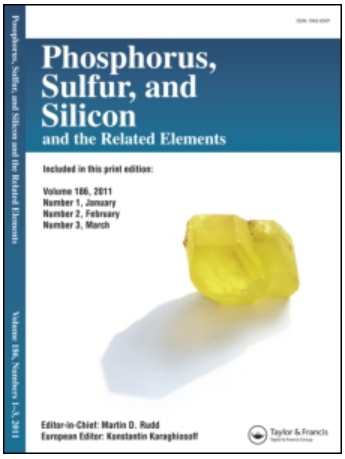

Phosphorus, Sulfur, and Silicon and the Related Elements

Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713618290

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To cite this Article Krause, Stephan O. , Gutheil, William G. , Simeonov, Anton M. , Matsunaga, Terry O. , Stauber, Margaret J. , Kenyon, George L. and Mckenna, Charles E.(1999) 'Perspectives on the Mechanism of ATP Hydrolysis by Nitrogenase', Phosphorus, Sulfur, and Silicon and the Related Elements, 144: 1, 513 — 516
To link to this Article: DOI: 10.1080/10426509908546294
URL: http://dx.doi.org/10.1080/10426509908546294

Phosphorus, Sulfur and Silicon, 1999, Vol. 144-146, pp. 513-516
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Perspectives on the Mechanism of ATP Hydrolysis by Nitrogenase

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The chemical mechanism of ATP hydrolysis by nitrogenase is discussed in terms of results from isotopic exchange studies using [$^{18}O_4$]-Pi, b,g-[^{18}O]-ATP, and g-[$^{18}O_4$]-ATP as probes of reversibility (ATP = ADP + Pi) and of the freedom of rotation around the O-P_b bond in enzyme-bound ADP.

Keywords: Nitrogenase; ATP hydrolysis; ^{31}P NMR; HRMS; g-[$^{18}O_4$]-ATP; [$^{18}O_4$]-phosphate; PIX

INTRODUCTION

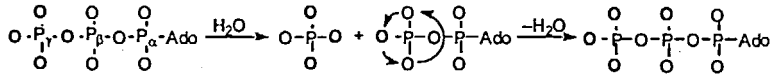
The catalytic reduction of N_2 by nitrogenase, the enzyme responsible for biological nitrogen fixation, requires two proteins: the iron protein (Fe protein) and the molybdenum-iron protein (MoFe-protein), plus an electron source and ATP, and under optimal conditions follows the idealized stoichiometry shown in Eq. 1.[1]

$$16\ MgATP + 16\ H_2O + 8\ H^+ + 8\ e^- + N_2 \rightarrow 16\ MgATP + 16\ Pi + H_2 + 2\ NH_3 \qquad (1)$$

It is generally accepted[1] that the exogenous reductant reduces the $[Fe_4S_4]^{2+}$ metal cluster of oxidized Fe protein (Fe_{Ox}) to its [3-] state, corresponding to reduced Fe protein (Fe_R), which binds two MgATP. Two $(ATP)_2$-Fe_R complexes interact with one molecule of MoFe protein, resulting in transfer of $2e^-$ to its $MoFe_7S_8$ metal cluster, and the hydrolysis of four ATP to ADP and Pi. Reduction of substrates is believed to take place at the molybdenum-iron center. The cycle is completed by dissociation of the $(ADP)_2$-Fe_{Ox} from MoFe protein which is thought to be rate-limiting overall, reduction to

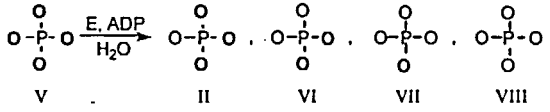
$(ADP)_2$-FeR, and release of ADP which proceeds more readily than from $(ADP)_2$-Fe_{Ox}. Although electron transfer to reduction substrates such as N_2, C_2H_2 and H^+ is linked to ATP hydrolysis, the latter reaction can also occur independently of reduction, e.g. when the Fe-protein:MoFe protein or Fe_{Ox}:Fe_R ratios are low.

The chemical mechanism of ATP hydrolysis by nitrogenase is still imperfectly understood. In a study on *A. vinelandii* nitrogenase, b,g-[^{18}O]-ATP was used to establish that the H_2O nucleophile attacks at P_g, leading exclusively to unlabeled oxygens in the product Pi (analyzed by MS after derivatization to trimethyl phosphate).[2] Experiments on the *C. pasteurianum* enzyme have shown that although g-S ATP hydrolysis (occurring at 1 % of the ATP rate) does not detectably couple to substrate reductions, stereochemical analysis of the S-Pi from hydrolysis of one enantiomer of g-S, g-[^{17}O, ^{18}O]-ATP in $H_2{}^{16}O$ found inversion at phosphorus. This result supports direct attack on the P_g of ATP by H_2O as opposed to hydrolysis of a first-formed phosphoenzyme intermediate, which would have given retention, possibly with racemization. Again utilizing *A. vinelandii* nitrogenase, b,g-[^{18}O]-ATP recovered from assays under fixing conditions at 30 °C was found not to have undergone significant positional isotope exchange[3] (PIX).[4] The PIX process is illustrated below for g-[$^{18}O_4$]-ATP **I** which was used in this work. Hydrolysis (with unlabeled H_2O) is postulated to generate enzyme-bound Pi **II** and ADP **III**. Rotation of the P_b followed by reversal of hydrolysis results in a 2/3 chance of the bridging [^{18}O] label in **I** moving to a non-bridging position in the reformed ATP IV.



