Motifs for Metallophosphatase Inhibition

Jason K. Myers, Stephen M. Antonelli, and Theodore S. Widlanski*

Department of Chemistry, Indiana University Bloomington, Indiana 47405

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Metallophosphatases have been shown to play an integral role in biological pathways such as hormone signaling, glycogen utilization, and immune activation.[1] The importance of these enzymes has made them prominent targets for inhibitor design. Although phosphonic acids are nonhydrolyzable and appear to be structural isosteres of phosphate esters, their potency as phosphatase inhibitors has been very disappointing. Despite much research, a general strategy for enhancing the inhibitory potency of phosphonic acids has not been achieved. This paper describes a new strategy for improving the inhibition of metallophosphatases by low molecular weight phosphonic acids. We demonstrate that alkylphosphonic acids containing functional groups capable of metal ligation bind more tightly to metallophosphatases than do unfunctionalized alkylphosphonic acids. Phosphonic acids with pendant thiol (**1** and **6**−**8**), carboxylate (**2** and **3**), and phosphonic acid (**4**) moieties are all tight-binding inhibitors (relative to the unfunctionalized phosphonic acid **5**) of both alkaline phosphatase and purple acid phosphatase. These results provide a paradigm for the design of metallophosphatases inhibitors that are easily prepared and may be elaborated to give more complex molecules.

Elegant work on calcineurin inhibition,[2] on the use of combinatorial chemistry,[3] on the development of irreversible inhibitors,[4] and on the use of phosphate mimics[5] represent four very diverse strategies that have been used to develop phosphatase inhibitors. Metal oxides such as tungstate and vanadate are the most common and reliable phosphatase inhibitors.[6] Organic phosphonic acids,[5a,b] sulfonic acids,[5c] and thiophosphates[5d] among others, have also been used as starting points for the design of phosphatase inhibitors. Although alkylphosphonic acids are among the most stable and easily prepared of the phosphatase inhibitors, they generally bind weakly to phosphatases, presumably because they have higher pK_a values than the corresponding phosphates,[7] and because replacement of the ester oxygen with a methylene group may compromise an interaction between the substrate and an active site acid or metal ion. A commonly used strategy for enhancing the inhibitory potency of phosphonic acids is to substitute the α-carbon with a halogen(s) as in α,α-difluoroalkylphosphonic acids. However, many heteroatom-substituted phosphonic acids function poorly as phosphate mimics (*vide infra*).[5d,8]

Metallophosphatases include alkaline phosphatase, purple acid phosphatase, the phosphoserine−threonine phosphatases, and several small molecule-specific phosphatases. All known metallophosphatases (with the exception of alkaline phosphatase[9]) employ a metal dyad to catalyze phosphate ester hydrolysis. However, the nature of these metals varies widely from one enzyme to another.[10] We felt that phosphonic acids containing pendant groups capable of metal ligation would bind more tightly to metallophosphatases than simple alkylphosphonic acids do because the missing ester oxygen−metal interaction might be replaced by a new, and potentially stronger, metal−ligand interaction with the pendant group of the inhibitor. Despite the logic of this strategy, adding a metal-chelating group to a known inhibitor of a metalloenzyme has not been used extensively, with the notable exception of inhibitors of zinc proteases such as angiotensin-converting enzyme.[11]

To test the applicability of this strategy to phosphatases, eight representative compounds (Figure 1, **1**−**8**) were tested as inhibitors of two unrelated metallophosphatases: bovine intestinal alkaline phosphatase (BAP) and kidney bean purple acid phosphatase (KBPuAP).[12] Thiol, phosphonic acid and carboxylate groups were chosen based on the known affinity of these groups for metal ions. Ethanylphosphonic acid was used to ascertain the intrinsic binding of an unfunctionalized phosphonic acid. Thiols **1** and **6**−**8**[13] were used to test the effect of inhibitor geometry on binding.

We were gratified to find that all but one of the bifunctional inhibitors tested bound to purple acid phosphatase more tightly than did the substrate (*p*-nitrophenylphosphate, *p*NPP) and up to 55 times tighter than did ethanylphosphonic acid. Thiol **1** and phosphonopropionic acid (**3**) were both tight binding but displayed complex kinetics. With the exception of (2-mercaptoethanyl)phosphonic acid (**7**), which bound relatively poorly, the enzyme did not discriminate strongly between the various thiols. It is unlikely that the observed inhibition arises from removal of the metal ions from the active site for several reasons. First, there is little or no change in V_{max} upon treatment of PuAP with these compounds. Second, the inhibitors show no time dependent inactivation. Lastly, since 5 days are required to remove the active site zinc ion using the strong chelator, EDTA, it is unlikely that a brief exposure to these compounds would lead to the rapid removal of the active site metals.

To determine whether the enhanced binding we observed with bifunctional phosphonic acids was due to ligation of the remote functional group to a metal ion, we measured the inhibition of PuAP in which the active site zinc ion had been replaced by a cobalt ion (CoPuAP).[14] In general, substitution by cobalt has the effect of weakening the interaction between the enzyme and

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(9) The zinc ions in the active site of AP interact directly with the substrate and can be thought of as a metal dyad. The magnesium ion, while important in catalysis, does not interact directly with the substrate (see ref 10a). The metal triad motif is more common in the phosphodiesterases.

