

# Promiscuity in the Enzymatic Catalysis of Phosphate and Sulfate Transfer

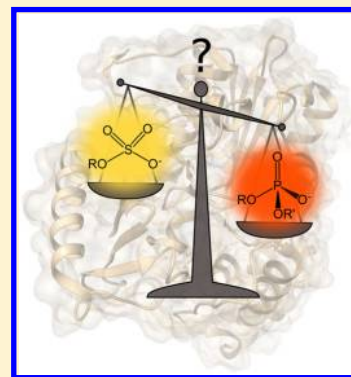
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**ABSTRACT:** The enzymes that facilitate phosphate and sulfate hydrolysis are among the most proficient natural catalysts known to date. Interestingly, a large number of these enzymes are promiscuous catalysts that exhibit both phosphatase and sulfatase activities in the same active site and, on top of that, have also been demonstrated to efficiently catalyze the hydrolysis of other additional substrates with varying degrees of efficiency. Understanding the factors that underlie such multifunctionality is crucial both for understanding functional evolution in enzyme superfamilies and for the development of artificial enzymes. In this Current Topic, we have primarily focused on the structural and mechanistic basis for catalytic promiscuity among enzymes that facilitate both phosphoryl and sulfonyl transfer in the same active site, while comparing this to how catalytic promiscuity manifests in other promiscuous phosphatases. We have also drawn on the large number of experimental and computational studies of selected model systems in the literature to explore the different features driving the catalytic promiscuity of such enzymes. Finally, on the basis of this comparative analysis, we probe the plausible origins and determinants of catalytic promiscuity in enzymes that catalyze phosphoryl and sulfonyl transfer.



Phosphoryl and sulfonyl transfer reactions are crucial to a wide range of biological processes.<sup>1–3</sup> In particular, phosphoryl transfer reactions play a central role in modulating cellular signaling processes, protein synthesis, and energy production (to name a few examples), whereas sulfonyl transfer reactions have also been implicated in cellular signaling pathways as well as in hormone regulation and cellular degradation.<sup>2</sup> Therefore, extensive research effort has been invested into understanding the mechanistic details of these processes as well as structure–function relationships among the enzymes that catalyze these reactions.

In solution, the rates of hydrolysis of both phosphate and sulfate monoesters are exceedingly slow,<sup>4–7</sup> with half-lives of up to millions of years, making the enzymes that facilitate these reactions some of the most proficient catalysts known to date. Moreover, despite superficial similarities in the geometries and kinetics of the uncatalyzed hydrolysis of the two esters, their physicochemical properties are quite distinct from each other.<sup>8</sup> Nevertheless, and despite these differences, it has been demonstrated that a large number of enzymes that are native catalysts of either phosphate or sulfate hydrolysis reactions can also catalyze the hydrolysis of the other substrate with varying degrees of proficiency,<sup>9,10</sup> and also that many of these enzymes show activity toward several other substrates in addition to phosphate and sulfate esters.<sup>10,11</sup>

Originally identified by Jensen in 1976,<sup>12</sup> this phenomenon, which is termed catalytic promiscuity, has become a topic of great interest in recent years, because of both its implications

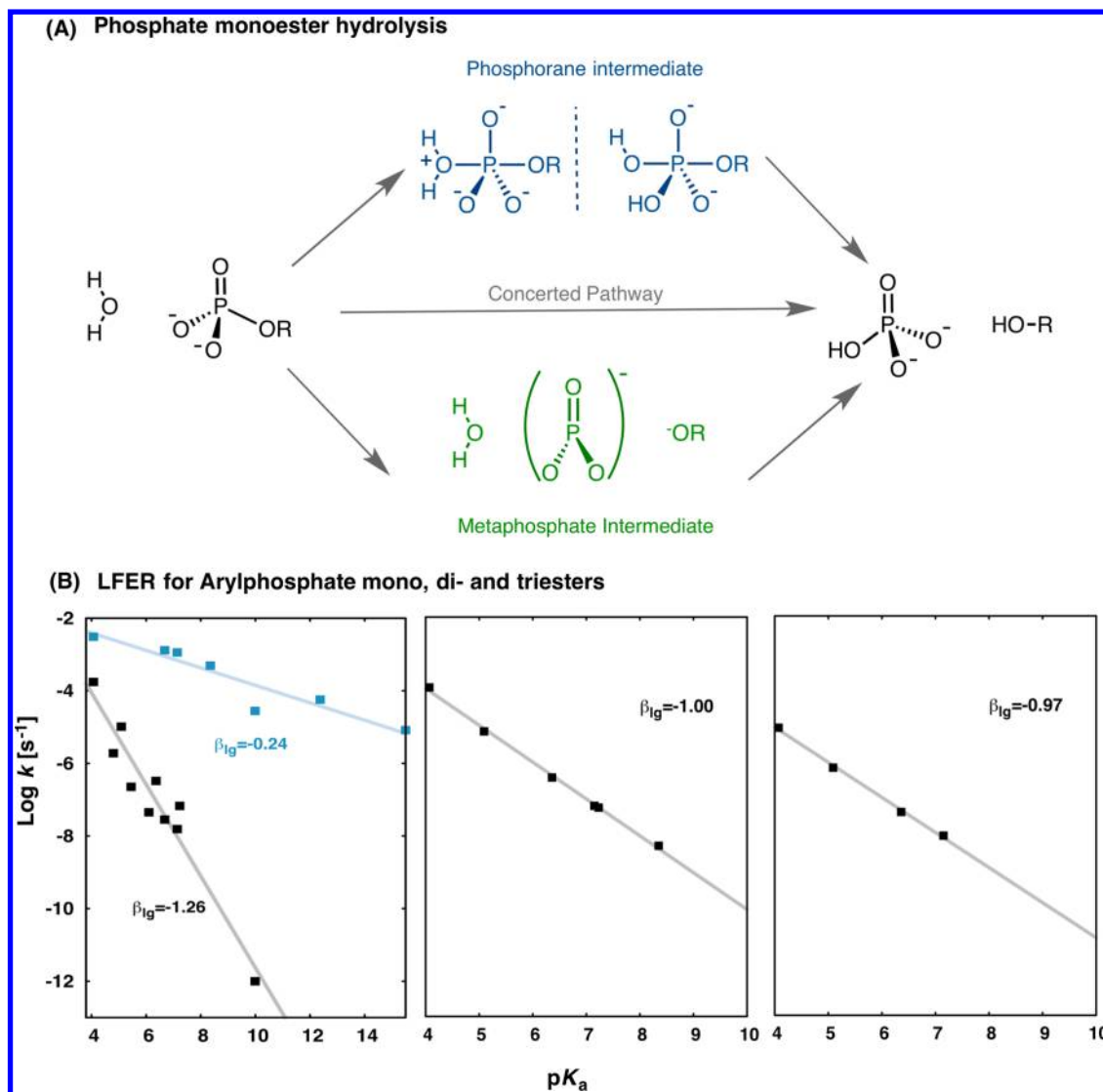
for understanding the evolution of enzyme function<sup>9,11</sup> and its utility for artificial enzyme design.<sup>13–15</sup> That is, these promiscuous side activities can provide a powerful starting point for the insertion of completely novel functionalities, as well as providing templates that can be used to learn how an enzyme acquires new catalytic abilities. It is important to note here that the term “promiscuity” is currently used to describe a wide range of different phenomena in enzyme catalysis, including condition promiscuity, substrate promiscuity, and catalytic promiscuity.<sup>11,16</sup> In this context, catalytic promiscuity can be understood as the ability of a single enzyme to catalyze multiple chemically distinct reactions, involving different bond making/breaking processes and proceeding through different transition states.<sup>16</sup> A number of detailed reviews have discussed various aspects of this phenomenon, including associated mechanistic issues,<sup>10,17</sup> evolutionary implications,<sup>11</sup> and its role in protein design.<sup>13–15</sup>

In this Current Topic, we will focus specifically on catalytic promiscuity among enzymes that catalyze phosphate and sulfate hydrolysis. As already demonstrated by O’Brien and Herschlag in 1999,<sup>9</sup> these enzymes are particularly prone to promiscuity, and a large number of experimental and theoretical studies of catalytically promiscuous phosphatases and sulfatases have provided valuable insights into not only the molecular origins of

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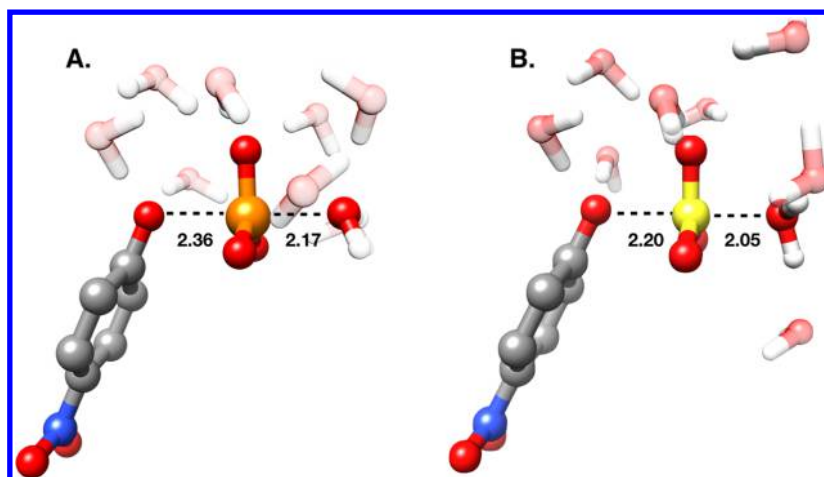


**Figure 1.** (A) Potential mechanisms for the hydrolysis of phosphate monoesters. (B) Experimentally observed linear free energy relationships for the spontaneous hydrolysis of phosphate monoester monoanions (blue, at 100 °C;  $\beta_{\text{lg}} = -0.24$ ) and dianions (black, 39 °C;  $\beta_{\text{lg}} = -1.26$ ),<sup>4,25</sup> phosphate diesters (39 °C;  $\beta_{\text{lg}} = -1.00$ ),<sup>26</sup> and phosphate triesters (39 °C;  $\beta_{\text{lg}} = -0.97$ ).<sup>27</sup>

promiscuity but also its role in the evolution of function within enzyme superfamilies.<sup>10,18,19</sup> Additionally, despite our primary focus on promiscuous phosphatases and sulfatases, we note that catalytic promiscuity is a phenomenon that is common to multiple enzyme superfamilies,<sup>11,20</sup> and many enzymes are capable of catalyzing radically different chemical reactions within the same active site. Examples of this include members of the mammalian paraoxonase (PONs) family, which are native lactonases with promiscuous esterase and/or phosphotriesterase (PTE) activities,<sup>21</sup> and members of the tautomerase superfamily [such as 4-oxalocrotonate tautomerase (4-OT)], which in addition to their isomerase activity can catalyze the breakdown of several different bonds, including C–H, C–C, and C–O bonds.<sup>22</sup> The multifunctionality observed in these systems suggests the existence of common features driving this phenomenon, including implications that the presence of multiple functional groups in the active site with charged or polar side chains or the presence of metal cofactors (which can increase the concentration of the deprotonated form of a nucleophile)<sup>11,16</sup> may enhance enzyme's ability to be catalytically promiscuous. Additionally, conformational flexibility has

also been suggested to play a role in enzyme promiscuity and evolvability.<sup>23,24</sup>

Clearly, understanding the factors shaping enzyme activity and functional evolution has broad implications for rational protein redesign, which has been one of the main driving forces for the recent explosion of interest in understanding catalytic promiscuity and enzyme multifunctionality.<sup>13–15</sup> In this Current Topic, we will start by discussing the mechanisms of uncatalyzed and enzyme-catalyzed phosphate and sulfate hydrolysis, to probe the chemical origins of catalytic promiscuity among phosphatases and sulfatases. Following from this, we will focus on a number of select model systems to explore the structural and catalytic features that allow these enzymes to accommodate multiple reactions within the same active site. Finally, on the basis of our comparative analysis, we will provide a summary of the most plausible origins of this phenomenon (catalytic promiscuity) and how they manifest among the different enzymes that are promiscuous catalysts of both reactions.



