experiments are proposed to resolve the question of a reversible ATPase for nitrogenase:

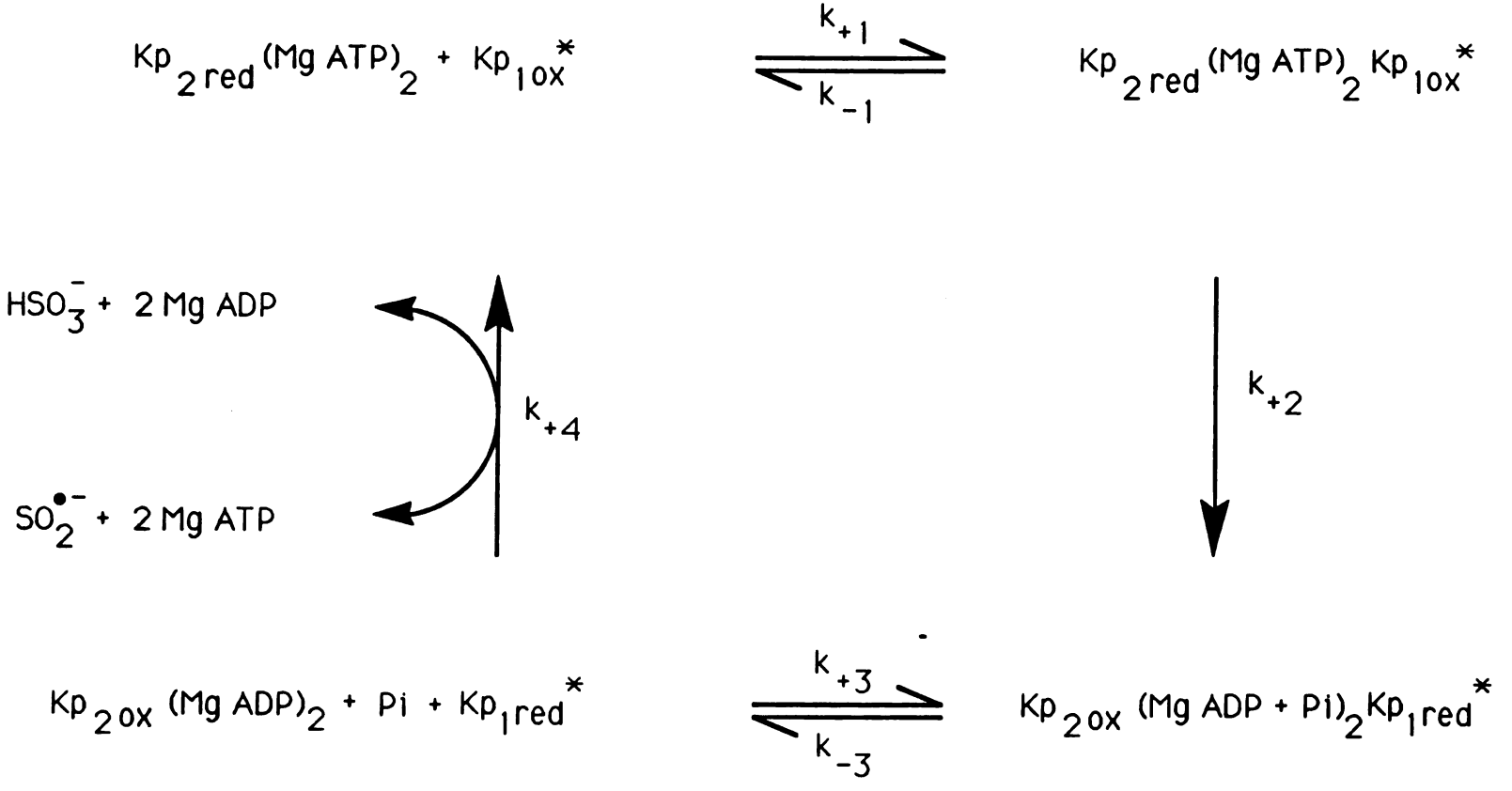
1) Repeat the Fe G33 control to determine if exchange exists with small amount of turnover. Note that this subunit is the only subunit associated with a significant amount of exchange. All other Fe-containing subunits exhibited insignificant exchange.

2) Conduct a high Fe : FeMo ratio fixing experiment (Fe : FeMo > 100) to determine if this will alter conditions so that exchange can be induced. This

