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Promiscuity and electrostatic flexibility in the alkaline phosphatase superfamily

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Catalytic promiscuity, that is, the ability of single enzymes to facilitate the turnover of multiple, chemically distinct substrates, is a widespread phenomenon that plays an important role in the evolution of enzyme function. Additionally, such pre-existing multifunctionality can be harnessed in artificial enzyme design. The members of the alkaline phosphatase superfamily have served extensively as both experimental and computational model systems for enhancing our understanding of catalytic promiscuity. In this Opinion, we present key recent computational studies into the catalytic activity of these highly promiscuous enzymes, highlighting the valuable insight they have provided into both the molecular basis for catalytic promiscuity in general, and its implications for the evolution of phosphatase activity.

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Introduction

The classical image of enzyme catalysis is that enzymes are highly specific, with each enzyme having exquisitely evolved to facilitate the turnover of a single substrate. There is an increasing body of evidence, however, that suggests that many (if not even most) enzymes are 'catalytically promiscuous', facilitating multiple, chemically distinct reactions within the same active site [1••]. Such promiscuity has been suggested to be important both for the *in vivo* evolution of enzyme function [2,3], as well as for artificial enzyme design [1••], as it provides a starting point for the accelerated acquisition of novel functionality. However, despite progress in this area (for reviews, see e.g. Refs. [1••,4,5]) our understanding of the underlying mechanistic basis for this promiscuity remains elusive. Here, the alkaline phosphatase (AP) superfamily provides a particularly attractive model system for both *in vitro* and *in silico* studies of enzyme promiscuity, as the individual superfamily members are not only catalytically promiscuous, but also exhibit crosswise promiscuity, catalyzing each other's native reactions [6•]. Although the vast bulk of work on this superfamily has been experimental (e.g. Refs. [7–18], among others), in recent years, computational studies have also started to make significant contributions to our insights into the molecular basis for the promiscuity of these enzymes [5,19••,20•,21,22,23••,24]. We have recently invested significant effort into exploring how both electrostatic cooperativity between different active site residues (where we define cooperativity as the electrostatic effect of changing two or more residues at once as being different from the sum effect of changing the individual residues) and the corresponding electrostatic flexibility such cooperativity provides affect enzyme specificity and promiscuity (e.g. Refs. [21,24]). In this manuscript, we will provide a review of some of the recent computational work by both ourselves and others, and illustrate the mounting body of evidence that electrostatic flexibility is a key driving force for catalytic promiscuity (and thus ultimately functional evolution) among not just alkaline phosphatases, but also quite possibly among phosphotransferases in general.

Structure–function relationships in the alkaline phosphatase superfamily

The AP superfamily comprises a family of highly promiscuous metallohydrolases, that are similar in active site architecture and substrate preference, but show limited sequence homology [6•]. The members of this superfamily catalyze the hydrolytic cleavage of P–O, S–O and P–C bonds in a range of phosphocarbohydrate, sulfo-carbohydrate and phosphonocarbohydrate substrates [6•], which often differ in their requirements for efficient catalysis, such as the nature of the transition state (TS) geometries, solvation or protonation patterns (Table 1). Common catalytic scaffolds employed by these enzymes (Figure 1) include one or more divalent metal ions (Zn^{2+}, Ca^{2+} or Mn^{2+}) that play important roles in nucleophile activation and substrate positioning [4,6•]. The nucleophile, in turn, is typically an alcohol or alkoxide (e.g. serine, threonine or formylglycine), depending on the particular superfamily member of interest. The members of this superfamily exhibit pronounced promiscuous (and cross-promiscuous) catalytic activities [6•]. Additionally, despite many similarities, there exist broad differences in their specific metal requirements, overall structure and choice of nucleophile, which can in turn

Table 1

Comparison of experimentally measured k_{cat}/K_M values (in M^{-1} s^{-1}) for a number of promiscuous members of the alkaline phosphatase superfamily[a]