**Figure 2.** Comparison of calculated transition states (using density functional theory) for the hydrolysis of (A) *p*-nitrophenyl phosphate and (B) *p*-nitrophenyl sulfate, optimized in the presence of eight water molecules (water molecules made more transparent here for the sake of clarity). This figure was prepared on the basis of the coordinates provided in the Supporting Information of ref 8.

## MECHANISMS OF NONENZYMATIC PHOSPHATE AND SULFATE HYDROLYSIS

To understand the parameters that facilitate and shape enzymes' catalytic promiscuity, it is important to first understand the intrinsic reactivity of the different compounds undergoing chemical transformations in the enzyme-catalyzed reactions, i.e., how similar or different the chemical properties of these different substrates and their associated transition state geometries actually are. In this way, it is possible to map the origin of any potential changes in how different compounds and transition states are recognized and catalyzed by the same enzyme.

The overall reaction mechanisms for the hydrolysis of both phosphate and sulfate esters involve in-line nucleophilic displacement reactions, with differences of only a single atom or functional group between the substrates involved (Figure 1).

Therefore, superficially, the fact that there are many enzymes that can catalyze both classes of reactions in the same active site may appear to be trivial. Appearances can be deceptive, however. For example, the hydrolysis of just a simple phosphate monoester can proceed through multiple different reaction mechanisms (Figure 1), with the precise pathway depending on the nature of the leaving group, pH, and local electrostatic environment (for example, in an enzyme active site), among other factors. Therefore, even in the supposedly simple case of the uncatalyzed hydrolysis of phosphate monoesters, the fine mechanistic details of these reactions have been highly controversial.<sup>3,8,28</sup> This controversy has, in particular, focused on the nature of the transition states (associative vs dissociative and concerted vs stepwise processes), as well as on any proton transfer processes involved. This makes the enzymes that catalyze these reactions extremely diverse, and the mechanistic details of biological phosphoryl transfer appear to be highly dependent on the system.<sup>3,18,28,29</sup>

Despite this, there do appear to be some very clear structural differences between the general mechanisms of hydrolysis of different phosphate esters, depending on esterification level, and how these compare to the hydrolysis of their sulfate ester counterparts.<sup>3,28</sup> Linear free energy relationships (LFERs) correlating the rate of hydrolysis of different homologous substrates to leaving group or nucleophile  $pK_a$ , for instance, have shown steep leaving group dependence for aryl phosphate

monoester hydrolysis with a Brønsted coefficient,  $\beta_{lg}$ , of  $-1.26$ .<sup>4,25</sup> This suggests a high sensitivity to leaving group  $pK_a$  and hence a loose dissociative transition state. This is also further supported by the observation of a near-zero measured entropy of activation,<sup>30,47</sup> and, in the case of *p*-nitrophenyl phosphate hydrolysis, also large kinetic isotope effects (KIEs) on the bridging oxygen and the nitrogen atom, and an inverse KIE on the nonbridging oxygens.<sup>51</sup> Note, however, that computational studies have suggested a shift to a more associative transition state with poorer alkyl leaving groups, which are very difficult to study experimentally because of the exceedingly low reactivities involved.<sup>4</sup>

Corresponding studies of the mechanisms of uncatalyzed phosphate diester hydrolysis have been complicated by the fact that these reactions are extremely slow.<sup>32</sup> However, where studies have been possible, shallower  $\beta_{lg}$  values (in the range of  $-0.97$  to  $-1.16$  for neutral hydrolysis<sup>26,27</sup> and  $-0.64$  to  $-0.94$  under alkaline conditions<sup>33,34</sup>) and normal but slightly smaller values of  $^{18}k_{bridge}$  and  $^{15}k$  compared to those observed for monoesters<sup>35,36</sup> have been reported in the literature, suggesting a tighter and plausibly still concerted transition state compared to that for phosphate monoester hydrolysis. Finally, in the case of phosphate triester hydrolysis, where experimental studies are available,<sup>27,37,38</sup> both stepwise and concerted pathways have been suggested depending on reaction conditions and functional groups involved. In all cases, however, the associated transition states have been suggested to become tighter with leaving group basicity.<sup>38</sup> Thus, the mechanistic differences between the uncatalyzed hydrolyses of phosphate mono-, di-, and triesters can be quite significant in terms of the nature of the transition states involved. Therefore, in the case of promiscuous phosphatases that can hydrolyze multiple different types of phosphate ester in the same active site, active site plasticity is clearly required to complement the steric and electrostatic requirements of the different substrates. This is the case with alkaline phosphatase (R166S AP) and wild-type nucleotide pyrophosphatase/phosphodiesterase (NPP),<sup>39–41</sup> *Pseudomonas aeruginosa* arylsulfatase (PAS),<sup>42</sup> and phosphonate monoester hydrolases (PMHs),<sup>43</sup> among other systems.

Although they have been far less studied than their phosphate ester counterparts, recent years have also seen a revival of interest in physical organic studies of the mechanisms

**Table 1. Comparison of Experimentally Measured  $k_{\text{cat}}/K_{\text{M}}$  Values for a Number of Enzymatic Systems Catalyzing Phosphoryl and Sulfuryl Transfer<sup>a</sup>**

enzyme <sup>a</sup>	activity	$k_{\text{cat}}$ (s <sup>-1</sup> )	$K_{\text{M}}$ (M)	$k_{\text{cat}}/K_{\text{M}}$ (M <sup>-1</sup> s <sup>-1</sup> )	ref
AP <sup>b</sup>	phosphate monoesterase	$3.6 \times 10$	$3.7 \times 10^{-6}$	<b><math>3.3 \times 10^7</math></b>	67
	phosphate diesterase	nd <sup>c</sup>	nd <sup>c</sup>	$5 \times 10^{-2}$	68
	phosphonate monoesterase	nd <sup>c</sup>	nd <sup>c</sup>	$3 \times 10^{-2}$	68
	sulfatase	nd <sup>c</sup>	nd <sup>c</sup>	$1 \times 10^{-2}$	48
	phosphorothioate monoesterase	nd <sup>c</sup>	nd <sup>c</sup>	$2.0 \times 10^4$	69
	phosphorothioate diesterase	nd <sup>c</sup>	nd <sup>c</sup>	$1.1 \times 10^{-3}$	70
NPP <sup>b</sup>	phosphate diesterase	nd <sup>c</sup>	nd <sup>c</sup>	<b><math>2.3 \times 10^3</math></b>	54
	phosphate monoesterase	nd <sup>c</sup>	nd <sup>c</sup>	1.1	54
	phosphorothioate monoesterase	nd <sup>c</sup>	nd <sup>c</sup>	0.2	71
	phosphorothioate diesterase	nd <sup>c</sup>	nd <sup>c</sup>	4.8	71
	sulfatase	nd <sup>c</sup>	nd <sup>c</sup>	$2 \times 10^{-5}$	71
	phosphate diesterase	5.8	$6.3 \times 10^{-4}$	$9.2 \times 10^3$	56
PMH <sup>b</sup>	phosphonate monoesterase	2.7	$1.9 \times 10^{-4}$	<b><math>1.5 \times 10^4</math></b>	56
	sulfonate monoesterase	$1.2 \times 10^{-2}$	$2.4 \times 10^{-4}$	$4.9 \times 10$	56
	phosphate monoesterase	$7.7 \times 10^{-3}$	$3.5 \times 10^{-4}$	$2.2 \times 10$	56
	sulfatase	$4 \times 10^{-2}$	$6.8 \times 10^{-2}$	$5.6 \times 10^{-1}$	56
	phosphate triesterase	nd <sup>c</sup>	nd <sup>c</sup>	$1.6 \times 10^{-2}$	56
	sulfatase	$1.4 \times 10$	$2.9 \times 10^{-7}$	<b><math>4.9 \times 10^7</math></b>	58
AS <sup>b</sup>	phosphate diesterase	$5.5 \times 10^{-1}$	$2.2 \times 10^{-6}$	$2.5 \times 10^5$	57
	phosphate monoesterase	$2.3 \times 10^{-2}$	$2.9 \times 10^{-5}$	$7.9 \times 10^2$	58
	phosphonate monoesterase	nd <sup>c</sup>	nd <sup>c</sup>	<b><math>8.2 \times 10^2</math></b>	72
PAP <sup>b</sup>	phosphate monoesterase	$8.5 \times 10^2$	$2.2 \times 10^{-3}$	<b><math>3.9 \times 10^5</math></b>	73
	phosphate diesterase	$5.4 \times 10^2$	$3.6 \times 10^{-1}$	$1.5 \times 10^3$	73
	phosphate diesterase	2	$9.0 \times 10^{-4}$	<b><math>2.1 \times 10^3</math></b>	74
GpdQ <sup>b</sup>	phosphonate monoesterase	1.6	$1.3 \times 10^{-3}$	$1.2 \times 10^3$	74
	phosphate monoesterase	nd <sup>c</sup>	nd <sup>c</sup>	5	74
	phosphate diesterase <sup>b</sup>	$6.0 \times 10^{-2}$	$3.8 \times 10^{-2}$	1.6	75
PTE	phosphorothioate diesterase	$7.2 \times 10^2$	$1.5 \times 10^{-3}$	$4.8 \times 10^5$	76
	phosphonate diesterase	$3.9 \times 10^2$		$7.2 \times 10^5$	77
	phosphate triesterase <sup>b</sup>	$8.6 \times 10^3$	$2.0 \times 10^{-4}$	<b><math>4.3 \times 10^7</math></b>	75
	phosphate diesterase	$2.0 \times 10^{-2}$	$2.6 \times 10^{-3}$	8.3	78
MPH	phosphorothioate diesterase	$2.8 \times 10$	$2.7 \times 10^{-5}$	<b><math>1.0 \times 10^6</math></b>	79
	phosphate triesterase	$5.0 \times 10^{-2}$	$2.1 \times 10^{-3}$	$2.1 \times 10$	78
	esterase	$2.0 \times 10^{-3}$	$5.2 \times 10^{-4}$	3.4	78
	fluorophosphate esterase	$2.1 \times 10^2$	$3.8 \times 10^{-6}$	$5.6 \times 10^4$	80
DFPase	fluorophosphonate esterase	nd <sup>c</sup>	nd <sup>c</sup>	$7.2 \times 10^5$	80
	phosphate triesterase	3	$0.5 \times 10^{-6}$	$0.6 \times 10^4$	81
PON1	lactonase (dihydrocoumarine)	$1.5 \times 10^2$	$0.1 \times 10^{-3}$	<b><math>1.2 \times 10^6</math></b>	21
	lactonase ( $\delta$ -valerolactone)	$2.1 \times 10^2$	$0.6 \times 10^{-3}$	$3.7 \times 10^5$	21
	lactonase (TBBL)	$1.9 \times 10^2$	$1.1 \times 10^{-3}$	$1.7 \times 10^5$	82
	arylsulfatase (phenyl acetate)	$7.0 \times 10^2$	$1.2 \times 10^{-3}$	$5.9 \times 10^5$	21

<sup>a</sup>Shown here are examples of representative substrates for each enzyme. The most efficient activity for each enzyme is highlighted in bold. <sup>b</sup>Data obtained from ref 10 and references cited therein. <sup>c</sup>Not determined.

of sulfate ester hydrolysis.<sup>7,8,29,44–46</sup> In addition, there have been a number of direct experimental<sup>7,46</sup> and computational studies<sup>8,44</sup> focusing on the kinetics, activation entropies, kinetic isotope effects, and calculated transition states for the hydrolysis of these compounds. These studies have shown that both *p*-nitrophenyl sulfate and *p*-nitrophenyl phosphate have virtually identical reaction rates (they correspond to  $2.5 \times 10^{-9}$  s<sup>-1</sup> for the sulfate<sup>47</sup> at 39 °C vs  $1.6 \times 10^{-8}$  s<sup>-1</sup> for the phosphate monoester at 35 °C<sup>30</sup>) and also virtually identical kinetic isotope effects.<sup>31,36</sup> Unsurprisingly, therefore, the two transition states would be expected to be very geometrically similar to each other, which has been corroborated by computational studies,<sup>8</sup> although theoretical calculations found a slightly looser transition state for the hydrolysis of

the phosphate monoester (Figure 2), whereas experiment has predicted a much steeper  $\beta_{\text{lg}}$  value of  $-1.79$  for the hydrolysis of aryl sulfate monoesters (obtained from data extrapolated to 25 °C) compared to the value for their aryl phosphate counterparts.<sup>7</sup> In addition, the alkaline hydrolysis of diaryl sulfate diesters appears to proceed through transition states that are similar in geometry to those of the hydrolysis of its phosphate diester counterparts.<sup>49</sup> Therefore, mechanistically, the hydrolyses of corresponding phosphate and sulfate monoesters follow similar pathways with similar transition states, yet the enzymes that are capable of catalyzing both hydrolysis reactions very clearly discriminate between the two substrates. To understand the origins of the selectivity among these enzymes in a meaningful way, one should therefore