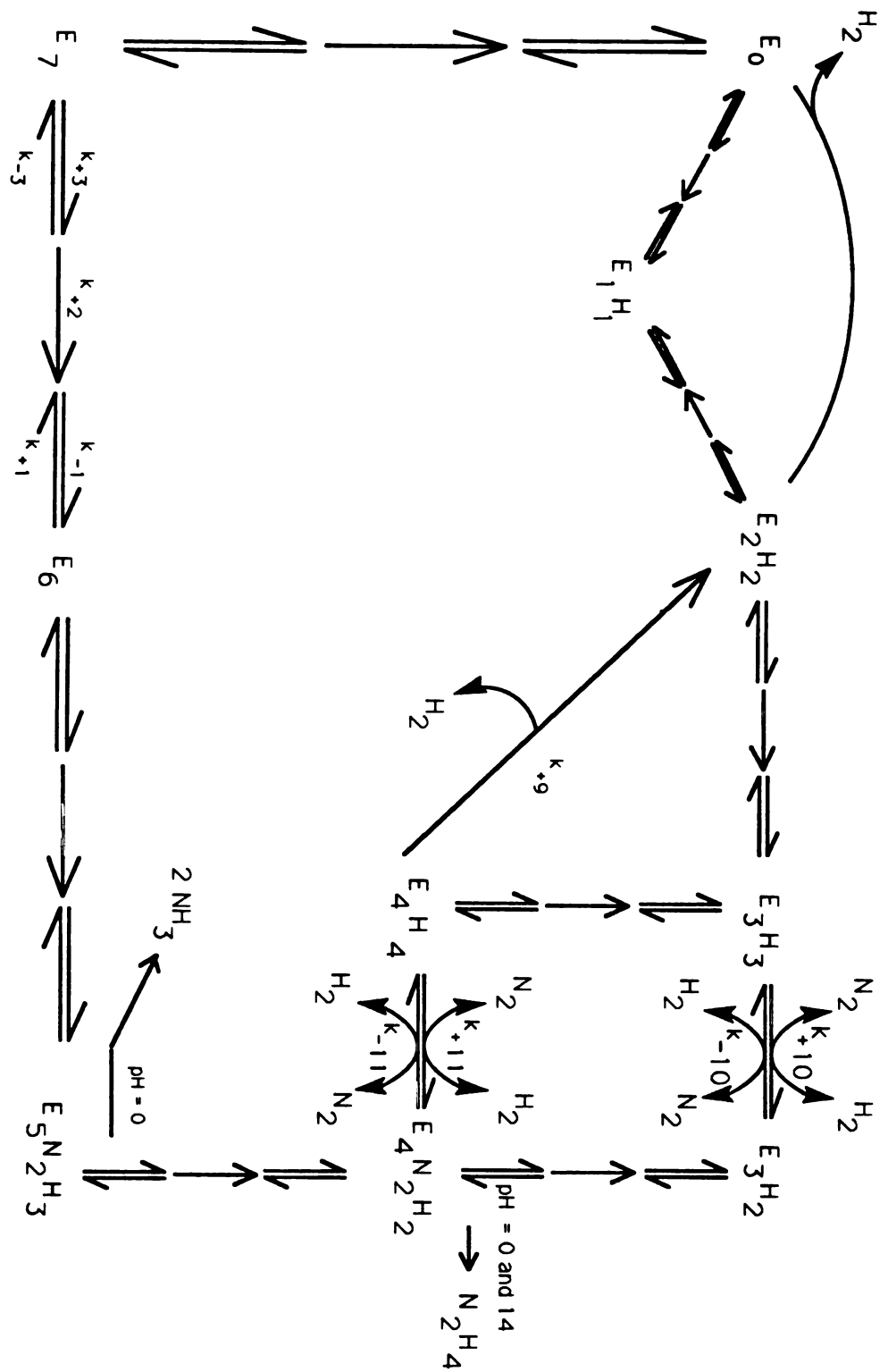
result (coupled to the result in 1) will verify whether the Fe G33 experiment (positive PIX) could be due to a high Fe : FeMo ratio.

3) Repeat CO inhibited experiments using the Fe "G36" subunit. This will help determine if CO is a significant cause of exchange. The appropriate control has already shown that the amount of exchange under C_2H_2 (and H^+) conditions is no more than 3% after 85% turnover. Personal communications with Professor Orme-Johnson at MIT have expressed the need for this experiment at different CO pressures because nothing is known about how CO alters the active site to inhibit C_2H_2 catalysis while still permitting (and possibly even promoting) H_2 formation.

4) Conduct fixing experiments with H+ as the sole substrate for fixing. Should there be evidence of exchange, this experiment should aid us in determining whether one or both substrates may or may not be associated with exchange.



Scheme 1. Oxidation - reduction cycle for the Fe protein.



Scheme 2. Mo-Fe protein cycle.

