Pergamon

Bioorganic & Medicinal Chemistry, Vol. 4, No. 9, pp 1515–1520, 1996

PII: S0968-0896(96)00144-7
0968-0896/96 $15.00 – 0.00

Quiescent Affinity Inactivators of Protein Tyrosine Phosphatases

William P. Taylor,[a] Zhong-Yin Zhang[b] and Theodore S. Widlanski[a,*]

[a]*Department of Chemistry, Indiana University, Bloomington, IN 47405, U.S.A.*

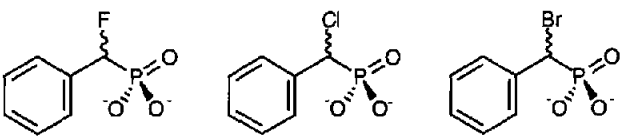
[b]*Department of Molecular Pharmacology, Albert Einstein College of Medicine, Yeshiva University, Bronx, NY 10461, U.S.A.*

Abstract—α-Halobenzylphosphonates were investigated as possible inactivators of protein tyrosine phosphatases (PTPases). These compounds inactivate the *Yersinia* PTPase (Yop51*Δ162) in a time- and concentration-dependent fashion. This inactivation is active-site directed and irreversible, and is surprisingly rapid in light of the stability of the α-halobenzylphosphonates toward nucleophiles in solution. The potential of these molecules for probing the stereochemistry of PTPase inactivation, as well as for providing a useful paradigm for the design of more potent PTPase inhibitors is discussed. Copyright © 1996 Elsevier Science Ltd

Introduction

The phosphorylation state of proteins is controlled by the joint action of phosphoprotein phosphatases, which catalyse the dephosphorylation of proteins, and protein kinases, which catalyse protein phosphorylation. The integration of the opposing activities of these two types of enzymes into signal transduction cascades provides higher organisms with an exquisite method for controlling cell function and growth.[1,2] Since protein tyrosine phosphatases (PTPases) play important roles in controlling a variety of cell activities, the development of methods to selectively inhibit these phosphatases is of substantial importance. Despite this, there exist few general strategies for the development of such inhibitors.[3–5] We now reveal a new methodology for the active site-directed, time-dependent inactivation of PTPases.

A variety of PTPases are characterized by substantial active site homology, including a highly conserved cysteine residue that is required for catalysis.[6–8] Compelling kinetic and physical evidence has led to the proposition that these PTPases catalyse phosphotyrosine hydrolysis via the formation of a phosphocysteine intermediate,[9,10] whose subsequent hydrolysis is largely rate determining.[11] Treatment of PTPases with iodoacetate leads to rapid enzyme inactivation and alkylation of this essential cysteine residue,[12,13] reminiscent of the inactivation of thiol proteases.[14] Thiol proteases are susceptible to inactivation by affinity reagents containing very weakly electrophilic groups that normally participate poorly in S_N2 reactions. These types of inhibitors have been termed 'quiescent affinity labels' because they are chemically reactive only when bound to the enzyme active site. This mode of inactivation is the basis for the development of potent and exceedingly selective thiol protease inhibitors.[15] The presence of a nucleophilic active site cysteine residue in PTPases prompted us to examine the possibility that these enzymes would behave like thiol proteases in that they would also be susceptible to affinity inactivation by molecules that are normally quite unreactive.



1 2 3

Three potential affinity reagents (**1–3**) shown above were synthesized and assayed for inhibition against Yop51*Δ162, a truncated recombinant form of the PTPase Yop51 from *Yersinia enterocolitica*.[16] These molecules were selected with the following rationale: they would mimic the geometry and charge of an aromatic phosphate ester and would deliver a normally unreactive electrophile, an α-halobenzylphosphonate, to the active site of the enzyme.

Results and Discussion

A direct comparison between the activity of the three α-halobenzylphosphonates toward Yop51*Δ162 (Fig. 1) shows that fluoride **1** causes little or no inactivation, chloride **2** gives modest inactivation rates and, as expected purely on the grounds of electrophilicity, bromide **3** is by far the best inactivator. This initial result led us to explore more fully the inactivation of Yop51*Δ162 by chloride **2** and bromide **3**.

