Purple Acid Phosphatase: A Diiron Enzyme that Catalyzes a Direct Phospho Group Transfer to Water

Eugene G. Mueller,[†,‡] Michael W. Crowder,[§] Bruce A. Averill,*[,§] and Jeremy R. Knowles*[,†]

Departments of Chemistry and Biochemistry and Molecular Biology, Harvard University Cambridge, Massachusetts 02138 Department of Chemistry University of Virginia Charlottesville, Virginia 22901

Received November 2, 1992

Purple acid phosphatases (PAPs) catalyze the hydrolysis of aryl phosphoric monoesters, phosphoric anhydrides, and phosphoproteins containing phosphoserine residues,[1] and these enzymes contain a mixed-valence binuclear iron center. Three mechanisms have been proposed: metal-catalyzed release of metaphosphate,[2] direct attack of a metal-coordinated hydroxide at phosphorus,[3] and attack by an enzyme nucleophile to produce a phosphoenzyme intermediate that is subsequently hydrolyzed.[4,5] This third possibility was supported by several lines of evidence, including the observation of a "burst" of *p*-nitrophenol, the appearance of transphosphorylation products upon incubation of PAP with *p*-nitrophenyl phosphate, and retention of ^{32}P by PAP after incubation with [γ-^{32}P]-labeled ATP. Such behavior is similar to that of other nonspecific phosphatases that form covalent phosphoenzyme intermediates.[6] To allow us to distinguish among the three mechanistic possibilities, the stereochemistry of phospho group transfer to water was determined for the reaction catalyzed by this enzyme. We find that PAP transfers the phospho group with overall *inversion* of the configuration at phosphorus. This result rules out a phosphoenzyme pathway and a long-lived metaphosphate intermediate and supports the direct transfer of the phospho group to water.

To probe the stereochemical course of the reaction, S_P-2′,3′-methoxymethylidene-ATP-γS$\gamma^{18}O\gamma^{17}O$ was synthesized.[7,8] 2′,3′-Isopropylideneadenosine was treated with [$^{18}O_2$]benzoic acid[9] under Mitsunobu conditions[10] to afford 5′-*O*-benzoyl-2′,3′-isopropylidene-[5′-^{18}O]-adenosine in quantitative yield. Deprotection[11] afforded [5′-^{18}O]adenosine in 90% yield. This compound