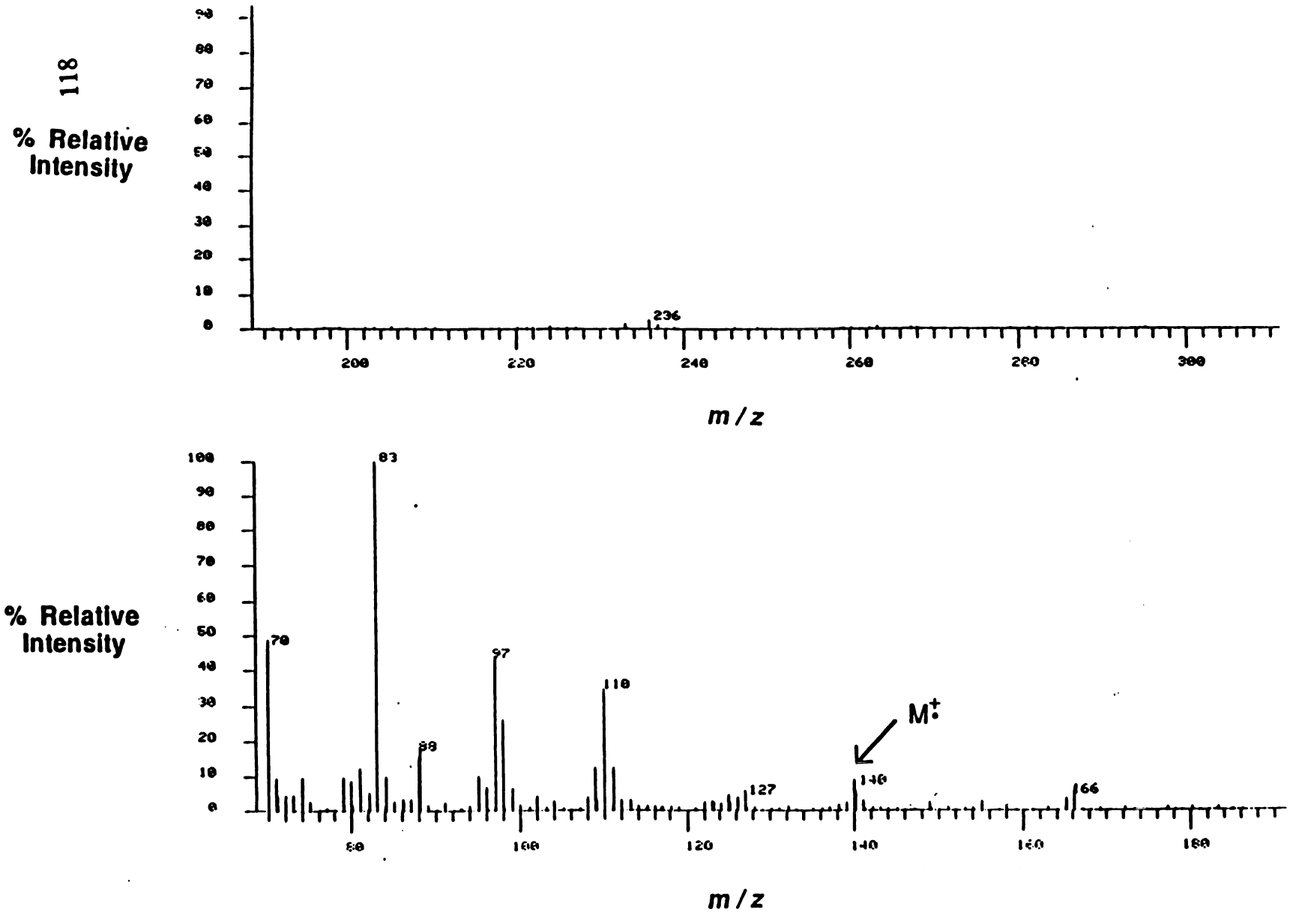


Figure 1. Electron impact mass spectrum of trimethyl phosphate.

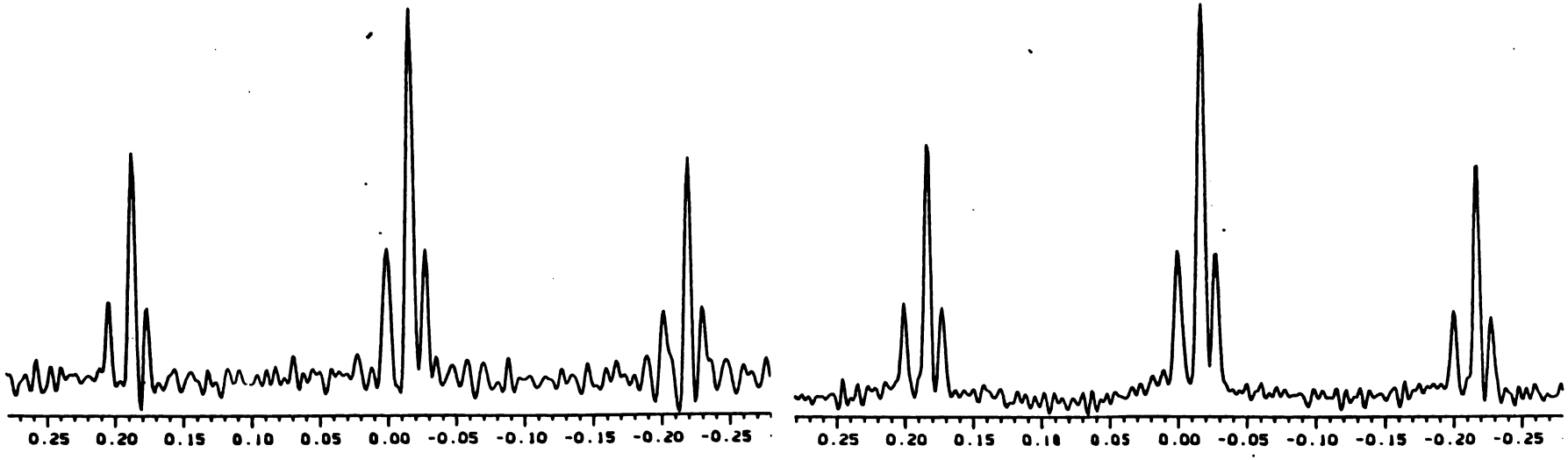


Figure 2. ^{31}P NMR at 97.57 MHz. of the β -phosphoryl resonances of ^{18}O- β,γ -ATP. Incubation conditions are described in text. Spectrum on the left contains only the Fe-Mo subunit of nitrogenase (control). Spectrum on the right contains both the Fe and Fe-Mo subunits of nitrogenase (experimental).

