Communications to the Editor

Inversion of Configuration during the Hydrolysis of D-1-S_p-*myo*-Inositol [^{17}O]Thiophosphate Catalyzed by *myo*-Inositol Monophosphatase

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Inositol monophosphatase (EC 3.1.3.25) catalyzes the hydrolysis of D-*myo*-inositol 1-, 3-, 4-, 5-, and 6-monophosphates.[1] This enzyme has attracted considerable attention because it plays a crucial role in the regulation of *myo*-inositol for the phosphatidyl inositol cell signaling pathway.[2] This pathway is thought to be overactivated in patients with manic depression, and inositol monophosphatase may be the in vivo target for its treatment with lithium.[3] The brain enzymes from a number of mammalian sources are very similar, comprising a homodimer of subunit $M_r \approx 30$ kDa, and the cloned human brain enzyme has been crystallized and the X-ray structure determined.[4] The structure of a catalytically inactive ternary (enzyme−substrate−metal) complex showed the active site to contain two metal ions, and the involvement of two metals in enzyme turnover has been confirmed by extensive kinetic investigation.[5,6] Notwithstanding this detailed structural and kinetic investigation, the fundamental details of the mechanism of this important enzyme remain obscure. Arguments based on these kinetic studies have been advanced to reject an in-line mechanism in favor of this enzyme catalyzing the hydrolysis of *myo*-inositol monophosphate via an adjacent attack involving a pseudorotation step.[6]

Extensive stereochemical studies on a wide range of phosphotransferases have established that single-step reactions occur with *inversion* of configuration, consistent with an in-line displacement reaction.[7] For enzyme-catalyzed phosphoryl-transfer reactions that occur with overall *retention* of configuration, there is usually strong independent evidence for a double displacement involving a phosphoenzyme intermediate.[7] In the case of *myo*-inositol monophosphatase, a phosphoenzyme appears to be ruled out on the basis of the detailed kinetic analysis and by virtue of the failure to identify an appropriately positioned enzyme nucleophile in the X-ray crystal structure. However, *retention* of configuration would also be observed for a mechanism involving direct phosphoryl transfer via a single pseudorotation step.[7a] Thus, we report here the stereochemical course of the hydrolysis of D-1-S_p-*myo*-inositol [^{17}O]thiophosphate catalyzed by recombinant bovine *myo*-inositol monophosphatase.

The methodology for the determination of the stereochemical course of enzyme-catalyzed phosphoryl-transfer reaction is well established.[7] For reactions catalyzed by phosphatases, it is necessary to resort to thiophosphate analogues in order to end up with a chiral product, viz., inorganic [^{16}O,^{17}O,^{18}O]thiophosphate. The synthesis of D-1-S_p-*myo*-inositol [^{17}O]thiophosphate is shown in Scheme 1.[8] It has already been established that *myo*-inositol monophosphatase catalyzes the hydrolysis of thiophosphate esters, albeit at reduced rates compared to the corresponding phosphate esters.[6d,9] In terms of the practicalities of determining the stereochemical course of the reaction, it is essential to optimize the conditions to allow accumulation of inorganic thiophosphate and to minimize the competing loss of sulfur that follows the enzymatic step. Hydrolysis of D-1-S_p-*myo*-inositol [^{17}O]-thiophosphate (90 μmol, added portionwise every 2 h over 32 h to minimize the substrate concentration to avoid substrate inhibition) in [^{18}O]water (4 mL, buffered with Tris-HCl at pH 9.0), catalyzed by recombinant bovine *myo*-inositol monophosphatase[10] (31 mg, previously lyophilised with [^{18}O]water), led to the isolation of inorganic [^{16}O,^{17}O,^{18}O]thiophosphate (ca. 20 μmol) of unknown configuration. The configurational analysis of this material was achieved by the chemical method developed by Lowe et al.[11]

