BIOORGANIC CHEMISTRY 17, 377–384 (1989)

Mass Spectrometric Analysis of Phosphate from β,γ-[^{18}O]ATP Hydrolyzed by *Azotobacter vinelandii* Nitrogenase: Direct Evidence for P_γ–OP_β Bond Cleavage[1]

CHARLES E. MCKENNA,*[,2] WILLIAM G. GUTHEIL,* GEORGE L. KENYON,†[,2] AND TERRY O. MATSUNAGA†[,3]

Department of Chemistry, University of Southern California, University Park, Los Angeles, California 90089-0744, and †Department of Pharmaceutical Chemistry, School of Pharmacy, University of California, San Francisco, California 94123

Received August 26, 1988

Nitrogenase catalyzes reduction-dependent hydrolysis of ATP to ADP and phosphate. It has been generally assumed that the catalytic process involves attack of a nucleophile at the P_γ of ATP by analogy with conventional ATPases, but no direct evidence verifying this assumption has been available. To determine which anhydride P–O bond is cleaved by the enzyme, β,γ-[^{18}O]ATP (75.4% specific enrichment; nonspecific enrichments of 6.0% nonbridging P_γ–^{18}O, 4.0% nonbridging P_β–^{18}O, and 2.0% P_β–^{18}O–P_α) was incubated with purified nitrogenase proteins from *Azotobacter vinelandii* under turnover conditions. The phosphate produced was isolated, derivatized to trimethyl phosphate, and analyzed by high-resolution mass spectrometry. The $C_3H_9O_4P/C_3H_9O_3{}^{18}OP$ mass (140/142) ratio found in the product phosphate derivative was 14.0 ± 2.0 (SE, n = 3). The calculated values for P_γ–OP_β and $P_\gamma O$–P_β bond breaking based on the known distribution of ^{18}O in the substrate were 15 and 0.23, respectively. The results establish nitrogenase hydrolysis of the ATP P_γ–OP_β linkage. © 1989 Academic Press, Inc.

Nitrogenase, the enzyme responsible for biological N_2 fixation, is constituted of two essential components, an $\alpha_2\beta_2$ protein containing several Fe-Mo-S and Fe-S clusters (FeMo or MoFe protein) and a γ_2 protein containing an Fe-S cluster (Fe protein) (*2*). *In vitro* reduction of N_2 and adventitious substrates such as C_2H_2, other small multiply unsaturated molecules, and small strained-ring alkene or azo compounds (*2–4*) requires an exogenous electron donor (usually dithionite) and is ATP-dependent. There is substantial evidence that ATP binds to the nitrogenase Fe protein, but hydrolysis of bound ATP to ADP and P_i does not occur unless the FeMo protein is present; concomitant reduction of the FeMo protein by the Fe protein generates the active substrate-reducing species (*2, 5, 6*). Virtually no

[1] Supported by U. S. Public Health Service Grants GM-31716 from the National Institute of General Medicine (C.E.M.), AR-17323 from the National Institute of Arthritis, Metabolism and Digestive Diseases (G.L.K.); Grant 84-CRCR-1-1106 from USDA-CRGO (C.E.M.); and Grant HF-67 from the H. Frasch Foundation (C.E.M.). A preliminary report of this work has appeared in abstract form (*1*).

[2] To whom correspondence should be addressed.

[3] Present address: Department of Chemistry, University of Arizona, Tuscon, AZ 85721.

0045-2068/89 $3.00
Copyright © 1989 by Academic Press, Inc.
All rights of reproduction in any form reserved.

unambiguous information is currently available on the *molecular* mechanism of this unique reduction-coupled ATP hydrolysis.