Activity	Charge	AP	NPP	PMH	AS
Phosphate monoesterase	−2	**3.3×10^7**	1.1	2.2×10^1	7.9×10^2
Phosphorothioate monoesterase	−2	2.0×10^4	0.2		
Phosphate diesterase	−1	5.0×10^{-2}	**2.3×10^3**	9.2×10^3	2.5×10^5
Phosphorothioate diesterase	−1	1.1×10^{-3}	4.8		
Phosphonate monoesterase	−1	3.0×10^{-2}		**1.5×10^4**	
Sulfatase	−1	1.0×10^{-2}	2.0×10^{-5}	5.6×10^{-1}	**4.9×10^7**
Sulfonate monoesterase	0			4.9×10^1	
Phosphate triesterase	0			1.6×10^{-2}	

[a] AP, NPP, PMH and AS denote alkaline phosphatase, nucleotide pyrophosphatase/phosphodiesterase, phosphonate monoester hydrolase and arylsulfatase respectively. The experimental data is summarized in Ref. [4], and obtained from references cited therein. The most proficient activity for each enzyme is highlighted in bold, demonstrating that AP shows a preference for dianionic substrates, whereas NPP, PMH and AS all show a preference for monoanionic substrates. Gaps in the column for a specific enzyme indicate that that activity has not been observed in that enzyme. Note that although the focus of the text is specifically on changes in catalytic activity, which are reflected in changes in k_{cat}, we present here for comparison k_{cat}/K_M values rather than k_{cat} values, because in many cases k_{cat} alone could not be determined for these enzymes (see Ref. [4]).

be mapped to differences in the specificity patterns between individual superfamily members [6•].

There is a wealth of available kinetic and structural data on several members of this superfamily, such that their specificity and promiscuity patterns are well defined [6•,7–9,11–16,18,25,26]. Tied in with this, a detailed, atomic-level comparison between particular AP superfamily members can provide insight into the factors responsible for substrate selectivity and promiscuity in those enzymes. In particular, a careful study of the structural and electrostatic features underlying the catalytic preferences for different substrates among the superfamily members can explain the features governing the specificity patterns of its individual members. This, in turn, would allow for a better understanding of structure-function relationships in the superfamily, provide more general insight into the molecular basis for catalytic promiscuity, and, considering the link between catalytic promiscuity and protein evolution [1••,27], ultimately aid in understanding the parameters shaping the evolution of different enzyme functions.