As shown in Figures 2 and 3, incubation of compounds **2** and **3** with the *Yersinia* PTPase leads to time-and concentration-dependent loss of enzyme activity. This loss of enzyme activity is apparently not a simple first

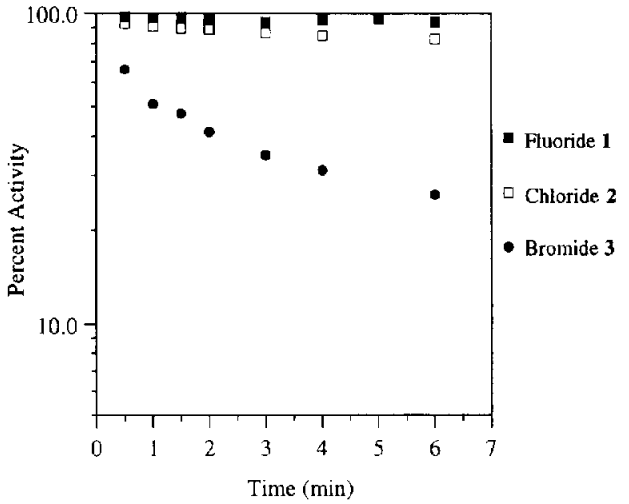


Figure 1. Comparison of the series of α-halobenzylphosphonates as inactivators of Yop51*Δ162. Residual enzyme activity following incubation with 5 mM fluoride **1** (■), chloride **2** (□), or bromide **3** (●).

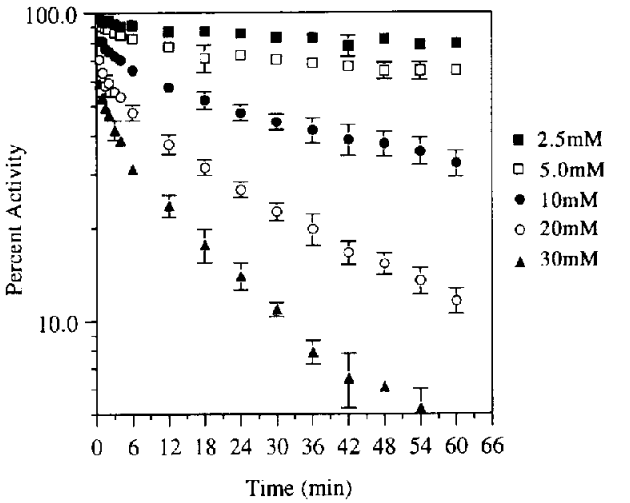


Figure 2. Time-dependent and concentration-dependent inactivation of Yop51*Δ162 by α-chlorobenzylphosphonate. Residual enzyme activity following incubation with chloride **2**: 2.5 mM (■), 5.0 mM (□), 10.0 mM (●), 20.0 mM (○), and 30.0 mM (closed triangles). Error bars represent the standard deviation of three separate inactivations. In many cases the error bars are smaller than the symbol used to represent remaining activity.

order process, as the inactivation kinetics are clearly biphasic. The reason for the biphasic nature of the inactivation is currently unknown.[17] To show that the inactivation is active site-directed, the enzyme was incubated with bromide **3** in the presence of substrate, *p*-nitrophenyl phosphate. As shown in Figure 4, the presence of substrate greatly reduces the rate of enzyme inactivation, thereby demonstrating that the inactivation is likely to be active site-directed. This conclusion is further supported by the observation that competitive inhibitors of the enzyme such as arsenate also slow the inactivation rate considerably. Substrate also protects Yop51*Δ162 against inactivation by the chloride **2** (data not shown). Enzyme activity is not recoverable after ultrafiltration and washing of the enzyme, demonstrating that the enzyme is irreversibly inhibited, presumably because a covalent bond is formed between the inhibitor and the enzyme.

Although the α-halobenzylphosphonates bear a superficial resemblance to the very reactive α-halobenzylcarboxylates, compounds **2** and **3** are remarkably unreactive toward nucleophilic substitution. For example, incubation of 10 mM bromide **3** with varying concentrations of the thiol nucleophile cysteine (up to a concentration of 50 mM), at either pH 5.5 or pH 7.5 gave none of the expected sulfide. Incubation of the α-bromobenzylphosphonate (30 mM) with the very potent nucleophile sodium azide (500 mM, pH 5.5) again failed to yield substitution products, even at incubation times of longer than 10 h. Indeed, we were unable to observe reaction of bromide **3** with any charged nucleophiles at pH between 5.5 and 7.5. NMR experiments revealed that the only products of the reaction result from a very slow solvolysis reaction (Fig. 5). Even incubation with a substantial level of an uncharged nucleophile (500 mM hydroxylamine, 30 mM bromide **3**, pH 7.5) gave only the solvolysis product.