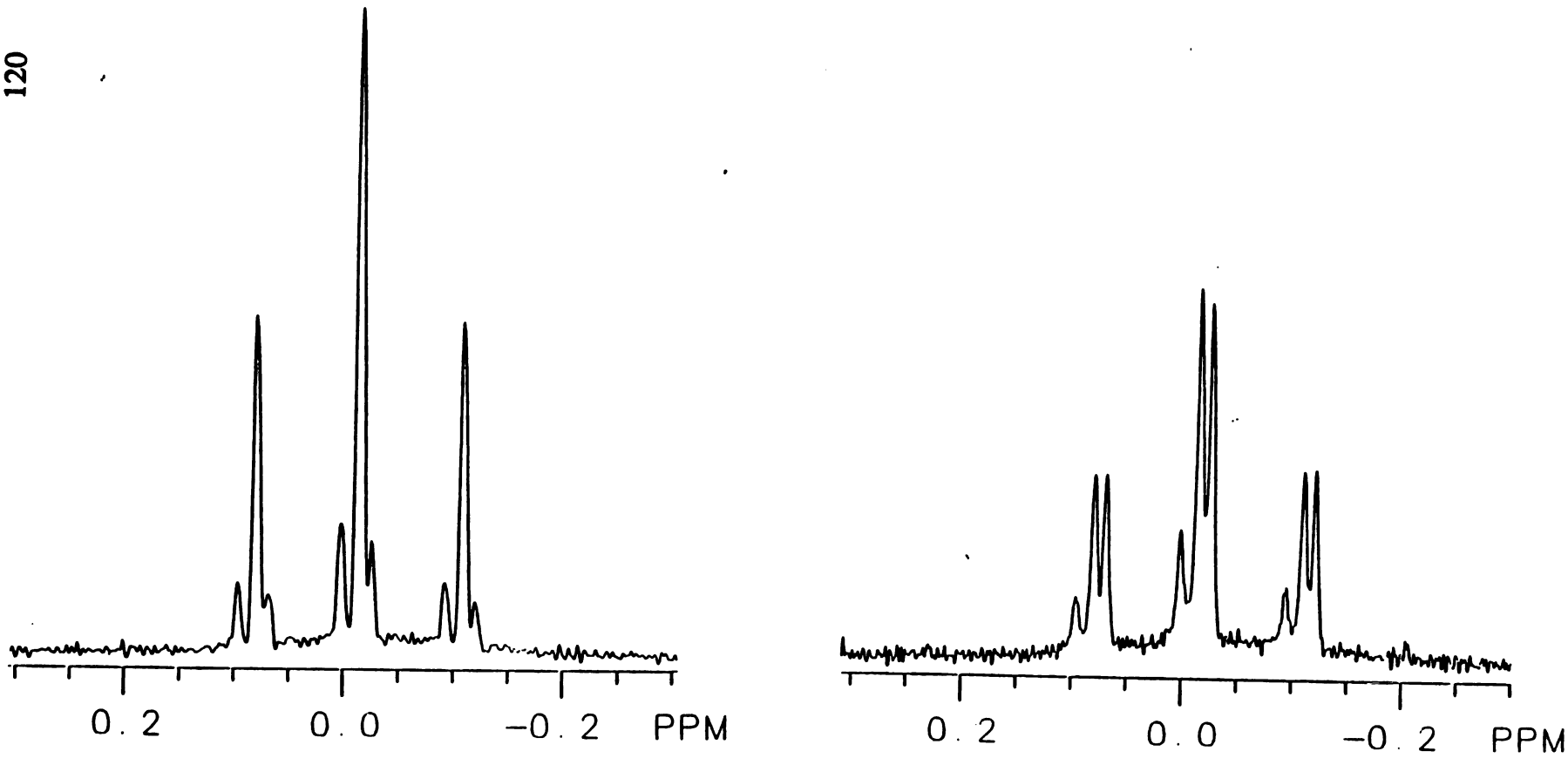


Figure 3. ^{31}P NMR at 202.5 MHz. of the β-phosphoryl resonances of ^{18}O-β,γ-ATP incubated with the enzyme nitrogenase in the presence of the non-competitive inhibitor carbon monoxide (CO). Spectrum on the left contains only the Fe-Mo subunit of nitrogenase (control). Spectrum on the right contains both the Fe and the Fe-Mo subunits of nitrogenase (experimental). Incubation conditions are described in text.