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(1) Majerus, P. W. *Annu. Rev. Biochem.* **1992**, *61*, 225.
(2) Potter, B. V. L.; Lampe, D. *Angew. Chem., Int. Ed. Engl.* **1995**, *34*, 1933.
(3) Atack, J. R. *Brain Res. Rev.* **1996**, *22*, 183.
(4) (a) Bone, R.; Springer, J. P.; Atack, J. R. *Proc. Natl. Acad. Sci. U.S.A.* **1992**, *89*, 10031. (b) Pollack, S. J.; Atack, J. R.; Knowles, M. R.; McAllister, G.; Ragan, C. I.; Baker, R.; Fletcher, S. R.; Iversen, L. L.; Broughton, H. B. *Proc. Natl. Acad. Sci. U.S.A.* **1994**, *91*, 5766. (c) Bone, R.; Frank, L.; Springer, J. P.; Pollack, S. J.; Osborne, S.; Atack, J. R.; Knowles, M. R.; McAllister, G.; Ragan, C. I.; Broughton, H. B.; Baker, R.; Fletcher, S. R. *Biochemistry* **1994**, *33*, 9460. (d) Bone, R.; Frank, L.; Springer, J. P.; Atack, J. R. *Biochemistry* **1994**, *33*, 9468.
(5) Takimoto, K.; Okada, M.; Matsuda, Y.; Nakagawa, H. *J. Biochem. (Tokyo)* **1985**, *98*, 363.
(6) (a) Leech, A. P.; Baker, G. R.; Shute, J. K.; Cohen, M. A.; Gani, D. *Eur. J. Biochem.* **1993**, *212*, 693. (b) Baker, G. R.; Gani, D. *Bioorg. Med. Chem. Lett.* **1991**, *1*, 193. (c) Cole, A. G.; Gani, D. *J. Chem. Soc., Perkin Trans. 1* **1995**, 2685. (d) Cole, A. G.; Wilkie, J.; Gani, D. *J. Chem. Soc., Perkin Trans. 1* **1995**, 2695. (e) Wilkie, J.; Cole, A. G.; Gani, D. *J. Chem. Soc., Perkin Trans. 1* **1995**, 2706.
(7) (a) Knowles, J. R. *Annu. Rev. Biochem.* **1980**, *49*, 877−919. (b) Buchwald, S. L.; Hansen, D. E.; Hassett, A.; Knowles, J. R. *Methods Enzymol.* **1982**, *87*, 279−301. (c) Eckstein, F. *Angew. Chem., Int. Ed. Engl.* **1983**, *22*, 423. (d) Frey, P. A. *Tetrahedron* **1982**, *38*, 1541. (e) Lowe, G. *Acc. Chem. Res.* **1983**, *16*, 244. (f) Frey, P. A. *Adv. Enzymol. Relat. Areas Mol. Biol.* **1989**, *62*, 119.
(8) (D/L)-1,2;4,5-Di-*O*-cyclohexylidene-*myo*-inositol was synthesized according to literature methods: Dreef, C. E.; Tulnman, R. J.; Lefeber, A. W. M. *Tetrahedron* **1991**, *47*, 4709. The assignment of the diastereomers was made by comparison with the product of phosphorylation of a sample of the resolved diol of known absolute configuration: Vacca, J. P.; deSolms, S. J.; Huff, J. R.; Billington, D. C.; Baker, R.; Kulagowski, J. J.; Maver, I. M. *Tetrahedron* **1989**, *45*, 5679; **1991**, *47*, 907 (corrigenda).
(9) Baker, G. R.; Billington, D. C.; Gani, D. *Bioorg. Med. Chem. Lett.* **1991**, *1*, 17.
(10) Dielh, R. E.; Whiting, P.; Potter, J.; Gee, N.; Ragan, C. I.; Linemeyer, D.; Schoepfer, R.; Bennet, C.; Dixon, R. A. F. *J. Biol. Chem.* **1990**, *265*, 5946.
(11) (a) Arnold, J. P. R.; Lowe, G. *J. Chem. Soc., Chem. Commun.* **1986**, 865. (b) Arnold, J. P. R.; Bethell, R. C.; Lowe, G. *Bioorg. Chem.* **1987**, *15*, 250.
(12) The predicted intensity ratios for the isotopomers arising from the configurational analysis of PS_i from the enzyme-catalyzed reaction assuming either inversion or retention can be calculated from the measured isotopic incorporation. Mass spectrometry of D-1-S_p-*myo*-inositol [^{17}O]thiophosphate established that the actual ^{17}O incorporation was 30%, with the remaining isotope composition being 69% ^{16}O and 1% ^{18}O. For the ^{18}O incorporation during the enzymatic hydrolysis, this was estimated to be 83%, with the remaining isotope composition 1% ^{17}O and 16% ^{16}O. Using these values, the predicted ratio for the resonances from the *trans* ester (A:B:C) for inversion of configuration is 40.6:34.9:24.5, whereas for retention of configuration the ratios would be 40.6:24.5:34.9. The experimental ratios A:B:C of 40.5:33.5:26 are in excellent agreement with complete inversion of configuration (the ratios for the *cis* esters are, of course, complementary).