Given the lack of reactivity of the α-halobenzylphosphonates, their ability to rapidly inactivate Yop51*Δ162 is noteworthy. Several factors must be operative in order for these molecules to be able to inactivate the *Yersinia* PTPase. First, binding to the enzyme undoubtedly removes at least part of the entropic barrier for the substitution. Second, the reduction of the charge about the phosphonate upon its binding to the active site (via hydrogen bonding) presumably facilitates the attack of a nucleophile. Third, the active site nucleophile is expected to be remarkably reactive. A comparison between the rate of enzyme inactivation by bromide **3** and the rate of reaction with nucleophiles such as thiol and azide may be instructive. In these inactivation assays, the enzyme

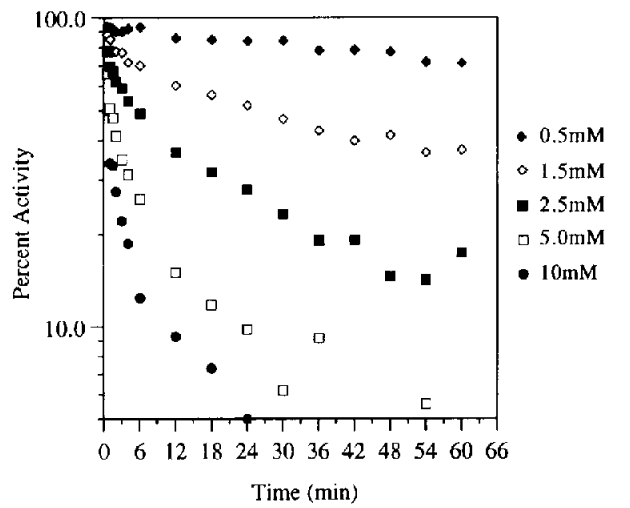


Figure 3. Time-dependent and concentration-dependent inactivation of Yop51*Δ162 by α-bromobenzylphosphonate. Residual enzyme activity following incubation with bromide **3**: 0.5 mM (◆), 1.5 mM (◇), 2.5 mM (■), 5.0 mM (□), and 10.0 mM (●).

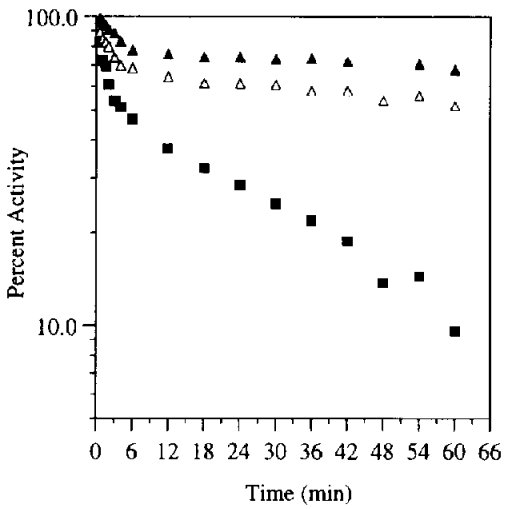


Figure 4. Inactivation of Yop51*Δ162 by α-bromobenzylphosphonate is slowed by the presence of substrate. Residual enzyme activity following incubation with 2.5 mM bromide **3** and 0 mM (■), 2 mM (△), or 4 mM *p*-nitrophenylphosphate (▲).

is present at about 50 pM. With the bromide at 10 mM, the half-life for inactivation is about 1 min. The half-life for nucleophilic substitution of 30 mM α-bromobenzylphosphonate by 0.5 M azide is greater than 10 h. (This is a lower limit since no reaction was observed.) Thus, alkylation of the enzyme by α-bromobenzylphosphonate is accelerated by greater than 10^{13} over reaction with azide. Although a direct comparison of reactivities is impossible due to the unknown nature and environment of the enzyme nucleophile, the inability of extraordinarily potent nucleophiles to participate in solution substitution reactions with α-halophosphonates suggests that the enzyme nucleophile is exceptionally reactive.