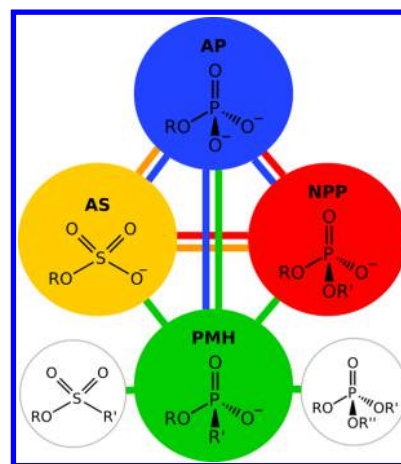
consider all available experimental markers of mechanism at the same time; however, despite the virtually identical reaction kinetics and KIEs for the hydrolysis of phosphate and sulfate monoesters, these compounds have shown very different activation entropies at  $-18.5$  and  $3.5$  e.u. for the hydrolysis of sulfate<sup>47,50</sup> and phosphate<sup>30</sup> monoesters, respectively (where 1 e.u. is equivalent to  $1 \text{ cal K}^{-1} \text{ mol}^{-1}$ ). Additionally, the differences in polarizabilities and S/P=O bond lengths between sulfur and phosphorus will lead to corresponding differences in charge distribution in otherwise apparently identical transition states (Figure 2).

This raises, therefore, two key questions: (1) How do these enzymes manage to discriminate between these two classes of substrate (which, although apparently very similar, are actually chemically different), and more importantly, (2) how do they even manage to accommodate these different substrates within the same active site? To explore this issue, the next section will discuss in detail the active site architectures and catalytic strategies utilized by a range of representative promiscuous phosphatases and sulfatases, highlighting both the commonalities and also some of the radical differences between these enzymes.

### ■ COMPARING SPECIFICITY AND PROMISCUITY PATTERNS IN REPRESENTATIVE PHOSPHATASES AND SULFATASES

**Alkaline Phosphatases.** The alkaline phosphatase (AP) superfamily is a family of structurally related metallohydrolases that primarily catalyze the hydrolysis of P–O, S–O, and P–C bonds. The members of this superfamily [which include the name-giving enzyme alkaline phosphatase (AP),<sup>51,52</sup> nucleotide pyrophosphatase/phosphodiesterase (NPP),<sup>53,54</sup> phosphonate monoester hydrolases (PMH),<sup>55,56</sup> and arylsulfatases (AS)]<sup>57,58</sup> exhibit broad catalytic promiscuity, with catalytic efficiencies toward their promiscuous substrates often reaching the efficiency observed for their native reactions<sup>10</sup> (Table 1). In addition to this, a high degree of cross-promiscuity is observed between the individual members of the AP superfamily, with a native substrate of one superfamily member often acting as a promiscuous substrate of another (Figure 3). Of particular interest to the topic examined herein is the ability of many members of this superfamily to hydrolyze both phosphate and sulfate esters in the same active site.<sup>56–58</sup>

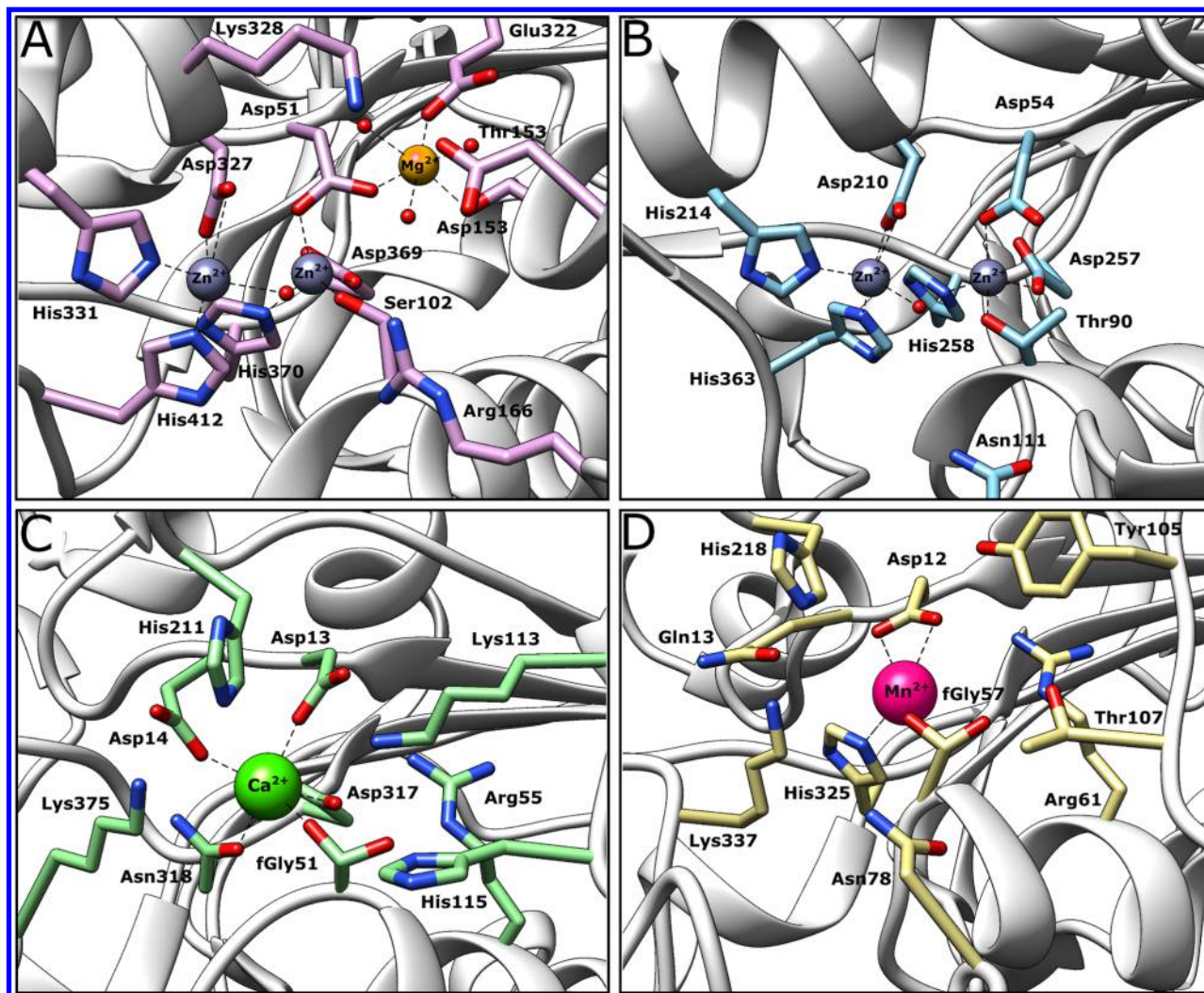
Despite limited sequence homology, the members of this superfamily share several common structural features and motifs. Specifically, they are generally globular, mixed  $\alpha/\beta$ -proteins, which are characterized by largely similar active site architectures.<sup>59</sup> They also show an absolute requirement for metal ions for their catalytic activities, employing a range of catalytic metal centers such as  $\text{Zn}^{2+}$ ,  $\text{Ca}^{2+}$ , and  $\text{Mn}^{2+}$ , as well as various alkoxide nucleophiles (serine, threonine, or formylglycine), to hydrolyze a broad range of phospho-, sulfo-, and phosphonocarbonyl substrates.<sup>10</sup> These systems have been extensively studied both experimentally<sup>55–58,60–62</sup> and computationally,<sup>17,39–43,63–66</sup> as a result of which the selectivity and specificity patterns of several individual members of this superfamily have been well-defined. However, even though their overall active site architectures (in terms of the availability and location of key ionizable residues, as well as their absolute dependence on catalytic metal centers) and substrate preferences are largely shared, the individual members of the AP superfamily differ between each other in their overall structure, choice of nucleophile, and specific metal require-



**Figure 3.** Schematic illustration of cross-promiscuity between selected members of the AP superfamily, where the native substrate of one enzyme (shown inside colored circles) is a promiscuous substrate of another (promiscuous activities represented by colored lines). The enzymes depicted here are alkaline phosphatase (AP), arylsulfatases (AS), nucleotide pyrophosphatase/phosphodiesterase (NPP), and a phosphonate monoester hydrolase (PMH). In addition to the main activities shown within the circles, BcPMH is thought to also hydrolyze phosphotriesters and sulfonate monoesters (an activity that is apparently not observed in other members of the AP superfamily that have been characterized to date). This figure was adapted from ref 10 and originally published in ref 17. Copyright 2013 Royal Society of Chemistry.

ments.<sup>19</sup> Detailed atomic-level analysis of the structural and electrostatic features of the individual superfamily members could therefore help to explain the differences in the specificity and promiscuity patterns observed within this superfamily and also aid in advancing our understanding of the structure–function relationships underlying the promiscuous behavior of these enzymes. This makes this superfamily a very attractive model system for studying the molecular basis for catalytic promiscuity, and its role in the evolution of phosphatase and sulfatase activities, and alkaline phosphatase, in particular, is treated as a prototype system for studying enzyme promiscuity.<sup>17,20</sup>

Figure 4 shows a comparison of the active site architectures of several key members of the AP superfamily. The catalytic scaffolds employed by these enzymes include the presence of one or more divalent metal ions that play a pivotal catalytic role, mainly through nucleophile activation (by lowering the  $pK_a$  of the alcohol nucleophile and thus increasing the concentration of the alkoxide available), as well as through positioning and activation of the substrate by polarization of the phosphate (or sulfate) ester bond, and stabilization of the negative charge buildup on the leaving group at the transition state,<sup>10</sup> all of which contribute to the remarkable rate enhancements observed for the reactions catalyzed by members of the AP superfamily (Table 1). We note here, however, that the substrates listed in Table 1 are all “generic” substrates for the different reaction classes with highly activated leaving groups, and thus, the rate of these enzymes toward their native/physiological substrates could be quite different, including even changes in rate-limiting step due to the use of highly activated substrates. The metal-coordinated alcohols/alkoxides, which serve as nucleophiles for the enzymes of this superfamily, are also highly reactive, which may result in low chemical selectivity and, in principle, promote the evolution of promiscuous



**Figure 4.** Comparison of the active sites of key members of the alkaline phosphatase (AP) superfamily, showing the catalytic architectures employed by these promiscuous enzymes. The figure illustrates the active sites of (A) alkaline phosphatase (AP, PDB entry 1ED9<sup>60</sup>), (B) nucleotide pyrophosphatase/phosphodiesterase (NPP, PDB entry 2GSN<sup>54</sup>), (C) *P. aeruginosa* arylsulfatase (PAS, PDB entry 1HDH<sup>95</sup>), and (D) a phosphonate monoester hydrolase from *Rhizobium leguminosarum* (PMH, PDB entry 2VQR<sup>55</sup>). Adapted from ref 17.

activities. In addition, a comparative study of alkaline phosphatase and three protein tyrosine phosphatases that do not use metal ions for catalysis has suggested that the positive charge of the metal ion is not the main driving factor for distinguishing between phosphoryl and sulfonyl transfer reactions.<sup>61</sup> However, this is in conflict with other studies that have demonstrated that binding of alternate metals can alter the activity levels of a metalloenzyme toward non-native substrates (including, also, organophosphate hydrolases from the metallo- $\beta$ -lactamase superfamily).<sup>83–94</sup>