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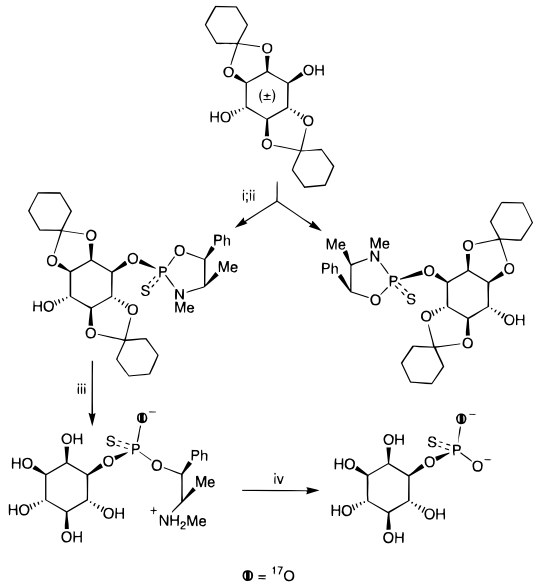
Scheme 1[a]



[a] Reagents: (i) NaH, DMF, (2*R*,4*R*,5*S*)-(+)-2-chloro-3,4-dimethyl-5-phenyl-1,3,2-oxazaphospholidine-2-sulfide (Aldrich); (ii) HPLC separation of diastereomers; (iii) THF, $(CF_3CO)_2O$, $H_2{}^{17}O$ (53% enriched); (iv) Na, liquid NH_3.

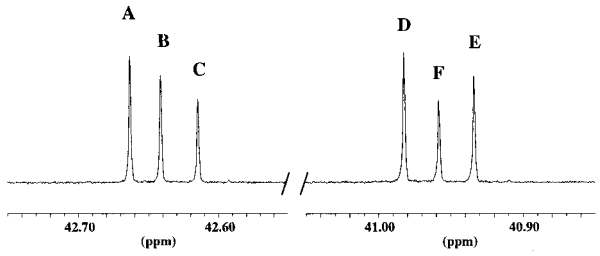
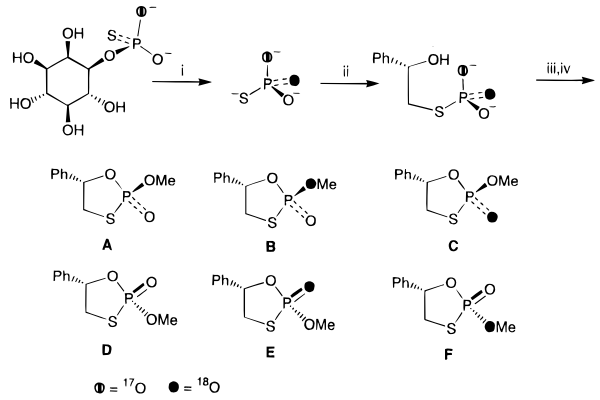