Although mono- and dihalophosphonates have previously been considered for use as nonhydrolysable phosphate ester analogues[18,19] their potential for use as affinity reagents has never been described. We assayed bromide **3** with alkaline phosphatase and prostatic acid phosphatase, two prototypical phosphatases with nucleophilic active site residues. In both cases there was little or no evidence of time-dependent inhibition, suggesting that the covalent inactivation caused by the α-halobenzylphosphonates is specific to PTPases. What is the source of this specificity? Although at the moment we can only speculate, it seems reasonable to assume that the reaction is related to the highly nucleophilic active site cysteine residue.

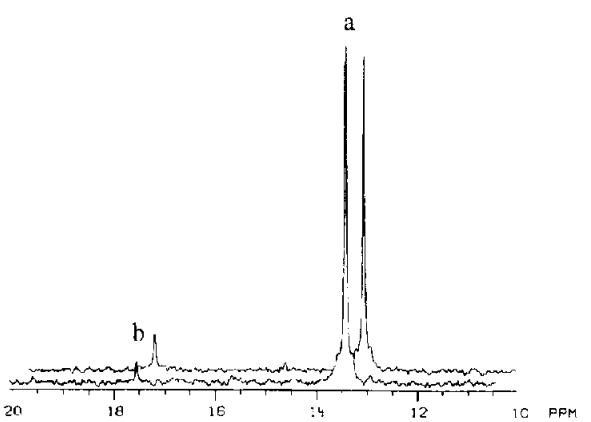
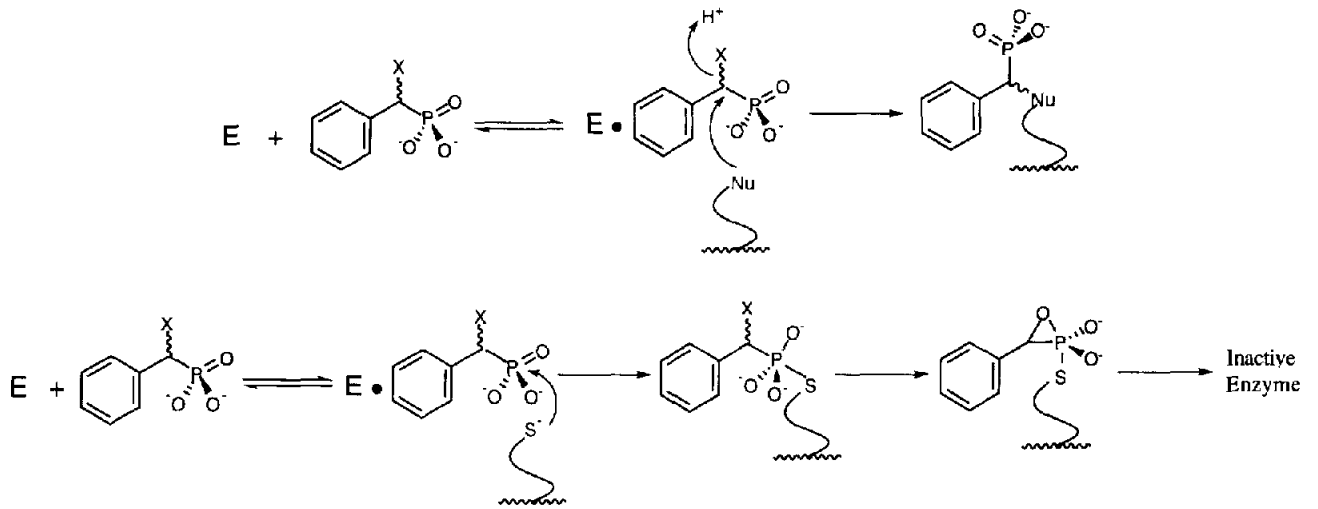


Figure 5. ^{31}P NMR spectra of the reaction of α-bromobenzylphosphonate and sodium azide. Spectra taken at 10 min (foreground) and 600 min (background) show only bromide **3** (signal a, 13.1 ppm) and solvolysis product (signal b, 17.4 ppm).