Apart from the role that metal ions play in promoting catalysis in the AP superfamily, interactions between the enzyme and heavily charged substrates have also been considered to make a major contribution to the rate acceleration observed in these enzymes. For example, in the case of AP, it has been suggested that a significant part of the discrimination in favor of stabilizing the transition rather than ground state of the reaction can be attributed to the destabilizing effect of the ground state charge repulsion between the negatively charged phosphate monoester substrate and the anionic side chain of Ser102, which acts as a nucleophile in this reaction.<sup>51,52</sup> Specifically, it was proposed

through combined binding, structural, and spectroscopic studies, including a quantitative comparison of the binding affinities toward inorganic phosphate when Ser102 is mutated to glycine or alanine, that a substantial destabilization of the binding of the phosphate ester dianion occurs due to the electrostatic repulsion from the anionic Ser102 nucleophile. Such ground state electrostatic destabilization exists in the enzyme–substrate and enzyme–product complexes but is absent in the transition state. The AP active site was thus suggested to be able to recognize the transition state of the phosphoryl transfer in an exceptionally specific and strong way, which highlights a potentially important role for anionic nucleophiles in the catalysis of phosphoryl transfer reactions. Following from this, several other hypotheses have also been put forward to rationalize the origins and mechanisms of promiscuity in this superfamily, including observations of the similarities between the native and promiscuous reactions, the availability of reactive and spacious active sites, and the presence of a recyclable nucleophile in all cases.<sup>19</sup> In addition, conformational flexibility, as expressed by the ability of an enzyme to reshape its active site through the movement of



flexible active site loops, has also been suggested as a key factor promoting enzyme promiscuity.<sup>23,24</sup>

As outlined above, the substrates hydrolyzed by the AP superfamily often differ in their requirements for efficient catalysis, as they have diverse solvation and/or protonation patterns, and also differ in the natures of the transition states involved (associative vs dissociative, and whether intermediates are present along the reaction pathway). This, in principle, should have a significant effect on how these reactions are catalyzed in the corresponding active sites of the AP superfamily members. That is, assuming that these enzymes have evolved for efficient and optimal stabilization of the transition states for the hydrolysis of their native substrates, one would expect poor catalytic efficiencies toward transition states with different structural and electronic characteristics. However, as shown in Table 1, some of these superfamily members can catalyze their native and promiscuous substrates with <10-fold discrimination in  $k_{\text{cat}}/K_{\text{M}}$  and depending on the enzyme, either of these parameters can contribute to the overall changes in  $k_{\text{cat}}/K_{\text{M}}$  between the different substrates (note, however, that for some of these superfamily members such as alkaline phosphatase,  $k_{\text{cat}}$  represents a nonchemical step).<sup>96</sup> This gave rise to the question of how different the transition states in solution and the enzyme active site actually are, i.e., whether the enzyme modifies these transition states to be very similar to each other in its active site or whether the same active site can stabilize various diverse transition states.

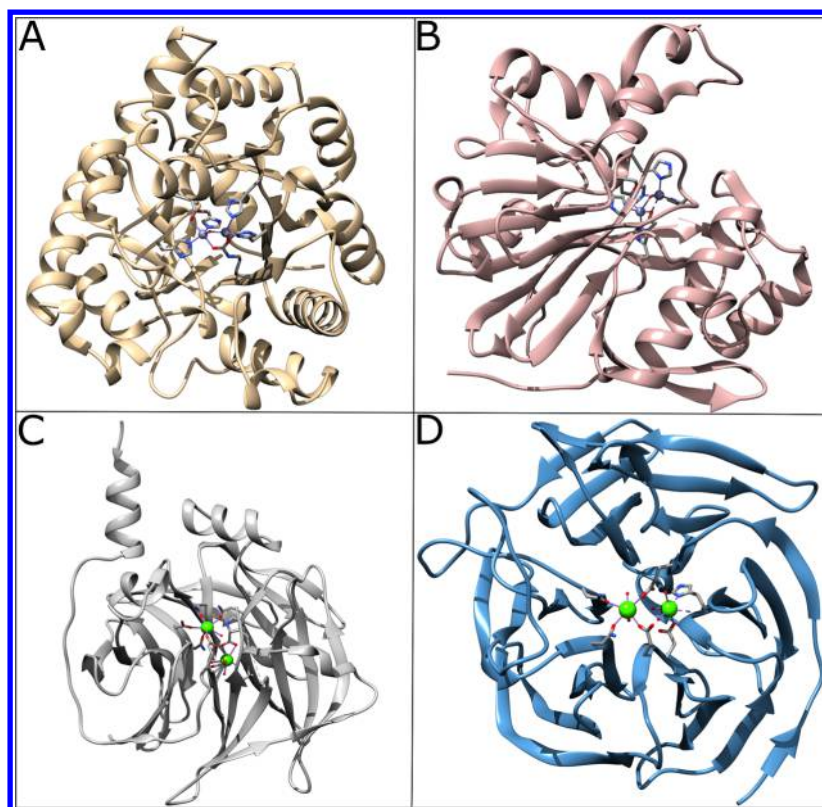
This hypothesis has been extensively explored through the use of both computational<sup>41,63,65,97</sup> and experimental approaches.<sup>34</sup> For example, linear free energy relationships (LFERs)<sup>34,98,99</sup> and kinetic isotope effects (KIEs)<sup>99,100</sup> obtained for both native and promiscuous reactions in the alkaline phosphatase active site suggested that, at least for this enzyme, the transition states of the catalyzed and uncatalyzed reactions are very similar to each other. Thus, the enzyme appears to be able to stabilize both the dissociative and associative transition states found in the hydrolytic pathways of the various reactions this enzyme catalyzes. Further computational studies characterizing the transition states for the hydrolysis of phosphate mono- and diesters by AP variants as well as nucleotide pyrophosphatase/phosphodiesterase (NPP) have presented a similar mechanistic picture.<sup>65</sup> That is, despite a slight tightening of the transition states observed for the enzymatic hydrolysis of phosphate diesters, the overall mechanisms were found to be practically unchanged when moving from the relevant reaction in solution to the enzyme's active site for a given substrate.<sup>41,65</sup> However, the transition states for different reactions were found to be quite different from each other.<sup>41</sup> The fact that both AP and NPP appear to be able to accommodate the different types of transition states found in phosphate mono- and diester hydrolysis, respectively, led the authors to suggest that the ability to recognize and bind differently charged substrates, accompanied by a high degree of solvent accessibility (i.e., active site plasticity), was an important factor shaping catalytic promiscuity in the AP superfamily.<sup>41,65</sup> Indeed, a similar phenomenon was observed in the case of another member of the superfamily, the arylsulfatase from *P. aeruginosa* (PAS),<sup>57,58</sup> where the active site plasticity is even more pronounced through the use of different general bases and reaction pathways for the different reactions catalyzed by this enzyme.<sup>42</sup>

A slightly different observation, however, was made in the case of the phosphonate monoester hydrolases from *Rhizobium*

*leguminosarum* (RIPMH) and *Burkholderia caryophili* (BcPMH).<sup>55,56</sup> The latter of these two enzymes is one of the most promiscuous hydrolases known to date, being able to catalyze at least five chemically distinct reactions, with the catalytic efficiency for the various substrates differing by up to 10<sup>5</sup>-fold.<sup>56</sup> This enzyme is structurally related to arylsulfatases, AP, and NPP and catalyzes the native reaction of all three classes of enzymes (see Table 1). In addition, these PMHs are the only non-sulfatases that have been characterized to date<sup>55,56</sup> that utilize an unusual formylglycine nucleophile generated by the post-translational modification of a cysteine or serine residue to an aldehyde, followed by hydration of this aldehyde to give a geminal diol.<sup>95</sup> This residue, which has been suggested to have both unique catalytic properties and biotechnological implication,<sup>101</sup> has also been proposed to be important to the promiscuity of these enzymes and related sulfatases, as it facilitates the degradation of all covalent intermediates formed during the hydrolyses of these compounds through a common pathway (hemiacetal cleavage), which is in turn much more energetically favorable than cleavage of a second P(S)–O bond through attack of water at the phosphate or sulfate intermediate.<sup>7</sup> Following from this, BcPMH is the only characterized enzyme that can degrade xenobiotic sulfonate esters through direct S–O bond cleavage.<sup>56</sup> The large binding site of this enzyme can potentially accommodate multiple substrates, in multiple binding modes, which could suggest that the most important factor underlying its high degree of promiscuity is the active site plasticity suggested for this and other members of the superfamily. However, a recent computational study of both RIPMH and BcPMH showed that in fact the transition states in various reactions catalyzed by two enzymes are very similar, despite differences in the shape and charge distribution of polarizability of individual substrates.<sup>43</sup> Hence, there seems to be no requirement for those enzymes to possess a high degree of active site structural plasticity. Instead, electrostatic flexibility, understood as the ability of the enzyme to adjust its electrostatic environment to meet the requirements of a specific substrate, seems to play a crucial role here.

Following from this, the clear dependence of the specificity patterns on the substrate charge determined for the phosphonate monoester hydrolases demonstrates that, in line with their well-established role in transition state stabilization,<sup>102,103</sup> electrostatic interactions are also important for determining the substrate specificity of those members of the AP superfamily. In addition, it has been observed that the same set of active site residues contributes to the hydrolysis of the various substrates catalyzed by PAS<sup>42</sup> and PMHs,<sup>43</sup> with contributions from individual residues varying quantitatively depending on the electrostatic requirements of a particular substrate. Such electrostatic cooperativity of the active site environment, which is related to the apparent catalytic backups proposed previously for a promiscuous organophosphate hydrolase serum paraoxonase 1,<sup>104</sup> could serve as an explanation for the differences in selectivity and promiscuity patterns for individual members of the AP superfamily. This electrostatic cooperativity between the active site residues in turn correlates with the electrostatic flexibility of a given active site and therefore appears to be the key factor underlying the catalytic promiscuity observed for these enzymes.

This observation was further corroborated by a detailed analysis of the structural and physicochemical properties of several other AP superfamily members, focusing in particular on



**Figure 5.** Comparison of the tertiary structures of four evolutionarily distinct organophosphate hydrolases, highlighting the different protein folds found among these enzymes, based on the structures available in the Protein Data Bank.<sup>132</sup> This figure depicts the structures of (A) the bacterial phosphotriesterase (PTE, PDB entry 1DPM<sup>133</sup>), (B) methyl parathion hydrolase (MPH, PDB entry 1P9E<sup>127</sup>), (C) serum paraoxonase 1 (PON1, PDB entry 1V04<sup>129</sup>), and (D) diisopropylfluorophosphatase (DFP, PDB entry 2GVV<sup>134</sup>).