Figure 1. ^{31}P NMR spectrum (Bruker ARX400; 162 MHz) of the sample from the configurational analysis of inorganic [^{16}O,^{17}O,^{18}O]thiophosphate derived from the hydrolysis of D-S_p-1-*myo*-inositol [^{17}O]thiophosphate in $H_2{}^{18}O$, catalyzed by recombinant bovine *myo*-inositol monophosphatase. Assignments of resonances **A**−**F** are given in Scheme 2. The spectrum was recorded at 0.038 Hz per point digital resolution and processed without resolution enhancement, with natural line widths of 0.27 Hz.

shown in Scheme 2. The high-field ^{31}P NMR spectrum of the resulting isotopomeric cyclic phosphothiolate triesters derived from the configurational analysis sequence is shown in Figure 1.[12] This establishes that the absolute configuration of the enzymatically derived inorganic [^{16}O,^{17}O,^{18}O]thiophosphate is R_p and that the reaction has proceeded with *inversion* of configuration. This result is entirely consistent with the expected in-line

Scheme 2. Outline of the Configurational Analysis of Inorganic [^{16}O,^{17}O,^{18}O]Thiophosphate, Shown for the R_p Enantiomer That Would Arise if the Enzymatic Step Proceeded with Inversion of Configuration[a]



[a] Only the isotopomers in which ^{17}O is lost during the cyclization step are shown since these are the only species with sharp resonances in the high-field ^{31}P NMR spectrum. Reagents: (i) recombinant bovine *myo*-inositol monophosphatase, $H_2{}^{18}O$ (97% enriched), pH 9.0, 25 °C; (ii) (*S*)-2-iodo-1-phenylethanol, DMF; (iii) $(PhO)_2P(O)Cl$ (1.1 equiv), tri-*n*-butylamine, DMF; (iv) CH_2N_2, CH_3CN.

nucleophilic displacement reaction and rules out an adjacent displacement reaction accompanied by pseudorotation. These observations are consistent with one of the models for the hydrolysis mechanism advanced by the Merck group on the basis of their X-ray structural work.[4] In this mechanism, the nucleophilic water is coordinated to Mg^{2+}-1 and is activated by Glu-70 and Thr-95, allowing approach in-line with the inositol leaving group. It is interesting to note that fructose 1,6-bisphosphatase also utilizes two magnesium cations and is sensitive to lithium, and this enzyme proceeds with inversion.[13]

This result is highly significant since it reestablishes and reinforces the generalization that single-step phosphoryl-transfer reactions proceed via in-line mechanisms. In addition, it has firmly ruled out a mechanism proceeding by a phosphoenzyme intermediate. To date, there are no examples of enzyme-catalyzed displacement reactions occurring via an adjacent attack involving a pseudorotation step. Indeed, in simple phosphate esters, particularly anions (mono- and diesters), there are few examples of simple chemical reactions involving pseudorotation.[14] This mechanism is principally encountered in displacement reactions involving phosphate esters held in small rings, where the ring dramatically stabilizes the trigonal bipyramidal intermediate. Hence, it would appear that in-line nucleophilic displacement is certainly the preferred pathway for phosphate mono- and diester anions in both chemical and enzyme-catalyzed reactions. This result may also assist in the design of inhibitors of inositol monophosphatase.

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(13) Domanico, P. L.; Rahil, J. F.; Benkovic, S. J. *Biochemistry* **1985**, *24*, 1623.

(14) (a) Thatcher, G. R. J.; Kluger, R. H. *Adv. Phys. Org. Chem.* **1989**, *25*, 99. (b) Hall, C. R.; Inch, T. D. *Tetrahedron* **1980**, *36*, 2059.