Scheme 1 shows two potential mechanisms for the inactivation process. The inactivation may be a simple S_N2 reaction in which an active site nucleophile displaces the halogen. The conserved active site cysteine is a possible candidate for alkylation, as observed in the inactivation of PTPases by iodoacetate. Although this is a tempting notion, it is unclear that the geometry of the phosphonate would permit this process to occur. If the normal hydrolysis reaction were to proceed via an in-line displacement at phosphorus, then the cysteine would be attacking phosphorus from the side opposite that of the leaving group phenol. Assuming that the phosphonate mimics the binding mode of the phosphate, this would place the benzylic position of the inhibitor at a considerable distance from the cysteine residue. Another, although less precedented, possibility would call for the addition of the cysteine to the phosphonate to give a phosphorane, followed by closure to a three-membered ring. One possible fate of this highly reactive molecule might be ring opening to an α-hydroxybenzylphosphonate containing a covalent bond to the active site cysteine. Alternatively, the ring might be opened by a second active site nucleophile. This mechanism bears some similarity to that suggested for the inactivation of chymotrypsin by α-haloketones.[20]

The initial rate of inactivation of Yop51*Δ162 by α-chlorobenzylphosphonate was analysed using a Kitz–Wilson double reciprocal plot (Fig. 6), which yielded values for K_I and k_{inact} of 20.5 mM and 0.140 min^{-1}, respectively. Similar analysis of the inactivation caused by bromide **3** does not yield a straight line (data not shown); the pronounced biphasic nature of the α-bromobenzylphosphonate inactivation (see Fig. 3) may preclude such simple kinetic analysis. The values obtained for the α-chlorobenzylphosphonate inactivation also require careful interpretation because the inactivator is delivered to the enzyme as a racemic mixture. If the enantiomers inactivate the enzyme with different kinetic constants, the values of K_I and k_{inact} determined above are an amalgamation of the values for each enantiomer. In the extreme case that one enantiomer acts as a competitive inhibitor, removal of that component of the inactivation mixture may significantly improve the apparent potency of these molecules. Synthesis and testing of the enantiomerically pure isomers should simplify interpretation of the kinetic data, as well as provide a means for probing the stereochemisty of the inactivation event.



Scheme 1.

Conclusion

Protein phosphorylation is a key regulatory element in the control of cellular processes as diverse as signal transduction, glucose metabolism, programmed cell death and cell transformation. The elucidation of roles of differing phosphatases in these processes may be facilitated by the development of phosphatase inactivators that display a high degree of specificity. The α-halobenzylphosphonates described above represent a first step toward the development of inactivators specific for protein tyrosine phosphatases. This specificity apparently arises from the highly reactive enzyme nucleophile (presumably a cysteine thiolate) overcoming the inherent stability of the inactivator. Additional specificity toward PTPases might be gained by incorporating the α-halobenzylphosphonate motif into peptides. Substrate specificity studies with a variety of PTPases have indicated a preference for acidic residues *N*-terminal to the phosphorylated tyrosine.[21-23] Although this preference may be a general feature of PTPase substrate recognition, individual PTPases appear to have unique substrate requirements for optimal catalytic activity.[24] Also, substitution of phosphonomethylphenylalanine for phosphotyrosine in peptide substrates has been shown to have little effect on the binding of these peptides to PTPases.[25] Thus, careful selection of the amino acid sequence of a α-halobenzylphosphonate-containing peptide may facilitate the development of potent inactivators with the ability to differentiate between specific PTPases.

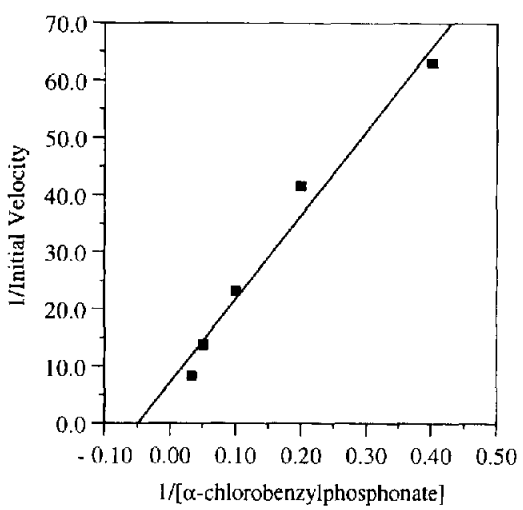


Figure 6. Kitz–Wilson analysis of the inactivation of Yop51*Δ162 by α-chlorobenzylphosphonate. Initial velocities were determined from a graph of the natural logarithm of the fractional activities with respect to time for the first 4 min of the inactivation shown in Figure 2.

Experimental

General

Bovine Serum Albumin (BSA), Fraction V powder, was obtained from ICN. Mono-sodium arsenate, *p*-nitrophenyl phosphate monocyclohexylammonium salt (*p*-NPP), and (L)-cysteine were from Sigma. Microcon-10 (10kD cutoff) centrifugal concentrators were from Amicon. 1,3-Bis[tris(hydroxymethyl)methylamino]propane (Bis-Tris-Propane) was from Aldrich. All other chemicals were ACS reagent grade.