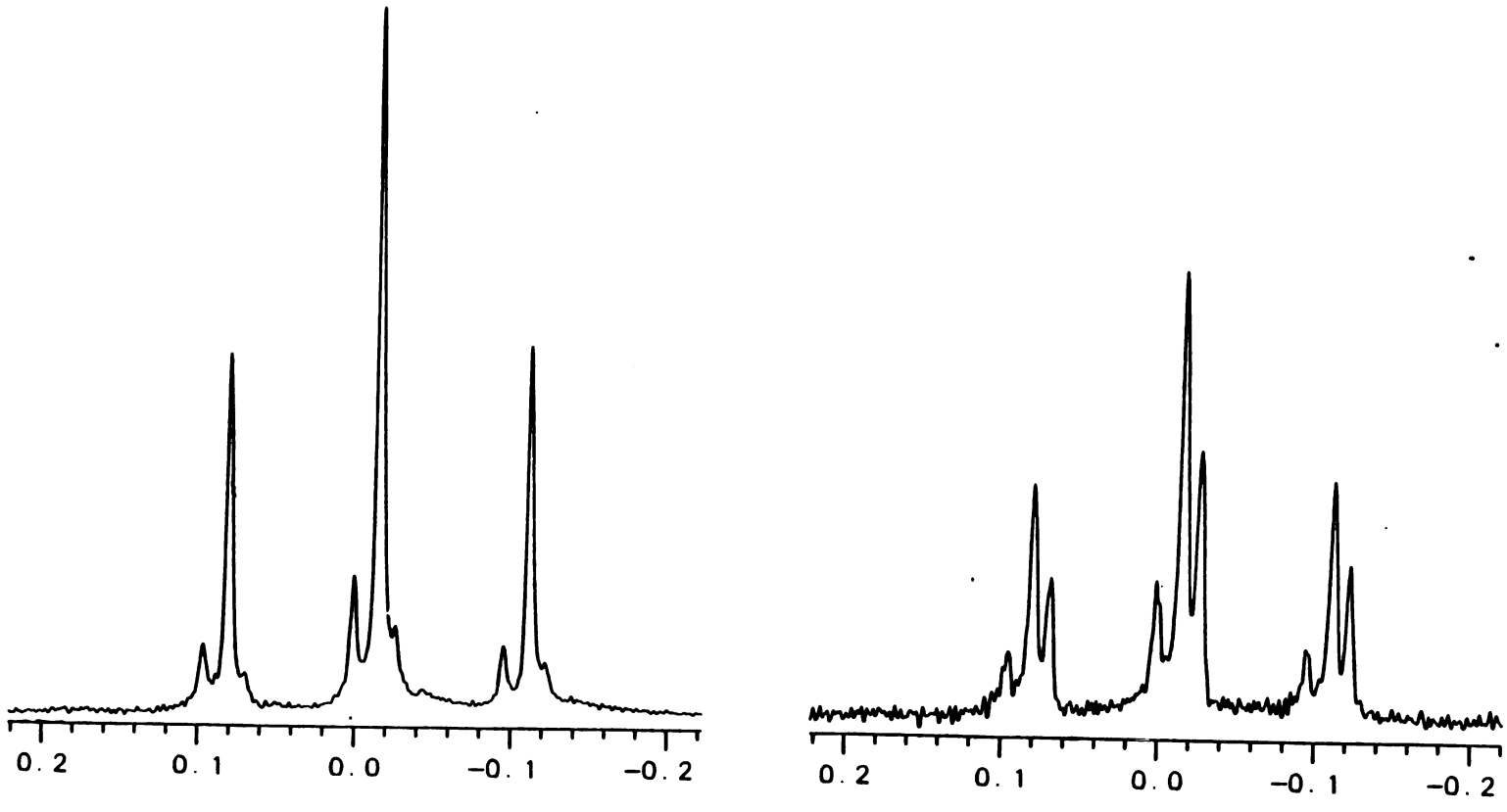


Figure 4. ^{31}P NMR at 202.5 MHz. of the β-phosphoryl resonances of ^{18}O-β,γ-ATP. Incubation conditions are described in the text. Spectrum on the left was after incubation in the absence of either subunit of nitrogenase. Spectrum on the right was after incubation in the presence of only the Fe subunit of nitrogenase (lot Fe G33).

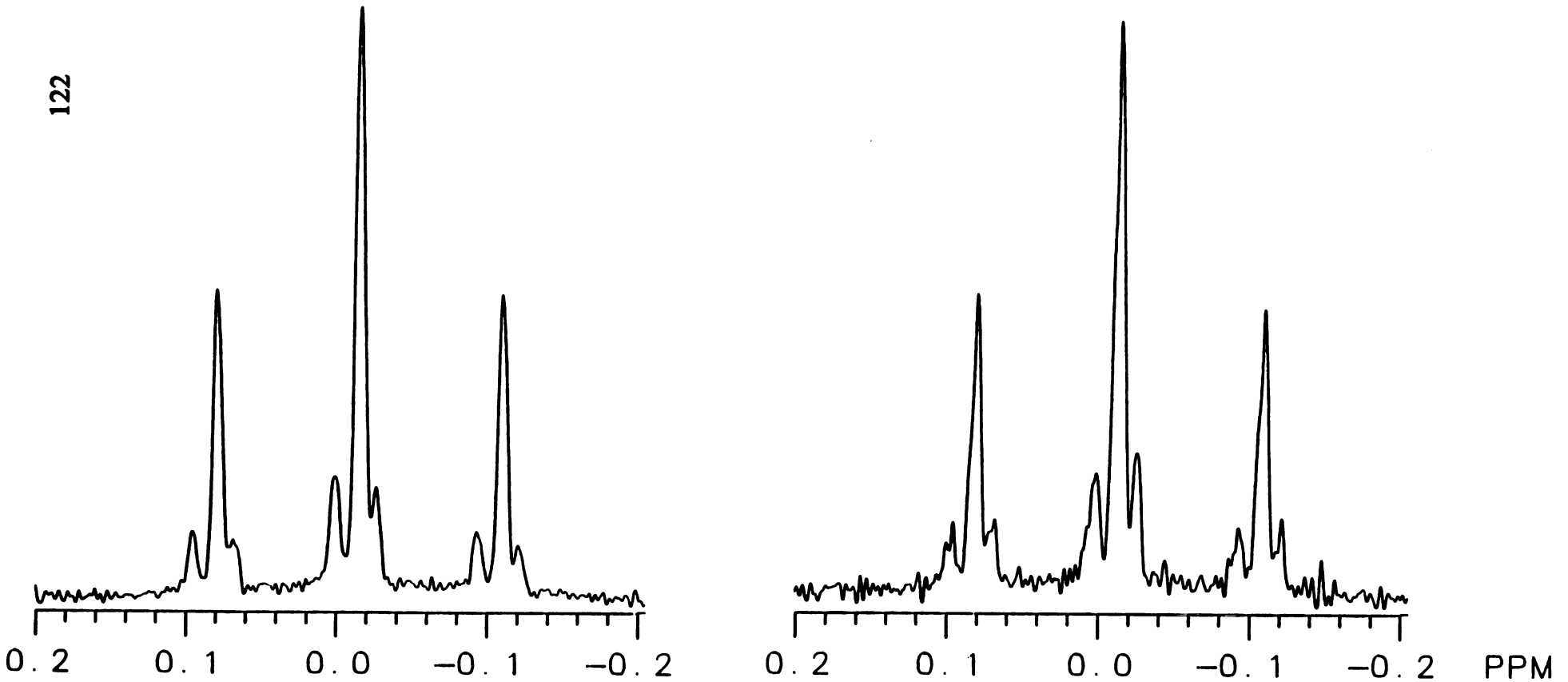


Figure 5. ^{31}P NMR at 202.5 MHz. of the β-phosphoryl resonances of ^{18}O-β,γ-ATP. Incubation conditions are described in the text. Spectrum on the left was after incubation in the presence of only the Fe subunit of nitrogenase after repurification on a Sephacryl S-200 exclusion column (control). Spectrum on the right was after incubation with both the Fe and Fe-Mo subunits of nitrogenase. Fe : Fe-Mo subunit ratio was equal to 0.8