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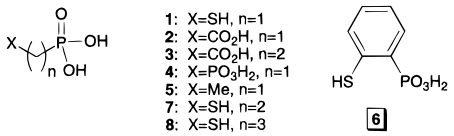

Figure 1. Structures of phosphonic acid inhibitors of metallophosphatases.

Table 1. Inhibition Constants and IC_{50} Values for the Inhibition of Bovine Alkaline Phosphatase, Purple Acid Phosphatase, Cobalt-Exchanged Purple Acid Phosphatase, and *E. coli* Alkaline Phosphatase[a]

	K_i			
inhibitor	(BAP) μM	(KBPuAP) μM	(CoPuAP) μM	IC_{50}, (EcAP) μM
1	41 ± 9	80[b]	34	1000
2	1100[b]	590 ± 60	2300	$\gg$500
3	200 ± 40	160[b]	570[b]	5000
4	750[b]	140 ± 10	760	N.D.
5	1100 ± 200	7600 ± 800	16000	N.D.
6	0.21	350[c]	350[b]	$\ll$10
7	11	3000[c]	N.D.	N.D.
8	3.9	160	N.D.	N.D.
*p*NPP	($K_m = 40$ μM)	($K_m = 700$ μM)	($K_m = 30$ μM)	($K_m = 11$ μM)

[a] Errors are the standard error of three measurements. N.D.-not determined. [b] IC_{50} values are reported because these compounds displayed nonlinear kinetics. [c] IC_{50}.

inhibitor (with the exception of thiol **1**). However the magnitude of the change varies considerably, suggesting that the pendant functional group of the inhibitor interacts directly with the metal ion.

To determine whether tight binding to metallophosphatases is a general property of these phosphonic acids, we repeated our inhibition assays with bovine alkaline phosphatase (Table 1). As with purple acid phosphatase, all of the compounds were tighter-binding inhibitors than **5**, with the best, thiol **6**,[15] binding over 5000 times tighter. In comparison, difluoromethylphosphonic acid is an extremely weak inhibitor of BAP ($IC_{50} > 2$ mM). Both thiophenol and phenylphosphonic acid were found to be extremely poor inhibitors of alkaline phosphatase ($IC_{50} > 2$ mM with BAP), demonstrating that tight binding requires the interaction of both groups with the metal ions. Again, it is very unlikely that the inhibitors are removing the metal from the active site because there is little or no change in V_{max} and because the assays are done in the presence of 1 mM magnesium chloride. If the inhibitors had a high affinity for the free metal ion, they would be sequestered by the large excess of free magnesium over enzyme. Since BAP is bound tighter to the longer phosphonopropionic acid **3** than to phosphonoacetic acid **2**, we synthesized and tested the longer aliphatic thiols **7** and **8**. Consistent with the results obtained with **2** and **3**, it was found that the potency of inhibition increased with increasing chain length.[16]

We were also interested whether we could generalize the binding of phosphonic acids **1**−**8** from one enzyme to closely related enzymes. We tested this hypothesis by measuring the inhibition of *Escherichia coli* alkaline phosphatase (EcAP), an enzyme with substantial homology to BAP. Surprisingly, we observed little or no inhibition with most of the inhibitors tested. The exception is thiol **6**, which inhibited EcAP completely at concentrations as low as 10 μM. While the reason for the different behavior of the *E. coli* and bovine enzymes is unclear, this result demonstrates that these inhibitors can display an impressive selectivity among even closely related enzymes.

This report demonstrates that the incorporation of metal-ligating groups into alkyl phosphonic acids can greatly improve their binding to metallophosphatases. Several observations attest to the usefulness of this strategy. First, the addition of simple functional groups to a phosphonic acid can lead to dramatic increases in binding affinity. With both enzymes tested, the best inhibitors bound 2−4 orders of magnitude more tightly than ethanylphosphonic acid. Given that the compounds we tested contain only a "minimal"-binding motif, it is likely that further optimization can lead to even larger increases in affinity. For example, the use of libraries of nucleic acid, peptide, or organic molecules attached to one of the phosphonic acids used in this study may lead to the discovery of even tighter binding inhibitors of metallophosphatases. Second, while the strategy is general, selectivity can be achieved by changing the structure and geometry of the pendant group. Lastly, thiol **6** is the most potent small molecule inhibitor of BAP yet discovered,[17] surpassing the potency of both vanadate and vanadyl ion as AP inhibitors.[18]

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Supporting Information Available: Experimental procedures for the isolation, and metal ion exchange of PuAP, the inhibition of all of the enzymes in this work, graphical analysis of the data, and synthesis and characterization of all new compounds (40 pages). See any masthead page for ordering and Internet access instructions.

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(15) Compound **6** is a slow tight-binding inhibitor of both forms of AP. To obtain reaction rates, the inhibitor was preincubated with enzyme for 10 min prior to the addition of the substrate. It was also necessary to incubate the resulting solution for 1 min before measuring the rate. This ensured linear progress curves.

(16) We are grateful to the referee who suggested that we test compounds **7** and **8**.

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