Proton NMR spectra were recorded either at 300 MHz on a Varian XL-300 or at 400 Mhz on a Varian VXR400, using residual solvent signals as an internal reference. Carbon NMR spectra were recorded at 100 MHz on a Varian VXR400, using residual solvent signals as an internal reference. Phosphorous NMR spectra were recorded at 146 MHz on a Nicolet NT-360 using H_3PO_4 as an external reference. All reactions were conducted in flame-dried glassware under a dry nitrogen atmosphere. Methylene chloride was distilled from calcium hydride. Triethylamine was distilled from KOH. NCS and NBS were crystallized from water prior to use.

The *Yersinia* PTPase[16] and human prostatic acid phosphatase[26] were purified as previously described.

Alkaline phosphatase was Type VII-NT from Sigma. All buffers were prepared using doubly distilled and deionized water. All enzymology was performed at 25 °C. The following buffers were used: 100 mM sodium acetate, 15 mM NaCl, 25 μg/mL BSA, pH 5.0 (buffer 5.0); 100 mM sodium acetate, 2 mM EDTA, 1 mM BME, 25 μg/mL BSA, pH 5.5 (buffer 5.5); 25 mM Bis-Tris–Propane acetate, 5 mM EDTA, 10 mM BME, 25 μg/mL BSA, pH 8.0 (buffer 8.0).

Synthesis of inactivators

All compounds were synthesized by adaptations of existing methods. Proton NMR spectra of known compounds **1**, **4**, **5**, **6**, and **7** were in good agreement with those previously reported. Diethyl-α-hydroxybenzylphosphonate[27] (**4**) was prepared by treatment of benzaldehyde (1.0 equiv) with diethyl phosphite (1.1 equiv) and triethyl amine (2.2 equiv) at room temperature for 24 h. Addition of ether caused a white solid to precipitate. This solid was filtered, washed with cold ether, then recrystallized from hot ether. Diethyl-α-fluorobenzylphosphonate[28] (**5**) was prepared from compound **4** (1.0 equiv) in methylene chloride at 0 °C by the dropwise addition of diethylaminosulfur trifluoride (2.0 equiv). The reaction was maintained for 1 h before being warmed to room temperature and then diluted with diethyl ether. The resulting solution was washed three times with phosphate buffer, and the organic extract was concentrated in vacuo and purified by flash column chromatography (5:1 ether:hexanes). Diethyl-α-chlorobenzylphosphonate[29] (**6**) was prepared by treating compound **4** (1.0 equiv) with triphenylphosphine (2.2 equiv) and *N*-chlorosuccinimide (2.0 equiv) in methylene chloride at room temperature for 12 h. The reaction was quenched with methanol and concentrated in vacuo. The resulting material was purified by flash column chromatography (3:2, ethylacetate:hexanes). Diethyl-α-bromobenzylphosphonate[30] (**7**) was prepared in a manner similar to the preparation of compound **6** using *N*-bromosuccinimide in place of *N*-chlorosuccinimide. Compound **5** (1.0 equiv) was allowed to react with bromotrimethylsilane (4.5 equiv) in methylene chloride at room temperature for 16 h. The reaction mixture was concentrated in vacuo, then dissolved in toluene and the volatile components evaporated (three times total). The resultant oil was stirred with triethylamine (1.5 equiv) in methanol for 10 min and the volatile components evaporated. The triethylammonium salt was then dissolved in methanol and volatile components again evaporated (three times total) to obtain the monotriethylammonium salt of α-fluorobenzylphosphonic acid. This salt was passed down a AG 50W-X8 ion exchange column (sodium form) to yield sodium α-fluorobenzylhydrogenphosphonic acid (**1**).[28] Sodium α-chlorobenzylhydrogenphosphonic acid (**2**): ^{1}H NMR (D_2O): δ 4.84 (1H, d, J = 12.9 Hz), 7.26–7.31 (3H, m), 7.43 (2H, d, J = 6.6 Hz); ^{13}C NMR (CD_3OD): δ 58.5 (d, J = 143.4 Hz), 128.5, 128.9, 130.16, 130.21; ^{31}P NMR (CD_3OD): δ 12.94; and sodium α-bromobenzylhydrogenphosphonic acid (**3**): ^{1}H NMR (D_2O): δ 4.82 (1H, d, J = 11.3 Hz), 7.23–7.29 (3H, m), 7.48 (2H, d, J = 6.9 Hz); ^{13}C NMR (D_2O:CD_3OD, 4:1): δ 47.8 (d, J = 140.8 Hz), 129.1, 129.5, 130.09, 130.14; ^{31}P NMR (D_2O:CD_3OD 4:1): δ 12.87 were obtained in an identical fashion from compounds **6** and **7**, respectively.