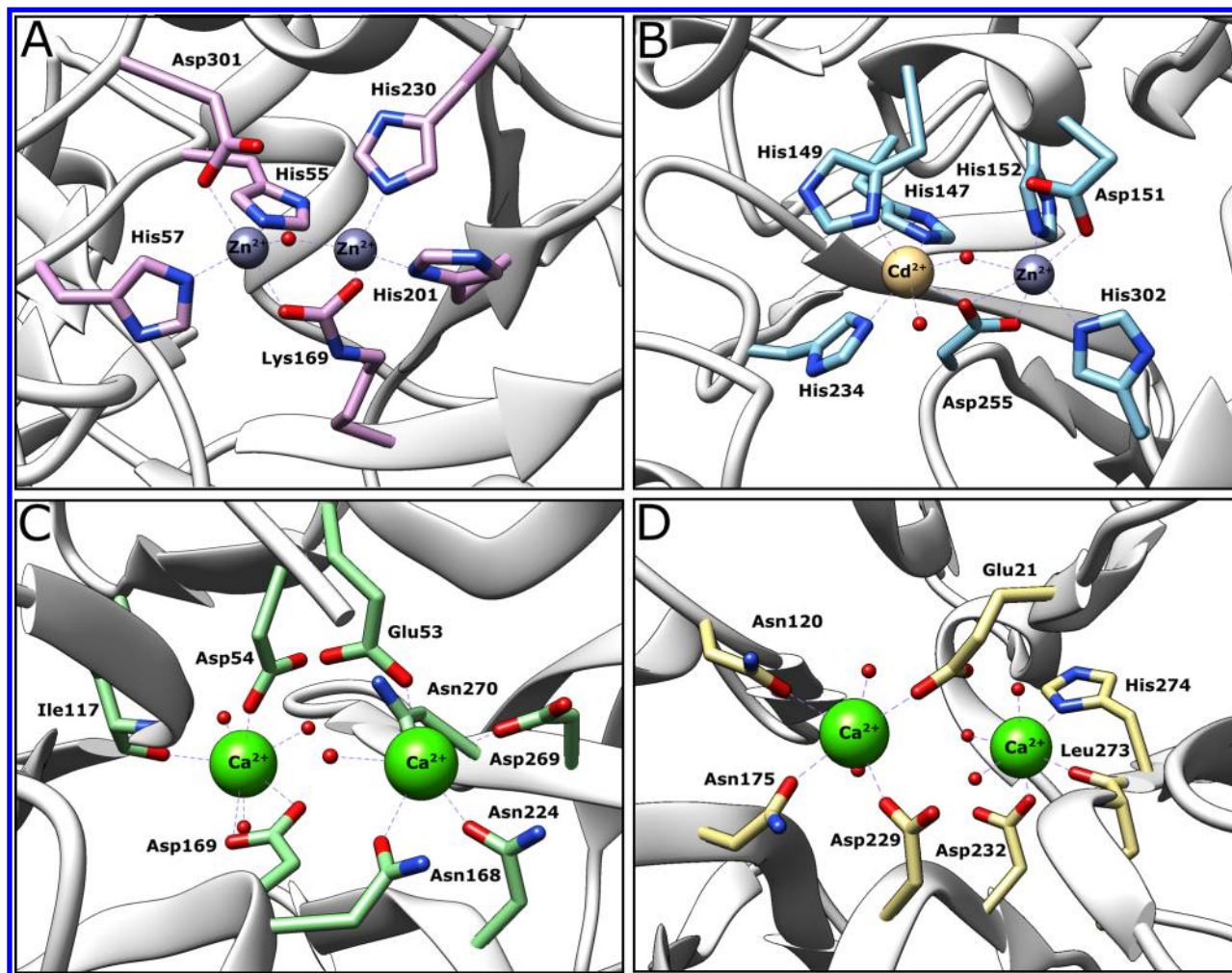
properties such as the active site volumes and polar solvent accessible surface areas (SASA) of these enzymes, and how they correlated with the number of promiscuous activities reported for each of the individual enzymes.<sup>43</sup> This study showed a general tendency for alkaline phosphatases with a more voluminous active site and a larger polar SASA to exhibit a broader spectrum of catalytic activities. This large active site, which is able to accommodate substrates of various shapes and sizes, appears to make an important contribution to the ability of these enzymes to be catalytically promiscuous. It should be noted, however, that a large active site volume is not sufficient for acquiring side activities on its own, because despite the potential ability to accommodate different substrates in multiple binding modes, the effective catalysis requires optimization of the productive binding conformations. If, however, a large binding pocket is at the same time characterized by a large polar surface, then the number of available electrostatic interactions allows the active site to adopt its electrostatic environment to various substrate requirements and ultimately bind and catalyze multiple, chemically different substrates. Hence, in this case, the prerequisite for these enzymes to be catalytically promiscuous appears to be that the number of electrostatic interactions available for transition state stabilization in the active site exceed the minimal number of interactions required for the stabilization of a given transition state (for more detailed discussion, see refs 43 and 105).

Finally, it should be noted that electrostatic interactions are not the only noncovalent interactions that can play a role in facilitating the turnover of various substrates by the AP superfamily. A large binding site can potentially allow for other

types of interactions, such as hydrophobic or hydrogen bonding contacts, that might be significant for substrate recognition. A good example of nonelectrostatic substrate discrimination is seen in NPP, where specific hydrophobic interactions with an ester functional group of the diester substrates make a major contribution to their preferential hydrolysis of these substrates.<sup>54</sup> Therefore, while it may be tempting to try to find a single-solution model for the promiscuity of these different enzymes, it is clear that even within an evolutionarily related superfamily, the observed specificity promiscuity is the interplay of multiple interrelated factors that manifest themselves in different ways in the different enzymes.

**Organophosphate Hydrolases.** Organophosphate pesticides, herbicides, and nerve agents pose a major human health hazard. These compounds are highly neurotoxic, as they inhibit the enzyme acetylcholine esterase, which plays an essential role in neurotransmission.<sup>106</sup> They are believed to be responsible for several hundred thousand fatalities worldwide annually, whether through accidental, suicidal, or malicious exposure.<sup>107</sup> As a result, the enzymes that can catalyze these reactions have been the focus of extensive research effort,<sup>108–111</sup> and enzymatic treatments for organophosphate poisoning are already under development.<sup>112</sup> In addition to their therapeutic applications, these enzymes are also interesting from a biochemical and evolutionary perspective, as they are able to hydrolyze human-made compounds that have been in widespread use only since the 1940s.<sup>113</sup> Therefore, these enzymes provide an excellent model system for understanding the parameters shaping enzyme functional adaptation and the





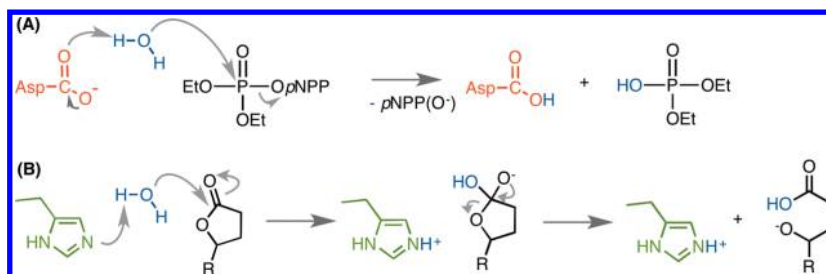
**Figure 6.** Comparison of the catalytic architectures of the four organophosphate hydrolases depicted in Figure 5, showing the first coordination spheres of the different bimetallic centers found in these enzymes. The figure illustrates the active sites of (A) the bacterial phosphotriesterase (PTE, PDB entry 1DPM<sup>133</sup>), (B) methyl parathion hydrolase (MPH, PDB entry 1P9E<sup>127</sup>), (C) serum paraoxonase 1 (PON1, PDB entry 1V04<sup>129</sup>), and (D) diisopropylfluorophosphatase (DFP, PDB entry 2GVV<sup>134</sup>). Shown here are also the crystallographic water molecules in the first coordination sphere of the metal ions.

complex structure–function relationships that determine how an enzyme chooses its preferred substrate(s).

Organophosphate hydrolases catalyze the hydrolysis of not only organophosphate nerve gases and pesticides but also cyclic and acyclic esters, among other substrates.<sup>114,115</sup> Therefore, they provide an interesting example of catalytic promiscuity involving phosphate hydrolysis. Additionally, and in contrast to the members of the AP superfamily, which share a conserved structural fold and similar catalytic mechanisms,<sup>20</sup> organophosphate hydrolase activity has convergently evolved from a broad range of evolutionarily unrelated enzymes.<sup>113,116</sup> This includes enzymes of both mammalian and bacterial origin.<sup>117–125</sup> In addition, and despite the chemical similarity of the reactions involved, the structures of these enzymes are highly diverse, encompassing a range of protein folds<sup>113</sup> (Figure 5 and Table 1), including TIM barrel folds (e.g., the bacterial phosphotriesterase, PTE<sup>126</sup>),  $\beta$ -lactamase folds (e.g., methyl parathion hydrolase, MPH<sup>127</sup>),  $\beta$ -propeller folds (e.g., DFPase<sup>128</sup> and serum paraoxonase 1, PON1<sup>129</sup>), and pita bread folds [e.g., the bacterial organophosphate acid anhydase (OPAA)<sup>130</sup>].

Figure 6, in turn, shows a comparison of the active site architectures of the different organophosphatases shown in Figure 5. From this figure, it can be seen that despite the overall structural diversity of these enzymes, they share many active site features in common both with each other and also to some extent with members of the AP superfamily (Figure 4). In particular, all these enzymes are metallophosphatases, and like AP/NPP from the AP superfamily, PTE, MPH, and OPAA possess two catalytic metal ions, which are on average 3.6 Å apart from each other (based on examination of available structures for these enzymes), with a bridging hydroxide ion located between the two metal centers. The native metal found in PTE is Zn<sup>2+</sup>, but high activity has also been found with Cd<sup>2+</sup>, Mn<sup>2+</sup>, or Ni<sup>2+</sup><sup>131</sup> (compared to Zn<sup>2+</sup> for MPH<sup>127</sup> and Mn<sup>2+</sup> for OPAA<sup>130</sup>).

PON1 and DFPase, in contrast, are Ca<sup>2+</sup>-dependent,<sup>104,135</sup> although DFPase has also been observed to be catalytically stimulated by Mg<sup>2+</sup><sup>136</sup> (the same does not hold true for PON1, which is instead inhibited by Mg<sup>2+</sup><sup>137</sup>). Both PON1 and DFPase have  $\beta$ -propeller folds with the active site located in the central tunnel of the  $\beta$ -propeller,<sup>128,129</sup> and while both enzymes have two metal ions in the central tunnel, they are 7.4 and 9.5 Å



**Figure 7.** (A) Mechanism for the general base-catalyzed hydrolysis of ethyl paraoxon (diethyl *p*-nitrophenyl phosphate), which has been suggested to proceed via a concerted pathway.<sup>142,143</sup> (B) Corresponding two-step mechanism suggested for the hydrolysis of lactones.<sup>144</sup>

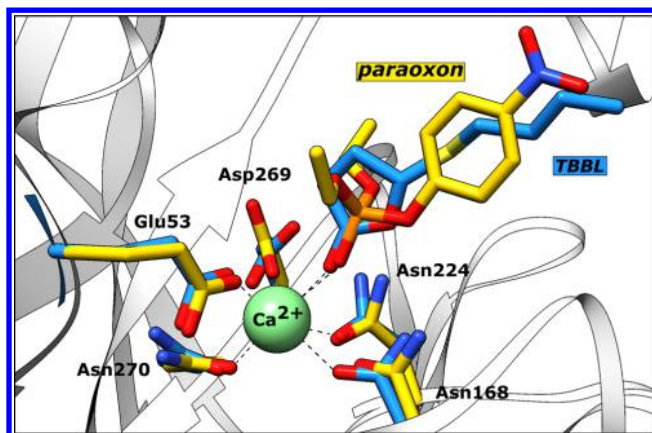
apart, respectively (again on the basis of examination of available structures for these enzymes), with only one of the two metals playing a catalytic role, and the other playing a crucial structural role instead.<sup>137,138</sup> Thus, their active sites are again reminiscent of members of the AP superfamily, especially so with PAS (Ca<sup>2+</sup>) and PMH (Mn<sup>2+</sup>), both of which utilize only a single metal ion in their active sites (Figure 4). However, unlike the members of the AP superfamily described above, these enzymes have an active site that is comparatively more hydrophobic in nature.<sup>139,140</sup> For example, via comparison of the different active site architectures and potential substrate positioning, there seems to be no analogue for the second metal ion or positively charged residue found in members of the AP superfamily, which plays a role in stabilizing the departing leaving group.

In addition to similarities in active site architecture and metal dependencies with those of AP members, the organophosphatases highlighted in Figures 5 and 6 also all have large binding pockets, allowing them to accommodate bulky organophosphate substrates, such as paraoxon. This is particularly important, as, where evolutionary analysis has been done on these systems, it appears that they have often evolved from a lactonase or related enzyme,<sup>21,113,115</sup> and many organophosphatases retain at least some level of lactonase and/or arylesterase activities.<sup>116</sup> Therefore, in contrast to alkaline phosphatases, in which both native and promiscuous substrates are hydrolyzed through similar in-line nucleophilic displacement mechanisms, here, the promiscuous substrates show completely different chemistries, which include the hydrolysis of not only organophosphate nerve gases and pesticides but also cyclic and acyclic esters.<sup>114,115</sup> That is, in the case of the organophosphatase activities, the reaction is again an in-line nucleophilic displacement reaction; however, in the case of the lactonase and arylesterase activities, the nucleophile instead attacks from a Bürgi–Dunitz angle of 102°<sup>141</sup> (Figure 7). Because of steric constraints, this creates a demand for significant structural plasticity to accommodate the geometric needs of both reactions in the same active site. Furthermore, it is possible that organophosphatase and lactonase activities are affected differently by the same mutation(s)<sup>11</sup> or stimulated differently by membrane association,<sup>82</sup> with even the possibility of multiple catalytic backups built into the same active site.<sup>104</sup> This then becomes a more extreme version of the electrostatic flexibility we have suggested for alkaline phosphatases,<sup>43</sup> because in addition to simple electrostatic backups in the active site, it means that multiple active site residues can play more than one catalytic role. At the same time, the same catalytic role can be played by multiple active site residues, complicating both the prediction and interpretation of mutational effects.