Such an event can be detected by high field ^{31}P NMR, owing to the difference in magnitude of the upfield chemical shifts produced by substituting [^{18}O] for [^{16}O] in bridging and non-bridging environments. A negative result in a PIX experiment is somewhat ambiguous, because it would be consistent with an undetectably slow reverse reaction, but also with a reversible reaction in which the P_b phosphate group is rotationally restricted at the enzyme binding site, preventing exchange.

Subsequently, it was reported that *K. pneumoniae* nitrogenase proteins catalyzed a MgADP-enhanced loss of isotopic label from [$^{18}O_4$]-Pi ('washout') to water, under conditions suggesting reversibility of reductant-independent MgATP cleavage by nitrogenase.[5] In a washout experiment, the labeled phosphate **V** is postulated to bind the enzyme at the ATP hydrolysis site. If unlabeled ADP also binds and ATP hydrolysis is reversible, $H_2{}^{18}O$ is formed during ATP synthesis. Dilution by the enormous excess of unlabeled H_2O in the solvent ensures that the label is effectively lost on re-hydrolysis of the ATP. In the *Klebsiella* nitrogenase experiment, a mixture of label washed-out Pi species (**II, VI, VII, VIII**) was detected.



V . II VI VII VIII

The exchange rate for the two proteins together was 34% more than the sum of the separate activities, and this rate doubled in the presence of ADP. As P^1, P^5-bis(adenosine-5')-pentaphosphate (Ap_5A) was added to the assays to inhibit any adenylate kinase contaminating the nitrogenase preparations, the origin of the background activities remained somewhat inconclusive. Bearing in mind that unified results from a single type of nitrogenase would be helpful, we have now undertaken additional labeled oxygen-exchange studies on *A. vinelandii* nitrogenase proteins using g-[$^{18}O_4$]ATP and [$^{18}O_4$]Pi.

EXPERIMENTAL PART

Synthesis

[$^{18}O_4$]-Phosphate was prepared by the method of Ray.[6] g-[$^{18}O_4$]-ATP was synthesized from ADP and [$^{18}O_4$]-phosphate.

Enzymology and Isotopomer Analyses

Fe and MoFe proteins were prepared, assayed and characterized as previously described.[7]. Some Fe proteins were further purified by chromatography on Sephacryl S-200 and Superdex 75HR 10/30 gel filtration columns. MoFe protein was further purified for washout experiments by HPLC (SAX 10/10 column) chromatography. Isotope exchange assays omitted the usual ATP regeneration system, and Ap_5A was not added. The incubation times were extended to maximize ATP hydrolysis (PIX experiments) or ^{18}O-label washout. Proteins were not pre-oxidized.

ADP and ATP from enzyme assays (quenched with MeOH) were separated on a DEAE-Sephadex A-25 column and analyzed by liquid secondary ion mass spectroscopy (LSIMS). Adenosine nucleotide were also analyzed by HPLC. PIX in g-[$^{18}O_4$]-ATP was determined by ^{31}P NMR. Distributions of ^{18}O-label isotopomers in phosphate isolated from washout experiments with [$^{18}O_4$]-phosphate were determined, after derivatization to trimethyl phosphate (diazomethane), by ^{31}P NMR and by HRMS.

RESULTS AND DISCUSSION

PIX Experiments

Confirming our earlier results with the b,g-labeled ATP, under the assay conditions there was no significant PIX observed (a small amount of exchange was attributed to a contaminant in the enzyme preparation). Analysis of both the ATP and ADP recovered from the second set of experiments by HRMS showed no difference from reference (ATP) or derived (ADP) values with respect to [$^{18}O_{4-0}$] (ATP) or [$^{18}O_{1-0}$] (ADP) isotope distributions. In summary, the re-isolated ATP showed little or no bridge-to-nonbridge isotope exchange, and the re-isolated ADP also showed no loss of label.

Washout Experiments

Washout experiments provide a test of reversibility that allows torsional restriction of the b-phosphate group in enzyme-bound ADP. Our findings may be summarized as follows: a) neither MoFe-protein nor Fe_{ox} catalyzed detectable washout (a small activity in MoFe-protein could be removed by additional purification); b) in the presence of ADP, the combined proteins catalyzed significant washout; c) this washout was not observed in the absence of ADP. The distribution of isotopomers is not consistent with a phosphorylated enzyme intermediate capable of undergoing multiple exchange reactions for each hydrolysis-resynthesis step.

Acknowledgments

We thank NIH, NSF, and NRICPG/CREES/USDA for grants supporting this research.

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