Inactivation of Yop51*Δ162

Stock enzyme (~7.5 mg/mL) was diluted 90,000-fold into Buffer 8.0 and stored at room temperature. A stock inactivator solution was prepared in water and stored on ice. A 20 μL aliquot of the enzyme solution was added to 920 μL buffer 5.5 and allowed to incubate for 3.5 min. The inactivation was initiated by the addition of a 60 μL sample of appropriately diluted inactivator for a final volume of 1.0 mL. Aliquots (60 μL) were removed from this mixture at appropriate time intervals, added to 1.0 mL 10 mM *p*-NPP in buffer 5.0, and allowed to react for 2.5 h. The activity assay was quenched with 100 μL 1.25 M NaOH and the absorbance at 405 nm was read. A control was performed by adding 60 μL water in place of inactivator and removing aliquots as before. Enzyme activity of the control mixture decreased 10–12% over the course of the assay (~1 h). The inactivation in the presence of *p*-NPP was performed by adding substrate to buffer 5.5 to yield a final concentration of 2 mM or 4 mM *p*-NPP after the addition of both enzyme and inactivator.

Kitz–Wilson analysis was performed on the data from the first 4 min of inactivation, representing the loss of between 6 and 21% of activity. Initial velocity was obtained from the slope of a plot of ln(remaining activity) versus time.

Inactivation of prostatic acid phosphatase and alkaline phosphatase

The inactivation of these phosphatases is identical to that of Yop51*Δ162 with the following exceptions. For human prostatic acid phosphatase the inactivation buffer was 100 mM sodium acetate, pH 5.0, and the inactivation assay was quenched after a 45 min incubation. For alkaline phosphatase the inactivation buffer was 50 mM Tris–Cl, 1 mM $MgCl_2$, pH 9.0, and the activity assay was performed in 5 mM *p*-NPP, 50 mM Tris–Cl, 1 mM $MgCl_2$, pH 9.0 for 10 min.

Ultrafiltration and washing of inactivated Yop51*Δ162

Stock enzyme (~7.5 mg/mL) was diluted 60,000-fold in buffer 8.0 and stored at room temperature. A stock solution of chloride **2** (500 mM) was prepared in water and stored on ice. A 30 μL aliquot of the enzyme solution was added to 1380 μL buffer 5.5 and allowed to incubate for 3.5 min. The inactivation was initiated by the addition of a 90 μL sample of inactivator for a final volume of 1.5 mL. A control was performed by adding 90 μL of water. The inactivation mixture was allowed to incubate for 1.5 h to ensure complete inactivation, at which time a 350 μL aliquot was placed into each of four Microcon-10 centrifugal concentrators.

The enzyme was concentrated by spinning for 45 min at 15,000 $\times g$. Enzyme was recovered from one of the concentrators, while the remaining filters were washed with 350 μL buffer 5.5 and concentrated again. Enzyme was recovered from one concentrator after each wash until all enzyme had been recovered. Enzymatic activity was assayed immediately after recovery by the addition of 700 μL 10 mM *p*-NPP in buffer 5.0. This was allowed to incubate for 30 min before quenching with 100 μL of 1.25 M NaOH and measurement of the absorbance at 405 nm.

Acknowledgments

This research was supported in part by NIH grant R01 GM47918-03, a Junior Faculty Award from the American Cancer Society (TSW), a Camille-Dreyfus Teacher-Scholar Award (TSW), and an A. P. Sloan Foundation Fellowship (TSW).