Despite these structural and mechanistic differences between the different classes of reactions catalyzed by these enzymes, there are still a number of significant similarities between substrate positioning and how the different reactions are catalyzed. First, unlike the members of the AP superfamily, which facilitate the catalysis of thermodynamically challenging P–O and S–O bond cleavage reactions,<sup>3,7</sup> the background hydrolysis of both organophosphates and arylesters/lactones is extremely fast, with rates typically on the order of 10<sup>–2</sup> s<sup>–1</sup> for the alkaline hydrolysis of organophosphates<sup>145</sup> and 10<sup>1</sup>–10<sup>–2</sup> s<sup>–1</sup> for lactones,<sup>146,147</sup> corresponding to activation barriers in the range of 15–19 kcal mol<sup>–1</sup> for lactones<sup>144,147</sup> (see also, e.g., refs 144 and 148–153, among others). Note that these values refer to the alkaline hydrolysis of these compounds, accounting for the fact that the nucleophile involved in the hydrolysis reactions catalyzed by the enzymes shown in Figure 6 is either a metal-activated hydroxide ion or a water molecule activated in a general base-catalyzed process. This makes the reactions involved much less demanding to catalyze on the part of the enzyme, reducing the evolutionary pressure on these systems, as the relevant reactions are already comparably fast even in the absence of an enzyme. Additionally, both compounds like paraoxon and the lactones hydrolyzed by these enzymes are neutral and hydrophobic substrates; thus, there is substantially less charge migration involved than in the hydrolysis of, for instance, a phosphate monoester dianion, and there are different catalytic requirements on the active site architecture. This partially explains the comparatively higher hydrophobicity of the active sites of these enzymes<sup>139,140</sup> (when compared to those of the members of the alkaline phosphatase superfamily). Finally, despite the differences in the angle of attack required for the nucleophile [and on the basis of examination of PON1 (Figure 8)], it is highly likely that the P(C)=O ester bonds of the substrate will fortuitously align perfectly on the metal,<sup>116</sup> and both sets of reactions have their ester bonds activated by the metal center in the same way, thus introducing greater chemical similarities between these substrates than would superficially be expected.

Following from this, in addition to being catalytically promiscuous, many enzymes are also metal promiscuous, with changes in the identity of the catalytic metal center playing a role in facilitating the switch from one activity to another.<sup>78,88,92,93,154–157</sup> Organophosphate hydrolases are no exception here, as several studies have demonstrated that metal ions play an important role in determining substrate selectivity among the enzymes that catalyze phosphoryl transfer. For example, Tawfik and colleagues have performed extensive biochemical, structural, and simulation analysis of both the wild-type enzyme and mutants in a catalytically crucial active site histidine, H115, in the PON1 active site,<sup>137</sup> and





**Figure 8.** Overlay of paraoxon and the chromogenic lactone substrate, TBBL, in the active site of serum paraoxonase 1 (PON1) after molecular dynamics simulations for 30 ns using the OPLS-AA force field,<sup>158</sup> extending the simulations described in ref 82. Despite the differences in the overall binding conformations of these two substrates, the P(C)=O ester bonds of these two substrates overlay almost perfectly, as also discussed in ref 116.

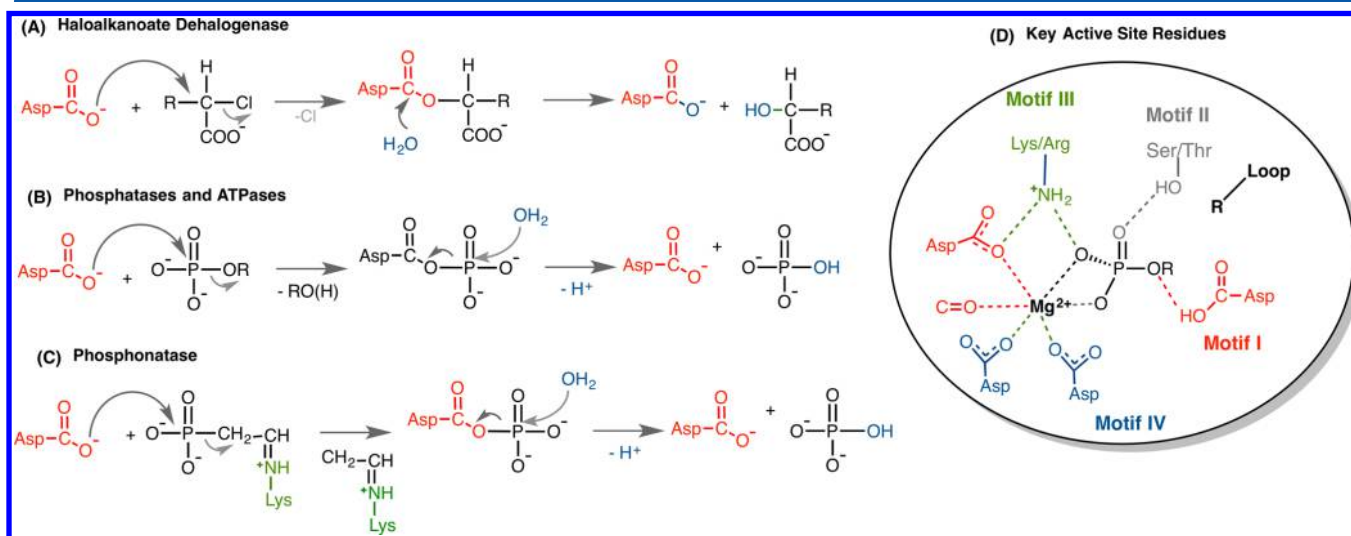
demonstrated that (1) mutations at this position diminish the lactonase activity of the enzyme while enhancing the organophosphate hydrolase activity and (2) these mutations are accompanied by substantial displacements of PON1's catalytic metal center. Therefore, they have argued that rearrangements in the catalytic metal ion affect not only the promiscuity but also the evolvability of the enzyme, in that the plasticity of the active site metal ions both permits the enhancement of latent promiscuous activities and provides a basis for the divergence of new enzymatic functions.<sup>137</sup> Following from this, Tokuriki and co-workers<sup>78</sup> have studied the effects of metal substitution on a broad range of promiscuous metallo- $\beta$ -lactamases (including methyl parathion hydrolase) and demonstrated clear metal-dependent specificity and promiscuity patterns upon comparison of different metal isoforms of the same enzyme. In addition, Warshel and colleagues have performed detailed empirical valence bond simulations of the bacterial phosphotriesterase<sup>159</sup> and quantified the effect of metal-metal distances

on the catalytic activity of PTE. Therefore, the identity and structural position of metal ions clearly appears to play a role in determining substrate specificity in these enzymes.

Finally, it is worth commenting on the role of substrate charge in substrate selectivity upon comparison of members of the AP superfamily and organophosphate hydrolysis. That is, despite differences in their native substrates, members of the AP superfamily preferentially hydrolyze mono- and dianionic substrates<sup>19,48,54,65</sup> (Table 1) and, where it has been measured, show minimal (or at least highly diminished) catalytic activities toward neutral substrates such as paraoxon or phenyl *p*-nitrophenyl sulfonate.<sup>56</sup> The converse is true for organophosphate hydrolases, which preferentially hydrolyze neutral organophosphates, lactones, and arylesters and show much lower activities toward anionic substrates [for example, the 10<sup>7</sup>-fold diminished phosphodiesterase activity of PTE<sup>75</sup> (Table 1)]. Thus, again, as was also the case for the enzymes in the AP superfamily,<sup>43</sup> the charge distribution at the transition state appears to be an important factor in discriminating between the different substrates.

**Haloacid Dehalogenases.** Haloacid dehalogenase hydrolases (HAD)-like represent one of the largest enzyme superfamilies characterized to date, with 33 major families distributed across the three superkingdoms of life.<sup>160</sup> Despite the fact that this superfamily is named after haloacid dehalogenases (C–Cl bond hydrolysis), most of its members are in fact involved in phosphoryl group transfer reactions,<sup>160</sup> including phosphonoacetaldehyde hydrolases (P–C bond hydrolysis), phosphomonoesterases (P–OC bond hydrolysis), ATPases (P–OP bond hydrolysis), and, to a lesser extent, phosphonatases and phosphomutases (such as  $\beta$ -PGM). HAD's biologically relevant substrates include sugars, nucleotides, organic acids, coenzymes, and small phosphodonors, which play a key role in primary and secondary metabolism, regulation of enzyme activity, cell housekeeping, and nutrient uptake.<sup>161</sup>

The members of the HAD superfamily that catalyze phosphoryl hydrolysis share some structural and functional similarities with the AP superfamily members and organophosphate hydrolases discussed above, including the absolute requirement for a catalytic metal ion and the use of an active site nucleophile to mediate the hydrolysis. However, in contrast



**Figure 9.** (A–C) Schematic representation of the different reactions catalyzed by HAD. (D) Key active site residues defining the different catalytic motifs.



to other superfamilies that use a wide range of catalytic metals and nucleophiles, all the HAD members use a highly conserved  $Mg^{2+}$  ion and an Asp residue. Here, the catalytic process is a two-step mechanism involving a covalent intermediate (Figure 9). In the first reaction step, the Asp nucleophile attacks the phosphoryl group and displaces the substrate leaving group. This is followed by a hydrolytic step, in which a water molecule attacks the phosphoaspartyl intermediate, releasing free phosphate and regenerating the catalytic Asp. However, in HADs that act as dehalogenases, there is no metal ion, and the nucleophilic attack of water takes place at the  $C=O$  group of the Asp residue rather than on the phosphoryl center (Figure 9A).

Extensive sequence analysis and crystallographic work has revealed that in addition to the conserved catalytic metal and nucleophile, practically all HAD-like members share four highly conserved sequence motifs that determine the catalytic machinery<sup>162</sup> (Figure 9D). These four motifs are located at the surface of a conserved Rossmannoid fold core domain, which also contain a mobile cap domain.<sup>163</sup> Motif I contains two Asp residues that coordinate the  $Mg^{2+}$  cofactor (one through the carboxylate group and the other one through the  $C=O$  backbone). The first Asp residue also acts as the nucleophile during the first step of the reaction. Phosphatases and mutases also contain a third conserved Asp residue that participates in general acid/base catalysis (protonating the leaving group in the first step and deprotonating the water nucleophile in the second).<sup>18</sup> In ATPases, the Asp that acts as a general acid/base in the reaction is replaced by a threonine, which acts instead as a hydrogen bond acceptor, but does not activate the nucleophilic water molecule. This leads to a reduction in the rate of aspartyl phosphate hydrolysis, which has been associated with a measured time lag needed to trigger a functionally important conformational transition that facilitates ion transport across the cell membrane.<sup>164</sup> Motifs II and III are in turn characterized by a highly conserved threonine/serine and lysine, respectively, which contribute to the stability of the reaction intermediates shown in Figure 9. Motif IV contains two conserved acidic residues, which, along with those in Motif I, coordinate the  $Mg^{2+}$  ion in the active site.