In the elucidation of ATP reactions catalyzed by other enzymes, the use of substrate specifically labeled with ^{18}O has frequently proved useful (*7*). Recently, the preparation of an ^{18}O-labeled ATP analog substantially enriched at the β,γ-bridge position was reported (*8*). This analog, originally designed as a probe for enzyme-catalyzed positional isotope exchange (PIX)[4] (*9*), is here used in a novel way to establish whether nitrogenase cleaves the P_γ–O anhydride bond in ATP, as do all conventional ATPases examined to date (*10, 11*). Attack by H_2O (or an enzyme nucleophile) at P_γ in β,γ-[^{18}O]ATP should lead to unlabeled product phosphate, whereas an abnormal attack at P_β would result in phosphate incorporating the labeled oxygen atom. To characterize the isotopic distribution actually resulting from hydrolysis by nitrogenase, phosphate liberated during turnover was isolated by ion-exchange chromatography, derivatized to trimethyl phosphate, and analyzed by high-resolution mass spectrometry (*9*). The experimental mass ratio of unlabeled : labeled derivative was then compared with the predicted ratios for P_γ–OP_β and $P_\gamma O$–P_β bond cleavage, taking into account the known distribution of ^{18}O in the ATP analog (for convenience, we refer to this overall procedure as positional isotope cleavage analysis (PICA)). The results provide the first direct evidence pertaining to the chemical mechanism of ATP hydrolysis by nitrogenase.

MATERIALS AND METHODS

Preparation of reagents. ATP, ADP, phosphocreatine (PC), creatine kinase (CK), and Hepes buffer were purchased from Sigma. β,γ-^{18}O-labeled ATP was synthesized from unlabeled AMP and specifically β,γ-^{18}O-labeled pyrophosphate (*8*) by a published method (*12*). The positional distribution of ^{18}O in the labeled pyrophosphate was determined by ^{31}P NMR as described previously (*8*), using a hybrid 97.571-MHz spectrometer equipped with a 5.6 T superconducting magnet (Cryomagnets, Inc.) and fitted with a thermostatted (20°C) 10-mm probe. The spectrometer was interfaced to a Nicolet Technologies 1180 computer and pulse programmer. Peak areas (*8*) were measured using the cut-and-weigh technique. Stock solutions of the labeled reagent were prepared by dissolving 210 mg of the mixed sodium/triethylammonium salt in 10 ml of 0.025 M Hepes (pH 7.4), readjusting the pH to 7.4 (NaOH), and diluting to 13.6 ml with additional Hepes buffer. The concentration of the solution in nucleotide (24.1 mM) was determined by measuring the absorbance at 259 nm (ε 15,400) of a sample diluted 400-fold with the same buffer. HPLC analysis of the stock solution showed that it contained 97% ATP and 3% ADP. $MgCl_2 \cdot 6H_2O$ was weighed into a volumetric flask and diluted with 0.025 M Hepes (pH 7.4) to make a 40 mM stock solution (pH 7.4).

[4] Abbreviations used: Av1, nitrogenase FeMo protein from *A. vinelandii*; Av2, nitrogenase Fe protein from *A. vinelandii*; PIX, positional isotope exchange; PC, phosphocreatine; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PICA, positional isotope cleavage analysis; CK, creatine kinase; TEA, triethylamine; TEA^+, triethylammonium; FID, flame ionization detector.

Sodium dithionite was obtained from Alpha (80–85%). Stock solutions of dithionite (0.08 M, pH 7.2–7.8) were prepared by adding 0.564 g of the salt under Ar to 40 ml of degassed 0.02 M NaOH; the solutions were maintained on ice and discarded after 4 h. Argon (99.9%) was freed from traces of O_2 by sparging through chromium–potassium sulfate solution in contact with amalgamated zinc. C_2H_2 (99.5%) was passed through a dry ice–isopropanol trap to remove acetone.

Purification and characterization of Azotobacter vinelandii nitrogenase. Nitrogenase components (Av1 and Av2 proteins) were purified from *A. vinelandii* by previously published methods (*13*). Specific activities (SA values) were measured using C_2H_2 (0.04 atm) as the reduction substrate (conditions and procedures given below). Protein concentrations were determined using the Biuret reagent. Av1 had an initial concentration of 20.4 mg/ml and a SA of 2000 nmol C_2H_4 mg^{-1} min^{-1}. Av2 had an initial concentration of 18.4 mg/ml and a SA of 1650 nmol C_2H_4 mg^{-1} min^{-1}. Specific activities were determined in the absence of flavodoxin and at optimal component ratios.