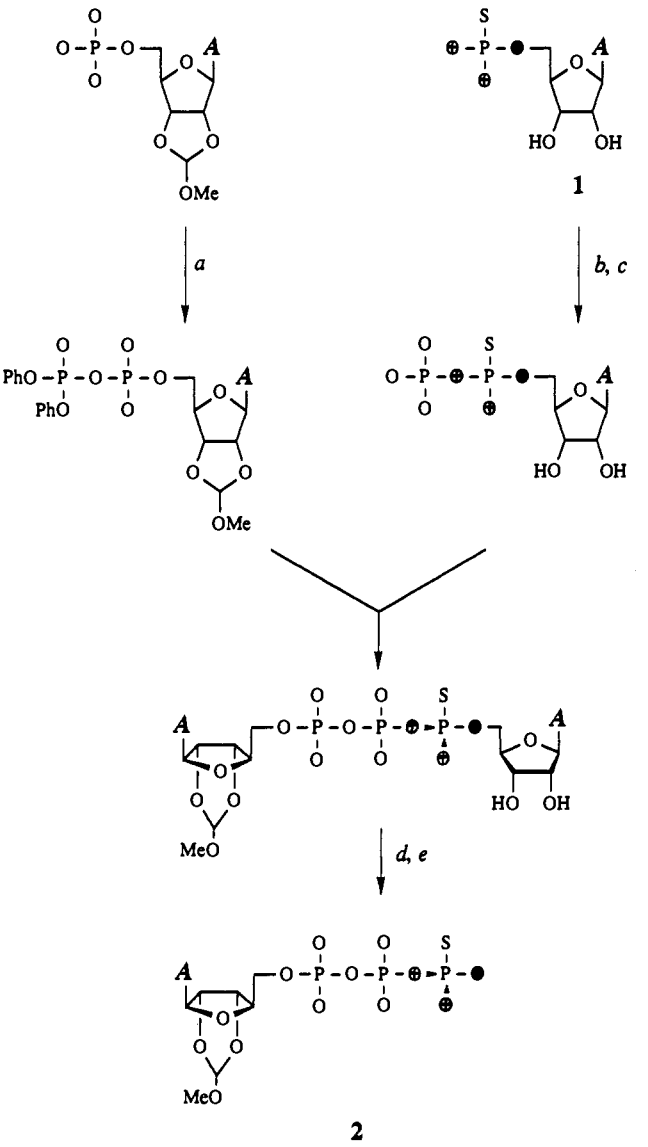

Figure 1. The synthesis of S_P-2′,3′-methoxymethylidene-ATP$\gamma^{17}O_2\gamma^{18}O\gamma$S (**2**) according to the method of Richard and Frey.[12] O = 16O, ⊕ = ^{17}O, and ● = ^{18}O; *A* = adenyl group. Reagents used: *a*, diphenyl chlorophosphate; *b*, adenylate kinase, pyruvate kinase, phosphoenolpyruvate; *c*, hexokinase, glucose; *d*, sodium periodate; *e*, 2-mercaptoethanol, pH 10, 50 °C. For simplicity, all multiple bonds and charges on the phospho groups are omitted.

was treated with trimethylorthoformate and *p*-toluenesulfonic acid to afford 2′,3′-methoxymethylidene-[5′-^{18}O]adenosine (92%), which was converted to AMP$\alpha^{17}O_2,\alpha^{18}O,\alpha$S (adenosine 5′-[$^{17}O_2$,$^{18}O$]phosphorothioate) (**1**) by sequential treatment with PCl_3, [^{17}O]H_2O, bis(trimethylsilyl)acetamide, and sulfur, followed by deprotection in aqueous solution at pH 2. Compound **1** was converted to S_P-2′,3′-methoxymethylidene-ATP$\gamma^{17}O_2\gamma^{18}O\gamma$S (2′,3′-methoxymethylideneadenosine 5′-(3-thio[3-$^{17}O_2$,^{18}O]triphosphate)) (**2**) by the method of Richard and Frey,[12] omitting the low pH incubation that removes the methoxymethylidene group (Figure 1).

Compound **2** was then incubated with PAP from bovine spleen,[13] and the configuration of [^{16}O,^{17}O,^{18}O]thiophosphate was analyzed

† Harvard University.
‡ Present address: Department of Chemistry, California Institute of Technology, Pasadena, CA 91125.
§ University of Virginia.

(1) Vincent, J. B.; Averill, B. A. *FASEB J.* **1990**, *4*, 3009–3014. Vincent, J. B.; Olivier-Lilley, G. L.; Averill, B. A. *Chem. Rev.* **1990**, *90*, 1447–1467. Que, L., Jr.; True, A. E. *Prog. Inorg. Chem.* **1990**, *38*, 97–200. Doi, K.; Antanaitis, B. C.; Aisen, P. *Struct. Bonding* **1988**, *70*, 1–26.
(2) David, S. S.; Que, L., Jr. *J. Am. Chem. Soc.* **1990**, *112*, 6455–6463.
(3) Dietrich, M.; Münstermann, D.; Suerbaum, H.; Witzel, H. *Eur. J. Biochem.* **1991**, *199*, 105–113.
(4) Vincent, J. B.; Crowder, M. W.; Averill, B. A. *Biochemistry* **1992**, *31*, 3033–3037.
(5) Vincent, J. B.; Crowder, M. W.; Averill, B. A. *J. Biol. Chem.* **1991**, *266*, 17737–17740.
(6) Frey, P. A. *Adv. Enzymol.* **1989**, *62*, 119–201.
(7) The PAP-catalyzed hydrolysis was shown to proceed via attack at Pγ by carrying out the reaction in a 1:1 mixture of [^{16}O]H_2O:[^{18}O]H_2O. Half of the resultant thiophosphate contained one atom of ^{18}O.
(8) This synthesis is an improvement over those reported earlier: Webb, M. R.; Trentham, D. R. *J. Biol. Chem.* **1981**, *256*, 4884–4887. Iyengar, R.; Cardemil, E.; Frey, P. A. *Biochemistry* **1986**, *25*, 4693–4698. Bethell, R. C.; Lowe, G. *Biochemistry* **1988**, *27*, 1125–1131.
(9) [^{18}O]H_2O (10 equiv; 95% atom excess ^{18}O) was used to hydrolyze benzoyl chloride. After prolonged incubation of the reaction mixture at 85 °C, the incorporation of ^{18}O into the product benzoic acid was complete.
(10) Shimokawa, S.; Kimura, J.; Mitsunobu, O. *Bull. Chem. Soc. Jpn.* **1976**, *49*, 3357–3358.
(11) The protected compound was treated with ammonia in methanol (5 M) and then with aqueous acetic acid (10% v/v, under reflux).
(12) Richard, J. P.; Frey, P. A. *J. Am. Chem. Soc.* **1982**, *104*, 3476–3481. 2′,3′-Methoxymethylideneadenosine 5′-phosphate was prepared according to the method of Webb, M. R. *Methods Enzymol.* **1982**, *87*, 301–316.
(13) Vincent, J. B.; Crowder, M. W.; Averill, B. A. *Biochemistry* **1991**, *30*, 3025–3034.