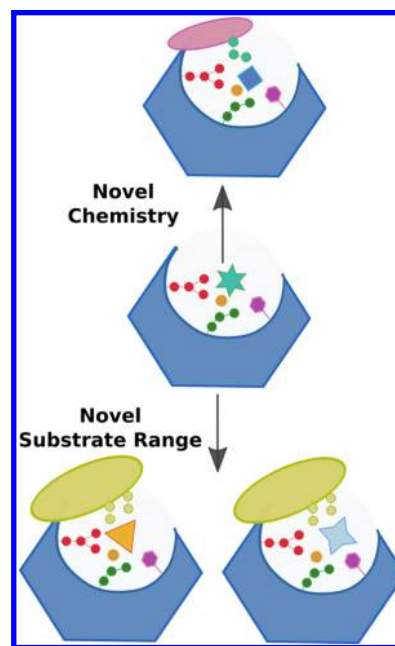
The position of insertion of the cap domain and its length provide a classification of the HAD members into three major structural classes (C0–C2).<sup>160</sup> the C0 members have the shortest or no inserts and are considered to be the primordial (older) HAD superfamily member.<sup>160</sup> C1 members have the cap insertion located between Motifs I and II and C2 members between Motifs II and III (Table 2).<sup>18,160</sup> It is hypothesized that the insertion of additional domains on a highly conserved core and on a very stable fold might have facilitated the acquisition of new functionalities (Figure 10). Examples of this are phosphonoacetaldehyde hydrolases, where the addition of a Lys residue (contributed by a cap domain near the active site) results in formation of a Schiff base, which provides the electron sink for catalysis of C–P bond cleavage.<sup>165</sup>

Given the dynamic properties of the cap domain, which can open and close during the catalytic process, it was previously assumed that C0 members possess a broader substrate range, due to the absence of residues that interact with the substrate leaving group, thus allowing it to vary in size, shape, and electrostatic surface.<sup>163</sup> However, more recently, it has been found that these cap domain insertions can, in fact, expand the substrate range by providing new interactions that can facilitate substrate binding.<sup>166,167</sup> For example, in the human cytosolic

**Table 2. Prominent Members of the Haloacid Dehalogenase Superfamily Sorted by Class and Reaction Type**

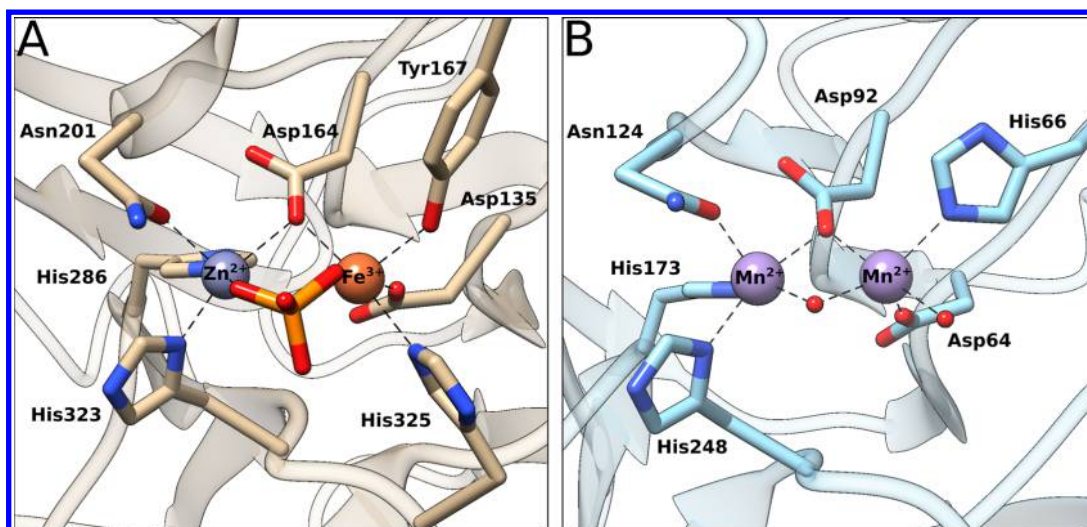
enzyme	cap	substrate
C–Cl Bond Cleavage		
2-L-haloalkanoic acid dehalogenase	C1	2-L-haloalkanoic acid
C–P Bond Cleavage		
phosphonoacetaldehyde hydrolase	C1	phosphonoacetaldehyde
CO–P Bond Cleavage		
phosphoserine phosphatase	C1	L-phosphoserine
mitochondrial 5'(3')-deoxyribonucleotidase	C1	dUTP
sucrose-6F-phosphate phosphatase	C2	sucrose-6F-phosphate
$Mg^{2+}$ -dependent phosphatase (MDP1)	C0	protein phosphotyrosine
8KDO phosphatase	C0	3-deoxy-D-manno-octulosonate 8-phosphate
CO–P Bond Cleavage/Formation (mutase)		
$\beta$ -phosphoglucomutase	C1	$\beta$ -glucose 1-phosphate II
phosphomannomutase	C2	$\alpha$ -mannose 1-phosphate
PO–P Bond Cleavage		
sarcoplasmic $Ca^{2+}$ -ATPase	C1	ATP
$Cu^{2+}/H^{+}$ -ATPase	C1	ATP

<sup>a</sup>Based on data provided in ref 18.

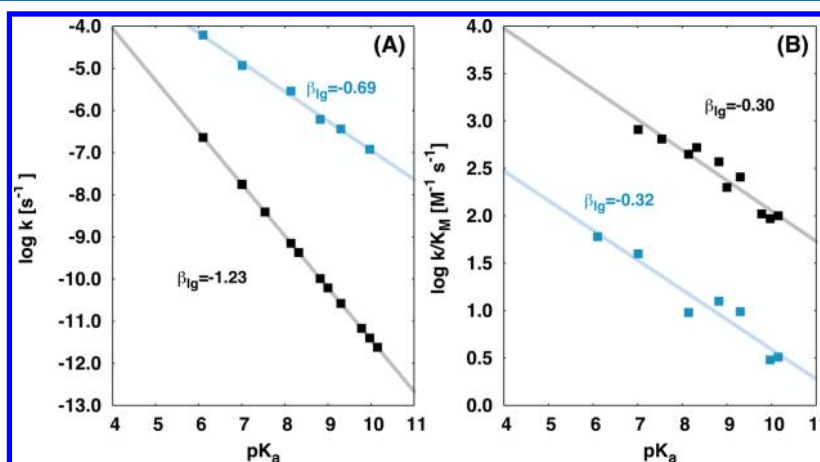


**Figure 10.** Acquisition of new functionalities via domain insertions. Addition of domain inserts into a highly stable core will introduce new stabilizing interactions that may lead to gain of novel chemistry or novel substrate range with simultaneous structural conservation of the core fold. This figure was adapted from ref 166. Copyright 2014 American Society for Biochemistry and Molecular Biology.

5'-nucleotidase II (cN-II) enzyme, which catalyzes the dephosphorylation of 5'-nucleotide monophosphates, different interactions between the cap domain and substrate were found to be present when either dGMP or UMP is the substrate.<sup>168</sup> This diversification of functionalities through cap insertion has been recently analyzed in prokaryotic organisms of this superfamily, using a customized library against >200 enzymes. More than 75% of these HAD members studied were found to



**Figure 11.** Comparison between the first coordination spheres of the catalytic metal centers in the active sites of (A) purple acid phosphatase (PAP, PDB entry 4KBP<sup>175</sup>) and (B) protein Ser/Thr phosphatase-1 (PP1, PDB entry 1FJM<sup>176</sup>).



**Figure 12.** Linear free energy relationships (LFERs) for (A) base-catalyzed hydrolysis of aryl methylphosphonates (black) and the spontaneous hydrolysis of phosphate monoester dianions (blue) and (B) protein phosphatase-1 (PP1)-catalyzed hydrolysis of methylphosphonates (black) and aryl phosphates (blue). Data taken from ref 72.

catalyze five or more substrates, thus confirming the high substrate promiscuity previously observed for members of this superfamily. Additionally, it was found that HAD members with minimal or no cap insertion (type C0) are equally efficient, but more specific than those with domain insertions (types C1 and C2),<sup>167</sup> suggesting that domain insertion can expand the chemical functionality and substrate range of these enzymes. This is associated with the lack of residues available in the C0 members to interact with different substrates. Conversely, in enzymes with cap domains, it is argued that the presence of extra residues might increase the number of interactions between the enzyme and substrates<sup>167</sup> and therefore their chemical scope. This superfamily contains both promiscuous and highly specific enzymes,<sup>169</sup> and therefore there are also examples of HAD members with this domain insertion that are highly specific.<sup>170</sup>

**Other Systems.** To conclude this section, we will briefly discuss three other promiscuous phosphatases, purple acid phosphatase (PAP), glycerophosphodiesterase (GpdQ), and protein phosphatase 1 (PP1); an overview of the active sites and catalytic activities is provided in Figure 11 and Table 1. As with the other metalloenzymes discussed to this point, these

three enzymes all have bimetallo active sites (Fe<sup>3+</sup> and either Zn<sup>2+</sup>, Mn<sup>2+</sup>, or Fe<sup>2+</sup> for PAP,<sup>171</sup> two Mn<sup>2+</sup> for PP1,<sup>172</sup> and Fe<sup>2+</sup>/Co<sup>2+</sup> and Fe/Zn<sup>2+</sup> for GpdQ<sup>173,174</sup>) and use an activated water molecule as a nucleophile. Once again, the metal coordination of GpdQ is very similar to that of MPH (Figure 6) and other metallo-β-lactamases, with a carboxylate bridging the two metal ions. Despite all three enzymes possessing bimetallo active sites with similar metal-coordinating ligands, PAP and PP1 are both phosphomonoesterases, with low levels of phosphodiesterase and phosphonate monoesterase activity, respectively,<sup>72,73</sup> whereas as the name suggests, GpdQ is a phosphodiesterase with equally high phosphonate monoesterase activity (in terms of  $k_{\text{cat}}/K_{\text{M}}$ ), similar to those of R1PMH and BcPMH from the AP superfamily, and very low phosphomonoesterase activity.<sup>74</sup> While the differences between the metal ions used in the active sites of these enzymes clearly play a role in the differences in specificity, PAP, PP1, and GpdQ are overall structurally very similar and belong to the functionally diverse α/β-sandwich family comprising monomeric or dimeric enzymes with two transition metals in the active site.<sup>174</sup>

Interestingly, Brønsted analysis of the hydrolysis of arylphosphate and phosphonate monoester substrates, as well

as heavy atom kinetic isotope effect studies of the hydrolysis of *p*-nitrophenyl phosphate and methyl-*p*-nitrophenyl phosphonate, has suggested that the structural and electrostatic features of the PP1 active site have a substantial effect on the transition states for the hydrolysis of these compounds.<sup>72</sup> That is, while the hydrolysis of aryl phosphate monoesters would be expected to proceed through much more dissociative transition states in aqueous solution, with a tighter transition state for the hydrolysis of their phosphonate counterparts, it appears that PP1 makes the transition state for the hydrolysis of the phosphonate monoester much tighter and almost diester-like, with a slightly looser but similar transition state for the hydrolysis of the corresponding phosphate monoester. Thus, the transition states for the two compounds appear to be much more similar to each other in the PP1 active site than in the corresponding uncatalyzed reaction in aqueous solution (Figure 12), which is similar to our computational observations for the reactions catalyzed by R1PMH and BcPMH<sup>43</sup> but quite different from, for example, the observations for alkaline phosphatase, which appears to easily accommodate multiple transition states in the same active site.<sup>34,41,48,61,68,69,97,177</sup>

In comparison to this, PAP is flexible in both its choice of catalytic metal center ( $\text{Fe}^{3+}/\text{M}^{2+}$ , where  $\text{M}^{2+} = \text{Fe}^{2+}$  in animal or  $\text{Zn}^{2+}/\text{Mn}^{2+}$  in plant PAPs) as well as in its corresponding choice of mechanism, allowing for greater flexibility in substrates catalyzed by this enzyme. Similarly, members of the glycerophosphodiesterase (GDPD) family of enzymes show catalytic activity with a wide range of divalent and trivalent metal ions, including  $\text{Zn}^{2+}$ ,  $\text{Mn}^{2+}$ ,  $\text{Fe}^{2+}$ , and  $\text{Fe}^{3+}$ ,<sup>174,178,179</sup> and the structure of GpdQ shows that while the  $\alpha$ -metal site of this enzyme is fully occupied the  $\beta$ -metal ion site is only partially occupied and the enzyme is catalytically active in the presence of both  $\text{Co}^{2+}$  ( $k_{\text{cat}}/K_{\text{M}} = 1.2 \times 10^3 \text{ M}^{-1} \text{ s}^{-1}$ ) and  $\text{Zn}^{2+}$  ( $k_{\text{cat}}/K_{\text{M}} = 3.7 \times 10^1 \text{ M}^{-1} \text{ s}^{-1}$ ).<sup>174</sup> Finally, comparison of GpdQ with other metallophosphodiesterases such as the novel phosphodiesterase from *Methanococcus janacchia*<sup>180</sup> and the glycerophosphodiesterase from *Agrobacterium tumefaciens*<sup>181</sup> shows a high degree of conservation of the central catalytic domain of these enzymes, but with structurally unrelated secondary domains at the entrance of the active site.<sup>174</sup> It has been suggested, therefore, that this is a common structural feature used by metallo-phosphodiesterases to constrain substrate specificity, thus preventing nonspecific phosphodiester hydrolysis.<sup>174</sup>

## OVERVIEW AND CONCLUSIONS

The focus of this Current Topic has been to explore the origin of catalytic discrimination between phosphate and sulfate hydrolysis in enzymes that can promiscuously catalyze both reactions, although we have also discussed other examples of promiscuous catalysts of phosphoryl transfer reactions for comparison. Since the rebirth of interest in catalytic promiscuity in the late 1990s,<sup>9</sup> there has been an explosion of studies addressing this topic from both experimental<sup>10,11,13–15,18,56,61,78,166,167,169</sup> and computational<sup>17,39–43,63,66,159</sup> angles. Because of their propensity for being catalytically promiscuous, the multitude of enzymes that are either native or promiscuous phosphatases and sulfatases have been at the center of the resurgence of interest in this topic.<sup>10,17–19,105</sup> Following from this, a broad range of hypotheses have been put forward to explain the origins of this phenomenon, which we will briefly summarize here.