Standard nitrogenase assays. Standard assays were performed at 30°C in 21.5-ml bottles containing 6 units of CK, 25 μmol of PC, 5 μmol of ATP, 5 μmol of $MgCl_2$, and 25 μmol of dithionite in 1 ml of 0.025 M Hepes buffer at pH 7.4 under Ar. C_2H_2 (1 ml) was injected into the gas phase with a gas-tight syringe, and Av1 (20 μl, 0.41 mg) was added to the assay solution. Injection of Av2 (5 μl, 0.092 mg) then initiated reaction, which was terminated by 0.5 ml of MeOH (*14*).

Gas chromatographic analysis of C_2H_2 and C_2H_4. C_2H_4 and C_2H_2 in 0.15-ml assay gas samples were separated on a Porapak N GC column (Varian 2440 GC), detected by flame ionization detection (FID), and quantitated by peak area integration using a Hewlett–Packard 3390A recording integrator. C_2H_2 was used as the internal standard (*15*). The FID response ratio for $C_2H_4 : C_2H_2$ was determined from a standard consisting of 50 μl C_2H_4 and 1.0 ml C_2H_2 in 22 ml Ar and was corrected for differential H_2O solubility of the two gasses.

Assays with β,γ-[^{18}O]ATP. A group of eight standard (1 ml) nitrogenase assay mixtures were prepared and run as described above using β,γ-[^{18}O]ATP (5 mM) in place of unlabeled ATP and omitting PC and CK. The following controls were run concurrently: (a) assay mixture minus Av1; (b) assay mixture minus Av2; (c) assay mixture minus both Av1 and Av2. After a 35-min reaction, the assay mixtures were cold-quenched, pooled, quick-frozen in liquid N_2, and stored at −20°C. Normal assay mixtures (containing unlabeled ATP, PC, and CK at their standard concentrations) were also run for comparison.

Isolation and methylation of phosphate. Samples were thawed at room temperature and loaded onto a DEAE-Sephadex A-25 anion exchange column (1 × 20 cm, HCO_3^- form, pH 7.8). The column was eluted with a linear gradient of TEA^+–HCO_3^- (0.07–0.7 M, pH 7.8, 400 ml in both mixing and reservoir chambers). Eluted nucleotide peaks (ADP, ATP) were measured by uv detection at 259 nm. All fractions (10 ml) were assayed for phosphate according to the molybdate method of Ames (*16*): those giving a positive test were isolated, concentrated under reduced pressure, washed with MeOH, and pumped under vacuum to remove solvent and residual TEA^+–HCO_3^-. Recovered phosphate (as the TEA^+ salt) was passed through Dowex AG-50 X-8 (H^+ form), reconcentrated under reduced pres-

sure, dissolved in 50 μl of 10% aqueous methanol, and treated with excess ethereal diazomethane (stirring) over 3 h (*9*). After removal of solvent under a stream of N_2 at room temperature, the oily residue (trimethyl phosphate) was stored for analysis.

Mass spectrometric analysis. Samples were analyzed by high-resolution electron impact mass spectrometry (70 eV) using a Kratos MS-50 mass spectrometer equipped with a DS-55 data system (located at the University of California, Berkeley). Three scans were collected and the parent ion peaks corresponding to unlabeled (140 mass units) and mono-^{18}O-labeled (142 mass units) trimethyl phosphate were quantitated. Low resolution mass spectrometery did not give reliable results on these samples due to interferences from other peaks.

RESULTS AND DISCUSSION

Nitrogenase reactions. It is well-known that ADP formed from nitrogenase-catalyzed ATP hydrolysis causes product inhibition of the enzyme. Normally, this difficulty is easily surmounted by adding an ATP regeneration system (PC/CK) to the assay mixture. However, the presence of the PC/CK system would vitiate a PICA experiment by diluting recovered phosphate product with unlabeled phosphate derived from the phosphocreatine. For this reason, the PICA experiments were run without the ATP generator. It was assumed that the fundamental hydrolysis mechanism would not be affected by the presence of inhibitory ADP, whose accumulation led to the incursion of nonlinear product formation kinetics well before sufficient phosphate for the mass spectrometric analysis had been released.