0002-7863/93/1515-2974$04.00/0 © 1993 American Chemical Society

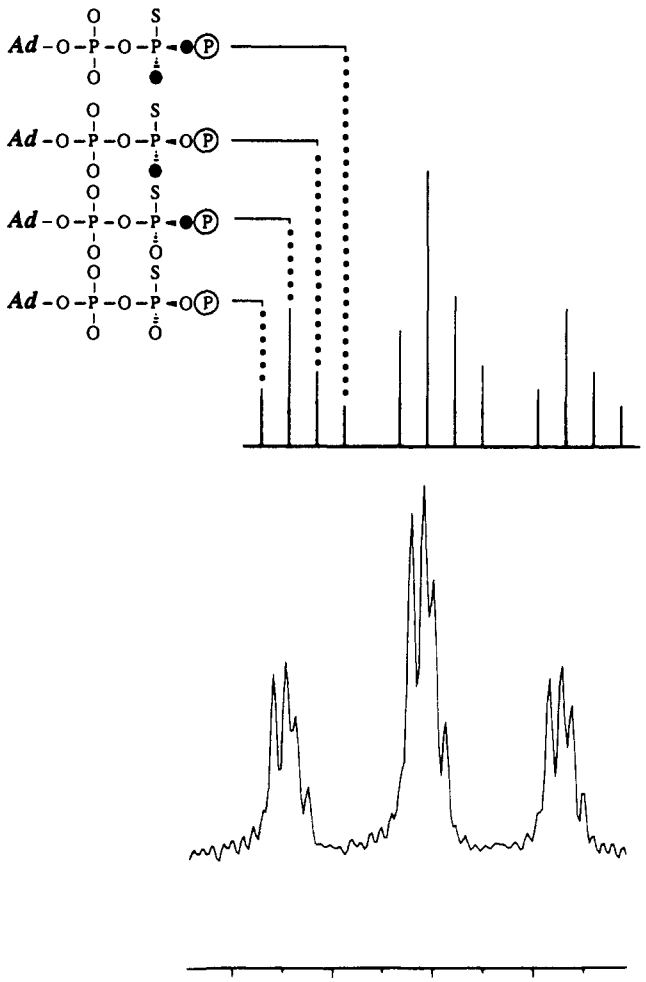

Figure 2. Theoretical (above) and actual (below) ^{31}P NMR spectra of Pβ of labeled ATPβS derived from *R*-[^{16}O,^{17}O,^{18}O]thiophosphate as predicted from the isotopic composition of the [5′-^{18}O]adenosine and [^{17}O]H_2O used in the synthesis.[17] ○ = ^{16}O, ⊕ = ^{17}O, and ● = ^{18}O. *Ad* = adenosyl group; Ⓟ = phospho group. The scale is 5 Hz/mark. The assignment of the resonances in the central and rightmost groups of four isotopomeric resonances is the same as indicated for the leftmost set of resonances. The lower spectrum was run on a Bruker WM-300 instrument at 121.50 MHz with a deuterium field lock and broad-band decoupling: spectral width 8064 Hz, acquisition time 2.03 s, pulse width 15.0 μs, number of transients 10 416, acquisition in 8K, Fourier transform in 32K. The chemical shifts of the resonances are 30.0552, 30.0350, 30.0198, 29.9991, 29.8288, 29.8085, 29.7932, 29.7731, 29.6025, 29.5827, 29.5664, and 29.5472 ppm.