The first and most obvious hypotheses have focused on the structural aspects of selectivity and promiscuity and, in particular, the role of conformational diversity and structural plasticity in allowing for both enzyme multifunctionality and substrate discrimination.<sup>11,23,41–43,57,114,182</sup> Specifically, the basis of this idea is that, in contrast to the traditional “one sequence—one structure—one function” paradigm,<sup>182</sup> one protein sequence can lead to multiple structures and functions.<sup>182</sup> This, in turn, has been suggested to expand the functional repertoire of proteins, as well as allowing for greater protein evolvability, as it not only facilitates the divergence of new functions within existing protein folds but also allows for the evolution of completely new protein folds within the same catalytic scaffold.<sup>160,163</sup> In addition, it has been argued that the greater the conformational diversity and flexibility (e.g., in the case of intrinsically disordered proteins), the greater the likelihood of the acquisition of new function.<sup>23,182</sup>

We have presented in this Current Topic examples of both the divergent and convergent evolution of promiscuous phosphatase and sulfatase activity, and it can be seen from the examples discussed (in particular in the case of organophosphate hydrolases) that despite some underlying structural themes, the link between the overall tertiary (or quaternary) structure and substrate selectivity is not immediately obvious. For example, there have been several structures linking the flexibility of conformational loops to active site reshaping and substrate selectivity.<sup>163</sup> Following from this, we have provided examples in which the conformational flexibility of side chains rather than the protein backbone leads to adaptable cooperative interactions between the different active site residues, resulting in a propensity for catalytic promiscuity but also dictating the discrimination between these different substrates.<sup>43</sup> In addition, there have been several studies discussing the link between protein conformational flexibility and evolvability,<sup>23,183–187</sup> and we have also recently demonstrated a direct link between correlated motions and changes in catalytic activity in engineered variants of a biocatalytically important aldolase, DERA.<sup>188</sup> Such conformational diversity has been suggested to be evolutionarily important in many other contexts, as well, for example, in the binding promiscuity of PDZ domains,<sup>189</sup> and in discriminating between neutral and disease-related single-amino acid substitutions.<sup>190</sup> Following from this, conformational diversity has been shown not only to modulate sequence divergence<sup>191</sup> but also to correlate with the evolutionary rate of proteins.<sup>192</sup> In even more examples, the molecular evolution of protein conformational changes has been linked to networks of evolutionarily coupled residues,<sup>193</sup> and in the case of  $\beta$ -lactamases, the evolution of conformational dynamics has been suggested to be important in the conversion of these proteins from ancestral generalist to modern specialist enzymes.<sup>184</sup> Clearly, therefore, a large body of work implicating the importance of conformational diversity and protein flexibility in protein evolution exists, and even if it does not necessarily provide the full picture of how an enzyme can discriminate between two different reactions it is able to catalyze, it at least provides a compelling rationale for how some proteins can accommodate so many distinct reactions in their active sites in the first place, and how they can evolve so quickly. It is also of direct relevance to the systems of interest to this Current Topic, as it has been suggested that interconnected networks of amino acids with distinct functional modes of cooperativity are important in determining the function in alkaline phosphatase.<sup>62</sup>



While structural factors can explain how these enzymes can accommodate different substrates in the first place, the question of how they discriminate between phosphate and sulfate transfer remains, in particular in light of the apparent similarity between the transition states for the two reactions.<sup>3,8,31</sup> This is particularly relevant because of both the cross-promiscuity between the two reactions, such that many native phosphatases carry promiscuous sulfatase activity<sup>10</sup> (and vice versa), and the fact that, despite the apparent chemical similarities between the two substrates, native sulfatases tend to be far more proficient phosphatases than native phosphatases are sulfatases (e.g., the examples listed in Table 1). We have discussed herein representative examples of the broad wealth of experimental (and, in particular, kinetic and biochemical) characterization of these systems, combined with computational studies, and demonstrated that there is no “one size fits all” answer to this question, as even in the same superfamily, there appears to be diversity in the role of metal ions, the nature of the different transition states in the same enzyme, and how the different enzymes use trade-offs between plasticity and rigidity to stabilize the different transition states. For example, while alkaline phosphatase appears to be able to flexibly accommodate multiple transition states within the same active site,<sup>34,41</sup> and only minimally perturbs their structure compared to those of the uncatalyzed counterparts,<sup>34,41</sup> other enzymes such as PP1 or BcPMH appear to substantially modify the transition states of the enzyme-catalyzed reaction to resemble each other far more than they resemble the corresponding uncatalyzed transition states.<sup>72</sup> Therefore, any links between transition state size [defined by the sum of the P(S)–O distances to the nucleophile and leaving group] and selectivity patterns appear to be tenuous at best for general applicability, because of the variations seen among these different promiscuous phosphatases and sulfatases.

The main unifying factor among these different enzymes, however, appears to be discrimination on the basis of substrate charge. As just three examples, BcPMH preferentially hydrolyzes monoanionic phosphate diesters and phosphonate monoesters and shows much lower catalytic activity with phosphate monoester dianions and minimal activity toward neutral organophosphates and sulfonates.<sup>56</sup> In contrast, PTE is a highly proficient organophosphatase and easily accommodates other neutral substrates such as paraoxon and parathion (Table 1)<sup>76</sup> but shows greatly diminished activity toward monoanionic phosphodiester.<sup>75</sup> Finally, the bacterial arylsulfatase from *P. aeruginosa*, PAS, shows comparably similar activity toward the *p*-nitrophenyl sulfate monoester and the much bulkier bis-*p*-nitrophenyl phosphate diester, which share the same monoanionic charge, and much lower activity toward the dianionic *p*-nitrophenyl phosphate monoester, despite the similarities in geometry between the two substrates.<sup>57</sup> This charge discrimination agrees with studies of magnesium and aluminum fluoride transition state analogues for different phosphoryl transfer enzymes, which show these enzymes to preferentially discriminate by charge rather than transition state analogue (TSA) geometry.<sup>194</sup> We have also shown in the case of BcPMH that differences in measured  $k_{\text{cat}}/K_{\text{M}}$  with different substrates can be directly linked to the amount of change in charge upon moving from the Michaelis complex to the transition state for the different reactions this enzyme catalyzed.<sup>43</sup>

On the basis of this, it is highly plausible that the geometric similarities between the two substrates are what allow their

binding to the same active site, whereas the subtle difference in charge distribution at their respective transition states (relative to the Michaelis complex) due to the differences in polarizability of phosphorus versus sulfur is the main reason for the discrimination between the two substrates. In addition to this, experimental and computational studies of the corresponding uncatalyzed reaction show that sulfate hydrolysis is, in general, a much more difficult reaction to catalyze than phosphate hydrolysis.<sup>7,8,42</sup> This is due to the more limited mechanistic possibilities for sulfate than for phosphate hydrolysis, as well as the fact that the sulfate esters are mono- rather than dianionic and thus have a stronger requirement for precision in the binding of the less charged substrate. This is in contrast to the case for phosphate monoesters, where strong electrostatic interactions between the heavily charged phosphate and surrounding enzyme may be enough to account for the tremendous rate acceleration. Thus, it is perhaps not surprising that native sulfatases are far more proficient phosphatases in relative terms than the other way around. That is, once an active site has been optimized to facilitate sulfate hydrolysis, the same catalytic machinery can then be easily extended to accommodate the phosphate ester counterparts. However, an active site that has evolved to facilitate phosphate hydrolysis it not necessarily suitable also for sulfate hydrolysis (in part because of the greater mechanistic versatility of phosphate than sulfate esters, and the fact that the enzyme could therefore be using a mechanistic solution that is not available to the sulfate ester counterpart).

Finally, the key conclusion that can be drawn from extensive structural studies of the different enzymes that catalyze phosphate and sulfate hydrolysis<sup>41,43,59,62,104</sup> is that active site architecture is crucial in facilitating promiscuity, in that once there are a greater number of available interactions than there are necessary interactions to stabilize the transition state of the native reaction, an enzyme can much more easily also be a promiscuous catalyst of other substrates and/or reaction classes. This is then further supported by the apparent structural and/or electrostatic flexibility of the active sites of many of these promiscuous enzymes,<sup>41,43,59,62,104</sup> which will facilitate the binding of a larger number of substrates and transition states, at the expense of specificity in binding interactions. In addition, as suggested by a reviewer of this Current Topic, there could plausibly exist evolutionary pressure to create such a scenario, as it leads to some redundancy in the active site, which could prevent the failure of the enzyme as a consequence of a single mutation (see, for example, the discussion of catalytic backups and redundancies in the case of serum paraoxonase 1<sup>104</sup>). Therefore, overall, it would appear that catalytic promiscuity in enzymes that catalyze phosphate and sulfate hydrolysis is an opportunistic phenomenon, with “piggy-backing” promiscuous substrates exploiting the architecture that already exists for catalyzing the native reaction, but yet with ultimate substrate discrimination on the basis of active site electrostatics. In addition, because of structural and electrostatic flexibility, once bound, these substrates can potentially “mold” the active site in different ways due to enzyme–substrate interactions, which we appear to observe in the case of phosphonate monoester hydrolases<sup>43</sup> and has also to some degree been observed in the case of serum paraoxonase 1.<sup>104</sup> Overall, this is clearly a highly complex problem, but the superficial similarity between these two class of substrates and their nonlinear mapping onto enzyme selectivity patterns can teach us a lot about the subtle balance among flexibility, active

site architecture, and electrostatics that leads an enzyme to choose its mechanism.

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## ABBREVIATIONS

AP, alkaline phosphatase; DFP, diisopropyl fluorophosphate; DFPase, diisopropyl fluorophosphatase; dGMP, deoxyguanosine monophosphate; GpdQ, glycerophosphodiesterase; HAD, haloacid dehalogenase hydrolase; KIE, kinetic isotope effect; MPH, methyl parathion hydrolase; LFER, linear free energy relationship; NPP, nucleotide pyrophosphatase/phosphodiesterase; OPAA, organophosphate acid anhydrase; PAP, purple acid phosphatase; (P)AS, (*P. aeruginosa*) arylsulfatase; PDB, Protein Data Bank; PMH, phosphonate monoester hydrolase; PON1, serum paraoxonase 1; PP1, protein phosphatase 1; PTE, phosphotriesterase; TBBL, thiobutyl butyrolactone; TIM, triosephosphate isomerase; TSA, transition state analogue; UMP, uridine monophosphate.

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