Reaction progress was conveniently monitored by C_2H_2 reduction. Under the conditions of the complete (Av1 + Av2) reactions with β,γ-[^{18}O]ATP, 420 nmol of C_2H_4 was formed after 15 min. Even allowing for the significant accompanying H_2 evolution observed under the assay conditions used, this was a fraction of the C_2H_2 reduction rate under the consistently linear turnover conditions expected with the PC/CK generator present, due to ADP inhibition as discussed above. During the remaining time of the experiment, the rate slowed further, but at termination (35 min), it was estimated that substrate reduction was more than 90% complete. C_2H_2 reduction in the controls lacking either or both nitrogenase components was negligible.

The corresponding amount of ATP hydrolysis was calculated directly from uv detection of ATP and ADP separated during isolation of phosphate from the assay reaction mixtures by DEAE-Sephadex ion exchange chromatography (Table 1). These data show that significant hydrolysis occurred only in the complete system with both nitrogenase components present (Experiment 1 in Table 1), which was also confirmed qualitatively by the molybdenum blue test for phosphate. A minimal ratio of 4 ATP/2e$^-$ has been documented for ATP hydrolysis-dependent nitrogenase reductions, with higher ratios observed under specific conditions, such as decreased electron flux through the enzyme when the concentration of Fe protein does not greatly exceed the concentration of FeMo protein (*2, 5, 6*). In separate control assays with unlabeled ATP, we observed a ratio of 6 $\pm$ 1 ATP/2e$^-$ using

TABLE 1

Extent of β,γ-[^{18}O]ATP Hydrolysis to ADP and P_i in Nitrogenase Assay Reactions[a]

Expt.	Nitrogenase protein added[b]	ATP hydrolysis[c] (%)	Detection of P_i[d]
1	Av1 + Av2	64	+
2	Av2	<5	–
3	Av1	<5	–
4	None	<5	–

[a] Performed as described under Materials and Methods.
[b] Amounts specified under Materials and Methods.
[c] ADP estimated by uv_{259} detection as a percentage of (ATP + ADP) recovered from the pooled assay reaction mixtures. Total nucleotide recoveries from the DEAE-Sephadex columns were 58–70% (Expts. 1–3) and 91% (Expt. 4). The variations were attributed to mechanical losses in the prechromatography work-up.
[d] Qualitative detection in eluted DEAE-Sephadex fractions using molybdate reagent (*16*).

conditions similar to those of Experiment 1 in Table 1. Taking the total electron flux as the sum of the C_2H_4 and H_2 produced under comparable conditions (*5*), we calculated that the β,γ-[^{18}O]ATP initially present in the assay mixtures should be 68 ± 15% hydrolyzed on termination of the reaction period. This is in reasonable agreement with the actual extent of hydrolysis given for Experiment 1 in Table 1.

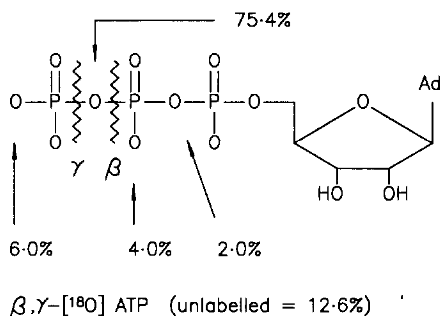
Mass spectrometric results. Expected and experimental mass spectrometric analytical results for the derivatized phosphate are presented in Table 2. The expected results were calculated on the basis of the known initial distribution of

TABLE 2

Predicted and Experimental Mass Spectrometric Results for Derivatized P_i from β,γ-[^{18}O]ATP Hydrolysis by Nitrogenase[a,b]