by the method of Webb and Trentham,[14] which entails the enzymatic incorporation of [^{16}O,^{17}O,^{18}O]thiophosphate into S_P-ATPβS (adenosine 5′-(2-thiotriphosphate)). The differential effects on the ^{31}P NMR resonances of phosphorus nuclei substituted with ^{17}O or ^{18}O in place of ^{16}O allow the configuration of the [^{16}O,^{17}O,^{18}O]thiophosphate to be deduced.[15,16] Figure 2 shows the theoretical and the actual ^{31}P NMR spectra of Pβ of labeled ATPβS derived from *R*-[^{16}O,^{17}O,^{18}O]thiophosphate as predicted from the isotopic composition of the [5′-^{18}O]adenosine and the [^{17}O]H_2O used in the synthesis.[17] The spectrum predicted for the *S* isomer of [^{16}O,^{17}O,^{18}O]thiophosphate would differ from that shown by the reversal of the relative intensities of the central pair of each group of four isotopomeric resonances.

From Figure 2, the product thiophosphate has the *R* configuration, indicating that the reaction catalyzed by PAP proceeds with *overall inversion at phosphorus*. It appears to be a general rule that a phospho group suffers inversion with every enzyme-catalyzed transfer.[18] The stereochemical result presented here therefore excludes any mechanism with a single covalent phosphoenzyme intermediate, for such a mechanism would lead to overall retention of configuration at phosphorus.[19] We must, therefore, conclude that, *in contrast to the other phosphatases of broad substrate specificity*[20] *and other acid phosphatases examined, purple acid phosphatase catalyzes the direct transfer of a phospho group to water.*

Acknowledgment. We thank the NIH for financial support (Grant GM32117 to B.A.A., GM21659 to J.R.K.). E.G.M. was a NSF predoctoral fellow. NMR spectra were recorded on instruments funded by the NIH (Grant 1-SIO-RR04870) and the NSF (Grant CHE88-14019); mass spectra were obtained at the Harvard University Chemistry Department Mass Spectrometry Facility (NIH grant 1-SIO-RR06716; NSF grant CHE-9020043).

Supplementary Material Available: Experimental procedures for the syntheses of all labeled compounds, the hydrolysis reaction, and the analysis of the [^{16}O,^{17}O,^{18}O]thiophosphate (14 pages). Ordering information is given on any current masthead page.

(14) Webb, M. R.; Trentham, D. R. *J. Biol. Chem.* **1980**, *225*, 1775–1778. Webb, M. R. *Methods Enzymol.* **1982**, *87*, 301–316.
(15) Tsai, M.-D. *Biochemistry* **1979**, *18*, 1468–1472.
(16) Cohn, M.; Hu, A. *J. Am. Chem. Soc.* **1980**, *102*, 913–916.
(17) The incomplete enrichment of the [^{17}O]H_2O (20.7% ^{16}O, 48.6% ^{17}O, 30.7% ^{18}O) and the [5′-^{18}O]adenosine (14% ^{16}O, 86% ^{18}O) results in four isotopomeric species that are visible in the ^{31}P NMR: an $^{16}O/^{16}O$ species, an $^{18}O/^{18}O$ species, and two $^{16}O/^{18}O$ species that differ in the position occupied by ^{18}O (bridging or nonbridging).
(18) Knowles, J. R. *Annu. Rev. Biochem.* **1980**, *49*, 877–919.
(19) Experiments are underway to resolve the apparent contradictions between the results of Vincent et al.[5] and the data presented here.
(20) Fructose 1,6-bisphosphatase also appears to catalyze the direct transfer of a phospho group to water (Domanico, P. L.; Rahil, J. F.; Benkovic, S. J. *Biochemistry* **1985**, *24*, 1623–1628).