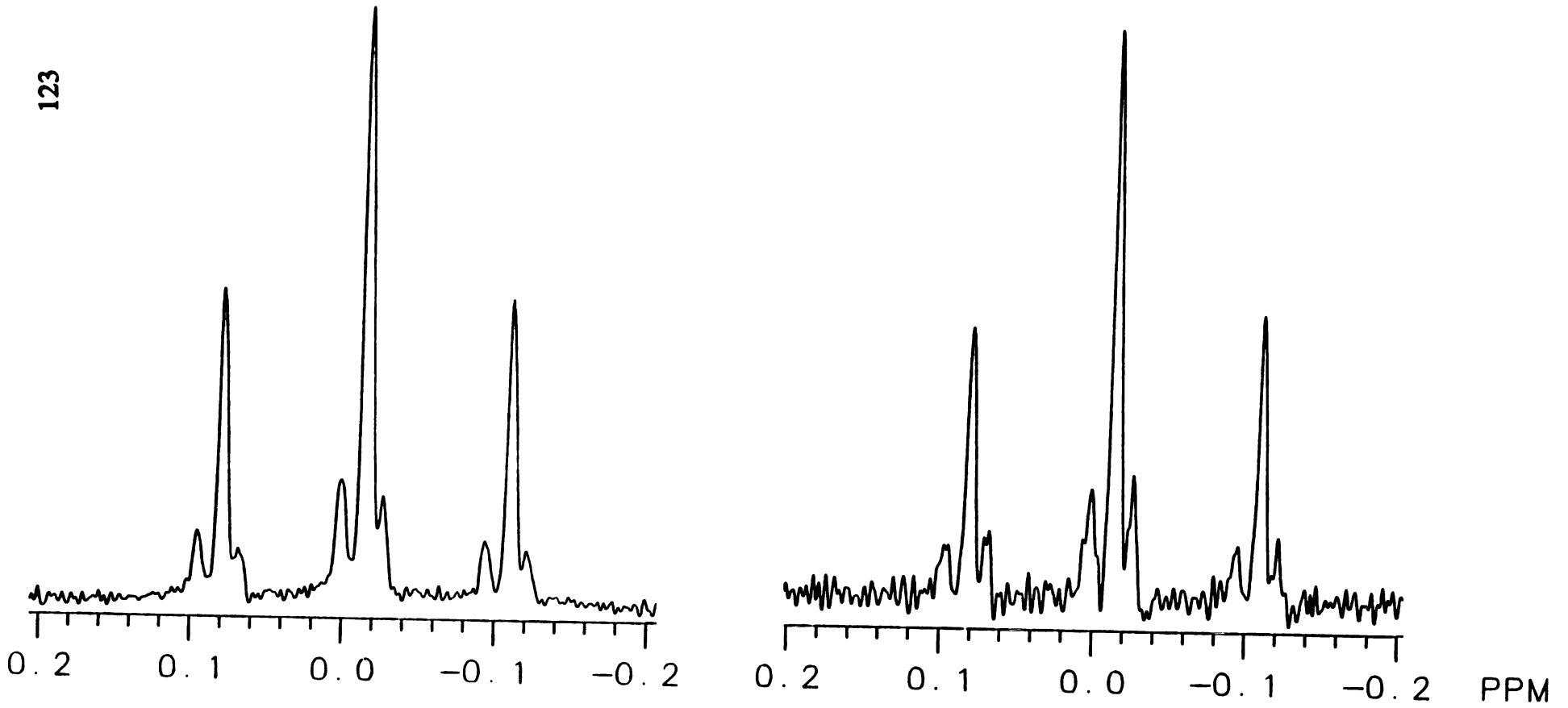


Figure 6. ^{31}P NMR at 202.5 MHz. of the β-phosphoryl resonances of ^{18}O-β,γ-ATP. Incubation conditions are described in the text. Spectrum on the left was after incubation in the presence of only the Fe subunit of nitrogenase after repurification on a Sephacryl S-200 exclusion column (control). Spectrum on the right was after incubation with both the Fe and Fe-Mo subunits of nitrogenase. Fe : Fe-Mo subunit ratio was equal to 1.3.

Table 1. Nitrogenase PIX Data

Fe Code	Conc.	FeMo Code	Conc.	Fe / FeMo	Δ T(min)	≈ Turnover ATP	CO	PIX	Error
Fe G29	1.5 uM	FeMo S29	1.84 µM	0.82	150	70 - 80%	NO	(-)	±4%
Fe G33	3.5 uM	FeMo S30	2.7 µM	1.29	300	90 - 95%	YES	26%	±4%
Fe G33	3.5 uM	FeMo S30	2.7 µM	1.29	105	85 - 90%	YES	12 %	± 4%
Fe G33	3.5 uM	FeMo S30	2.7 µM	1.29	105	85 - 90%	NO	17%	±10%
Fe G33	3.5 uM	FeMo S30	3.16 µM	1.11	105	80 - 90%	NO	32%	± 6%
Fe G33	3.5 uM	FeMo S30	3.16 µM	1.11	15	55%	NO	≈35%	±10%
Fe G33	3.5 uM	(-)	(-)	(-)	105	≈ 5 - 10%	NO	24%	± 5%

Table 1. Nitrogenase PIX DATA (cont.)

Assay Volume	Ethylene Activity	Hydrogen Activity	Total Activity	ATP Hydrolyzed	ATP/2e- ratio
25.2 mL	563 nm / mL	361 nm / mL	924 nm / mL	142.6 µ mole	6.12
71.6 mL	11.8 nm / mL	1160 nm / mL	1170 nm / mL	507.7 µ mole	6.06
34.8 mL	12.2 nm / mL	1200 nm / mL	1210 nm / mL	221.9 µ mole	5.26
34.8 mL	530 nm / mL	443 nm / mL	993 nm / mL	222.6 µ mole	6.57
46.4 mL	579 nm / mL	475 nm / mL	1054 nm / mL	210.1 µ mole	4.32
23.2 mL	462 nm / mL	289 nm / mL	751 nm / mL	130.9 µ mole	2.61

Table 1 (Part 2) Nitrogenase PIX Data.