	Predicted results from cleavage of		Experimental results[c]
	P_γ–OP_β	$P_\gamma O$–P_β	
% Mass 140	93.8	18.6	—
% Mass, 142	6.2	81.4	—
Ratio, 140/142	15	0.23	14.0[d]

[a] Substrate (ATP) ^{18}O positional distribution as shown in Scheme 1.
[b] $C_3H_9O_4P$: mass, 140.023; $C_3H_9O_3{}^{18}OP$: mass, 142.028.
[c] Data for sample from Expt. 1, Table 1.
[d] $n = 3$; SE = 2.0. Preliminary analysis of the same data (1) gave 13.8.



β,γ-[^{18}O] ATP (unlabelled = 12·6%)

SCHEME 1

[^{18}O] in the unreacted ATP substrate, which was bridging P_γ–[18]O, 75.4%; nonbridging P_γ–[18]O, 6.0%; nonbridging P_β–[18]O, 4.0%; and bridging P_β–[18]O–P_α, 2.0% (Scheme 1). Given this isotopic distribution, the percentage of [18]O in the product P_i was then predicted for the two alternative bond-breaking processes. The isotope content values immediately give the corresponding mass 140/142 ratios expected to be observed for each hydrolytic pathway (Table 2), assuming 100% selectivity in either case. A correction of +0.20% in the [18]O content of phosphate derived from hydrolysis at P_γ was made to allow for the naturally abundant [18]O contributed by H_2O. The mass 140/142 ratios predicted for exclusive P_γ–OP_β and $P_\gamma O$–P_β cleavage were 15 and 0.23, respectively. The mass 140/142 ratio calculated from the experimental mass spectral data was 14.0 with a standard error of 2.0 (three determinations).

Some conceivable sources of error in the interpretation of these results are (a) product formation contributed by a hydrolysis process not involving nitrogenase; (b) catalyzed PIX in the β,γ-[^{18}O]ATP substrate. In regard to possibility (a) it has been shown (*17*) that a small (7%) amount of P_β attack by H_2O leading to $P_\gamma O$–P_β cleavage of ATP occurs in dilute HCl, although at pH 8.3 cleavage of the P_γ–OP_β bond occurs exclusively. However, nonenzymatic ATP hydrolysis under usual nitrogenase assay conditions is negligible and was undetectable in our experiments (Table 1).

The second possibility (b), nitrogenase-mediated PIX in the starting ATP, can be analyzed as follows: for simplicity, 100% specific [18]O labeling in the bridging P_γ–O–P_β oxygen is assumed. In the case of attack at P_γ accompanied by PIX that is rapid relative to release of P_i, the label will randomize among its original bridge position and the two unlabeled nonbridging P_β oxygen positions. Since the P_γ attack is signaled by the absence of label in P_i formed from hydrolysis, this PIX process would have no effect on the observed mass 140/142 ratio of the derivatized product phosphate: similar reasoning applies in the case of P_β attack accompanied by competing PIX that randomizes the P_γ–O–P_β bridging label oxygen with the nonbridging P_γ oxygens. However, if both P_γ and P_β attack were to happen concurrently with PIX processes, interpretation of the mass spectrometric results would no longer be straightforward. For example, a PIX label randomization occurring from attack at P_γ, followed by complete hydrolysis via an attack at P_β,

would decrease the expected ^{18}O content of the product phosphate by 2/3. The absence of PIX in the β,γ-[^{18}O]ATP substrate at a level that would significantly affect the PICA experiment assay has been verified separately for the Av1 and Av2 used in these experiments, at the component ratio indicated under Materials and Methods (*1, 14*).

CONCLUSION

Our PICA results clearly demonstrate hydrolysis of the ATP P_γ–O anhydride linkage by nitrogenase,[5] establishing it to be a normal ATPase in this respect. They support any mechanism involving attack at P_γ, but do not distinguish between, e.g., one involving direct cleavage by H_2O (*18*) and one postulating a phosphoenzyme intermediate (*19*).