References

1. Cyert, M. S.; Thorner, J. *Cell* **1989**, *57*, 891.

2. Fischer, E. H.; Krebs, E. G. *Biochim. Biophys. Acta* **1989**, *1000*, 297.

3. Myers, J. K.; Widlanski, T. S. *Science* **1993**, *262*, 1451.

4. Myers, J. K.; Cohen, J. D.; Widlanski, T. S. *J. Am. Chem. Soc.* **1995**, *117*, 11049.

5. Wang, Q.; Dechert, U.; Jirik, F.; Withers, S. G. *Biochem. Biophys. Res. Comm.* **1994**, *200*, 577.

6. Streuli, M.; Krueger, N. X.; Thai, T.; Tang, M.; Saito, H. *EMBO J.* **1990**, *9*, 2399.

7. Pot, D. A.; Woodford, T. A.; Remboutsika, E.; Haun, R. S.; Dixon, J. E. *J. Biol. Chem.* **1991**, *266*, 19688.

8. Guan, K. L.; Dixon, J. E. *Science* **1990**, *249*, 553.

9. Cho, H.; Krishnaraj, R.; Kitas, E.; Bannwarth, W.; Walsh, C. T.; Anderson, K. S. *J. Am. Chem. Soc.* **1992**, *114*, 7296.

10. Guan, K. L.; Dixon, J. E. *J. Biol. Chem.* **1991**, *266*, 17026.

11. Zhang, Z.-Y.; Malachowski, W. P.; Van Etten, R. E.; Dixon, J. E. *J. Biol. Chem.* **1994**, *269*, 8140.

12. Zhang, Z.-Y.; Dixon, J. E. *Biochemistry* **1993**, *32*, 9340.

13. Pot, D. A.; Dixon, J. E. *J. Biol. Chem.* **1992**, *267*, 140.

14. Halász, P.; Polgár, L. *Eur. J. Biochem.* **1976**, *71*, 563.

15. Krantz, A. *Methods in Enzymol.* **1994**, *244*, 656.

16. Zhang, Z.-Y.; Clemens, J. C.; Schubert, H. L.; Stuckey, J. A.; Fischer, M. W. F.; Hume, D. M.; Saper, M. A.; Dixon, J. E. *J. Biol. Chem.* **1992**, *267*, 23759.

17. We have observed that Yop51*Δ162 undergoes some aggregation during the inactivation reactions. This may account for the biphasic kinetics since the inactivation rate of the aggregated enzyme may be different than that of the free enzyme.

18. Burke, T. R. Jr; Smyth, M. S.; Nomizu, M.; Otaka, A.; Roller, P. P. *J. Org. Chem.* **1993**, *58*, 1336.

19. Smyth, M. S.; Ford, H. Jr; Burke, T. R, Jr. *Tetrahedron Lett.* **1992**, *33*, 4137.

20. Kreutter, K.; Steinmetz, A. C. U.; Liang, T.-C.; Prorok, M.; Abeles, R. H.; Ringe, D. *Biochemistry* **1994**, *33*, 13792.

21. Zhang, Z.-Y.; Thieme-Sefler, A. M.; Maclean, D.; McNamara, D. J.; Dobrusin, E. M.; Sawyer, T. K.; Dixon, J. E. *Proc. Natl Acad. Sci. U.S.A.* **1993**, *90*, 4446.

22. Hippen, K. L.; Jakes, S.; Richards, J.; Jena, B. P.; Beck, B. L.; Tabatabai, L. B.; Ingebritsen, T. S. *Biochemistry* **1993**, *32*, 12405.

23. Ruzzene, M.; Donella-Deana, A.; Marin, O.; Perich, J. W.; Ruzza, P.; Borin, G.; Calderan, A.; Pinna, L. A. *Eur. J. Biochem.* **1993**, *211*, 289.

24. Zhang, Z.-Y.; Dixon, J. E. *Adv. Enzymol.* **1994**, *68*, 1.

25. Zhang, Z.-Y.; Maclean, D. M.; McNamara, D. J.; Sawyer, T. K.; Dixon, J. E. *Biochemistry* **1994**, *33*, 2285

26. VanEtten, R. L.; Saini, M. S. *Clin. Chem.* **1978**, *24*, 1525.

27. Froneman, M.; Modro, T. A.; Qaba, L.; Vather, S. M. *Tetrahedron Lett.* **1987**, *28*, 2979.

28. Blackburn, G. M.; Kent, D. E. *J. Chem. Soc. Perkin Trans. 1* **1986**, 913.

29. Gajda, T. *Synthesis* **1990**, 717.

30. Gajda, T. *Phosphorus, Sulfur Silicon Related Elem.* **1990**, *53*, 327.

(*Received in U.S.A.* 5 *October* 1995)