Fe Code	Conc.	FeMo Code	Conc.	Fe / FeMo	T(min)	≈ Turnover ATP	CO	PIX	Error
(-)	(-)	S 30	3.16 uM	(-)	105	(-)	NO	< 4%	±16%
G33	3.5 uM	(-)	(-)	(-)	105	6 - 9%	NO	< 0.9%	± 4%
G36	3.6 uM	(-)	(-)	(-)	120	11.7%	NO	6%	±4%
"G36"	3.6 uM	(-)	(-)	(-)	130	9 %	NO	<0.9%	±4%
"G36"	3.6 uM	S 30	2.73 uM	1.3	55	74%	NO	< 3%	±4%
"G36"	3.6 uM	S30	4.37 uM	0.8	55	85%	NO	< 3%	± 4%

Table 1 Nitrogenase PIX data (part 2) cont.

Assay Volume	Ethylene Activity	Hydrogen Activity	Total Activity	ATP Hydrolyzed	ATP/2e- ratio
4 mL	4.6 nmole / mL	77 nmole / mL	81.6 nmole / mL	2.26 µmole	7.00
4 mL	3.5 nmole / mL	75 nmole / mL	78.5 nmole / mL	2.63 µmole	8.23
6 mL	57.1 nmole / mL	30.9 nmole / mL	88 nmole / mL	5.19 µmole	9.83
4 mL	0.4 nmole / mL	-0-	0.4 nmole / mL	2.66µ mole	1660
34.8	630 nmole / mL	346 nmole / mL	976 nmole / mL	193 µmole	5.68
34.8	558 nmole / mL	423 nmole / mL	981 nmole / mL	223 µmole	6.53

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APPENDIX 1

The aforementioned spectra for ^{18}O-β, γ-ATP and ^{18}O bridge-labeled pyrophosphate were generated as designated by the Zeta list printed below.

```
PYRO      .066
TOM       18JUN86

O-18 ATP FROM EXPT 74 FOR QUANTITATION.

ONE PULSE SEQUENCE

P2        =   90.00 USEC
D5        =  500.00 USEC
NA        =      200
SIZE      =    32768
ADC       =       14
AI        =       10
RG        =       10
EM        =          .03
PA        =    354.1
PB        =    119.4
LOCK      =     4.78
T(C)      =     18.0
OF        =    -5.48
SW        =  +/- 500.000  HZ
DW        =  1000 USEC
DE/DW     =    .60
AT        =   16.38  SEC
SF      =  202.4275256
F2      =  500.0719200

OBS HI PWR =  63
OBS LO PWR = 2047
DEC PWR    =   0
DEC SCHEME =  4
SCALE         8.09 HZ/CM
      =       .0400 PPM/CM

FROM       11.04
TO      10.22 PPM
```

```
ATP074    .001
TOM       18JUN86

O-18 ATP FROM EXPT 74 FOR QUANTITATION

ONE PULSE SEQUENCE

P2        =   90.00 USEC
D5        =  500.00 USEC
NA        =      200
SIZE      =     8192
ADC       =       14
AI        =       10
RG        =       10
EM        =          .03
PA        =    354.1
PB        =    119.4
LOCK      =     4.78
T(C)      =     18.0
OF        =    -5.48
SW        =  +/- 500.000  HZ
DW        =  1000 USEC
DE/DW     =    .60
AT        =     4.10  SEC
SF      =  202.4275256
F2      =  500.0719200

OBS HI PWR =  63
OBS LO PWR = 2047
DEC PWR    =   0
DEC SCHEME =  4
SCALE         8.09 HZ/CM
      =       .0400 PPM/CM

FROM       5.56
TO      3.93 PPM
```

In addition, all spectra were resolution enhanced by apodizing the free induction decay (FID) by double exponential multiplication (DM routine). The essential effect of this routine is a combination of the "GM" routine with LB > 0 and "EM" routine with the LB < 0 in the GE-Nicolet 1280 software package. The GM routine with LB > 0 will convert the resonances to ones having more Gaussian lineshapes (less tailing) than Lorentzian. The EM routine with LB < 0 will alter the shape of the resonances such that the transformed spctra will produce narrower lines at the expense of smaller Signal : Noise. Importantly, the use of this routine can only be used under the assumption that the

linewidths, $w_{1/2}$, (and t_2 values) of the resonances being compared (in this case, the unlabeled vs ^{18}O labeled bridge and non-bridge β, γ ATP and pyrophosphate are identical. Should this not be the case, then multiplying the FID by this function will alter the different components of the FID by different amounts, allowing for erroneous quantitation of the resonances. We have controlled for this by measuring the linewidths (which are inversely proportional to t_2) and have found them to be identical (within ± 1 data point) as seen in Figure 4 of the Chemical Synthesis chapter. Meticulous care has been paid to this aspect of resolution enhancement as recent comments by von der Saal and Villafranca has questioned the ability of one to quantitate spectra that have undergone such enhancement.**

It should also be noted that if any resolution enhancement was performed on any spectrum, **it was performed in exactly the same manner as all other spectra so as to maintain consistency.**

Note: Pulse angle determined by the following formula:*

$t = \cos^{-1} e^{-(t_1/T)}$

t_1 = spin-lattice relaxation time (determined to be 2.57 sec. and 0.73 sec. at 97.57 MHz. and 202.5 MHz. respectively for the β phosphorus of ATP)

T = spectrometer acquisition time + pulse delay time.