ACKNOWLEDGMENT

We thank Prof. Arnold Falick for assistance in carrying out the mass spectrometric analyses.

REFERENCES

1. McKenna, C. E., Gutheil, W. G., Kenyon, G. L., and Matsunaga, T. O. (1985) *in* Nitrogen Fixation Research Progress (Evans, H. J., Bottomley, P. J., and Newton, W. E., Eds.), p. 635, Martinus Nijhoff, Dordrecht.
2. Orme-Johnson, W. H. (1985) *Annu. Rev. Biophys. Chem.* **14**, 419–459.
3. McKenna, C. E. (1980) *in* Molybdenum and Molybdenum-Containing Enzymes (Coughlan, M. P., Ed.), Chap. 14, pp. 439–462, Pergamon, Oxford.
4. McKenna, C. E., Stephens, P. J., Eran, H., Luo, G. M., Zhang, F. X., Ding, M., and Nguyen, H. T. (1984) *in* Advances in Nitrogen Fixation Research (Veeger, C., and Newton, W. E., Eds.), pp. 115–122, Martinus Nijhoff–W. Junk, The Hague.
5. Burris, R. H., and Hageman, R. V. (1980) *in* Molybdenum Chemistry of Biological Significance (Newton, W. E., and Otsuka, S., Eds.), pp. 23–38, Plenum, New York.
6. Thorneley, R. N. F., and Lowe, D. J. (1985) *in* Molybdenum Enzymes (Spiro, T. G., Ed.), Chap. 5, pp. 221–284, Wiley–Interscience, New York.
7. Boyer, P., Hackney, D., and Stempel, K. (1980) *in* Methods in Enzymology (Purich, D. L. Ed.), Vol. 64B, pp. 60–83, Academic Press, New York.
8. Reynolds, M. A., Oppenheimer, N. J., and Kenyon, G. L. (1983) *J. Amer. Chem. Soc.* **105,** 6663–6667.
9. Midelfort, C. F., and Rose, I. A. (1976) *J. Biol. Chem.* **251,** 5881–5887.
10. Cohn, M. (1959) *J. Cell. Comp. Physiol.* **54,** 17–31.
11. Knowles, J. R. (1980) *Annu. Rev. Biochem.* **49,** 877–919.
12. Hoard, D. E., and Ott, D. G. (1965) *J. Amer. Chem. Soc.* **87,** 1785–1788.
13. McKenna, C. E., Nguyen, H. T., Huang, C. W., McKenna, M. C., Jones, J. B., and Stephens, P. J. (1982) *in* From Cyclotrons to Cytochromes (M. D. Kamen Symposium) (Kaplan, N. O., and Robinson, A. B., Eds.), pp. 397–416, Academic Press, New York.

[5] It should be pointed out that the error of the mass ratio measurement does not allow us to distinguish exclusive from merely predominant P_γ–OP_β cleavage.

14. McKenna, C. E., Gutheil, W. G., Matsunaga, T. O., and Kenyon, G. L. (1989) to be submitted for publication.
15. McKenna, C. E., McKenna, M.-C., and Higa, M. T. (1976) *J. Amer. Chem. Soc.* **98,** 4657–4659.
16. Ames, B. N. (1966) *in* Methods in Enzymology (Neufeld, E. F., and Ginsberg, V., Eds.), Vol. 8, pp. 115–118, Academic Press, New York.
17. Meyerson, S., Kuhn, E. S., Ramirez, F., and Marecek, J. F. (1982) *J. Amer. Chem. Soc.* **104,** 7231–7239.
18. Mortenson, L. E., Webb, M., Bare, R., Cramer, S. P., and Morgan, T. V. (1985) *in* Nitrogen Fixation Research Progress (Evans, H. J., Bottomley, P. J., and Newton, W. E., Eds.), pp. 577–584, Martinus Nijhoff, Dordrecht.
19. Cordewener, J., ten Asbroek, A., Wassink, H., Eady, R., Haaker, H., and Veeger, C. (1987) *Eur. J. Biochem.* **162,** 265–270.