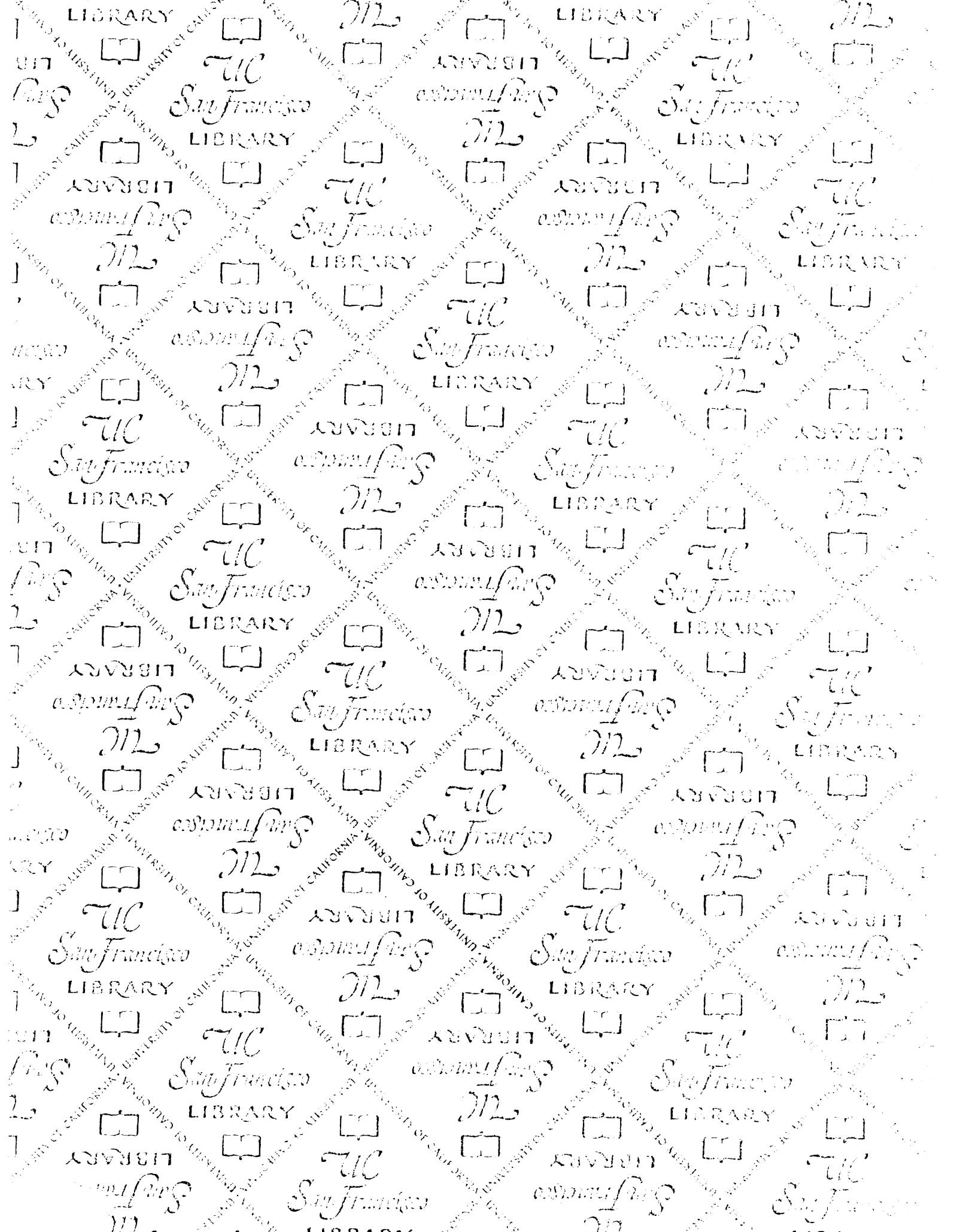
* Farrar, T.C., & Becker, E.D. Pulse Fourier Transform NMR Academic Press (1971)

**von der Saal, W. and Villafranca, J.J. (1986) Bioorganic Chemistry 14, 28

GLOSSARY OF ABBREVIATIONS

ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
Av_1	Azotobacter vinelandii dinitrogenase
Av_2	Azotobacter vinelandii dinitrogenase reductase
CK	Creatine phosphokinase
Cp_1	Clostridium pasteurianum dinitrogenase - Fe-Mo subunit
Cp_2	Clostridium pasteurianum dinitrogenase reductase - Fe subunit
Cr	Creatine
DEAE	Diethylaminoethyl Sephadex (Pharmacia)
DM	Appodization by double exponential multiplication
EM	Appodization by exponential multiplication
EPR	Electron paramagnetic resonance
	F6P Fructose-6 phosphate
F 1,6 dP	Fructose 1,6 bisphosphate
FID	Free induction decay
formyl-H_4 folate	Formyl tetrahydrofolate
GM	Appodization by Gaussian multiplication
HEPES	Hydroxyethyl piperazine ethanesulfonic acid
INDOR	Internuclear double resonance Spectroscopy

k_{rot}	Rotational rate constant (sec^{-1})
Kp_1	Klebsiella pneumonia dinitrogenase - Fe-Mo subunit
Kp_2	Klebsiella pneumonia dinitrogenase reductase - Fe subunit
NMR	Nuclear magnetic resonance
NOE	Nuclear Overhauser Enhancement Spectroscopy
PCr	Phosphocreatine
PGA	Pteroyltriglutamate
P_i	Inorganic phosphate
PP_i	Inorganic Pyrophosphate
PPi - PFK	Pyrophosphate-dependent Phosphofructokinase
PIX	Positional Isotope Exchange
SDS	Sodium dodecylsulfate
TAPS	Tris hydroxymethylaminopropane sulfonic acid
TM	Trapezoidal multiplication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