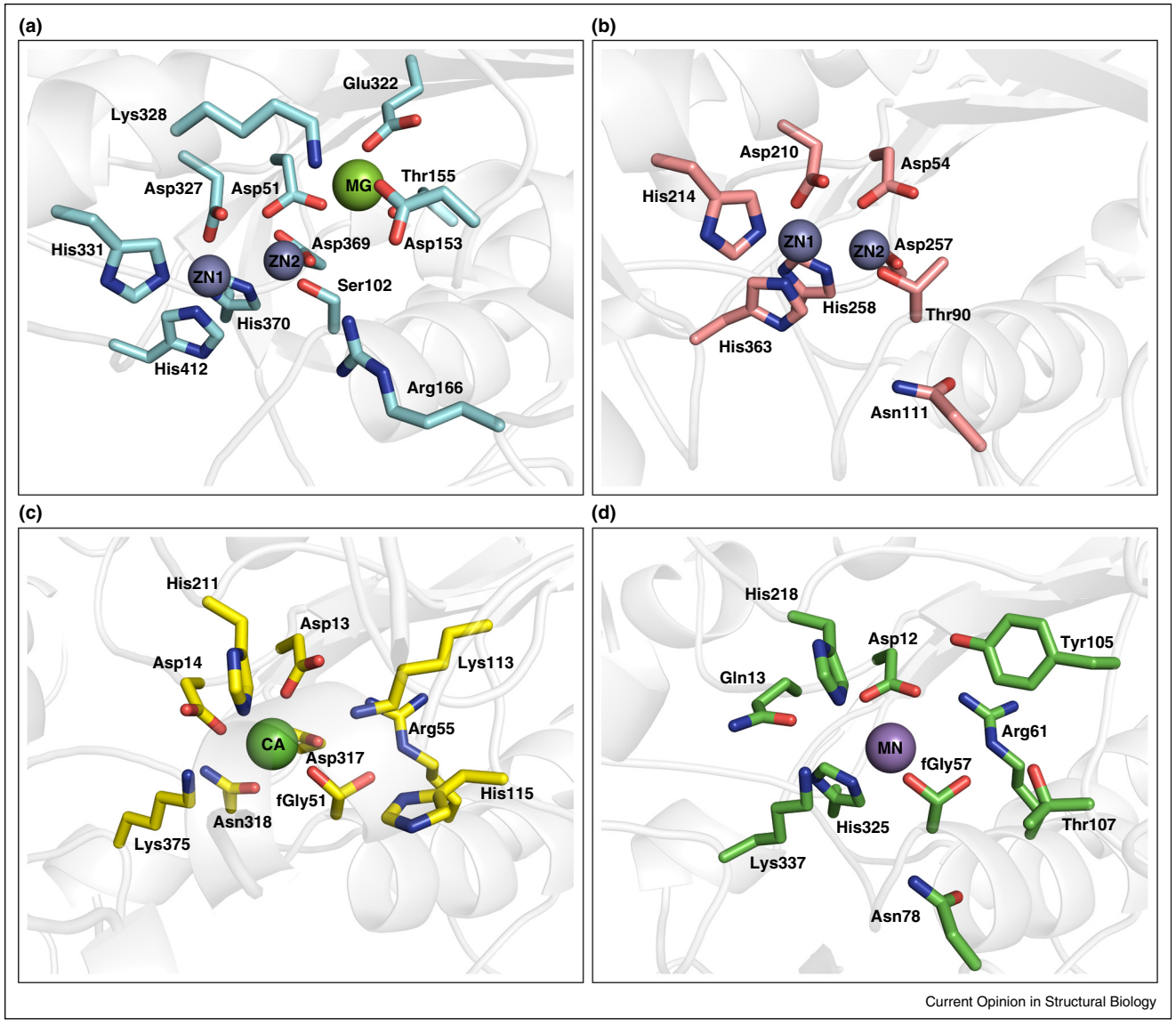
Examples of insights from recent computational studies

There are a number of inherent computational challenges involved in studying this particular superfamily, including but not limited to the complexity of the reaction mechanisms involved, the large system sizes, and the need for a reliable treatment of the metal ions [5]. These challenges, and current approaches to address them have been reviewed in detail elsewhere [5]. However, despite these specific pitfalls, theory has provided valuable insight into our understanding of the molecular details of specificity and promiscuity in these enzymes from both a structural and a mechanistic point of view [19••,20•,21,23••,24,28–30]. Some recent key studies will be briefly summarized here.

The uncatalyzed counterparts of the reactions facilitated by the alkaline phosphatase superfamily are highly diverse, with different transition states, protonation patterns and solvation requirements (see discussion in Refs. [31–33]). A key question, however, is whether these differences still exist in the relevant enzyme active sites, or if the enzymes in question alter the transition states to be more similar to each other. Experimental data, in particular linear free energy relationships [7,9,13,34] (LFER) and kinetic isotope effects [35] suggest that at least for alkaline phosphatase (AP), the enzyme-catalyzed transition states are apparently similar to their solution counterparts. This hypothesis has been further explored computationally by thorough characterization of the transition states for a range of reactions catalyzed by a number of members of the AP superfamily, in particular AP and NPP [19••,20•,23••,28,29]. For instance, Hou and Cui have performed a valuable comparative analysis of these two enzymes in their recent studies [20•,23••] of the chemical steps of the hydrolysis of phosphate mono- (pNPP^{2-}) and diesters (MpNPP^{-}) by AP variants as well as wild-type NPP. This was done using a QM/MM approach, in which the QM subsystem is described by the approximate Self-Consistent-Charge Density-Functional-Tight-Binding theory previously parametrized for phosphate hydrolysis (SCC-DFTBPR) [36]. The SCC-DFTBPR method offered general agreement between the reported calculations and experimental data, which supported its use for a semiquantitative analysis of phosphoryl transfer in AP and NPP.

Hou and Cui's studies demonstrated that apart from some slight tightening of the transition states in the enzyme active sites compared to aqueous solution, which was observed in the case of the hydrolysis of phosphate diesters, the relevant reaction mechanisms remained unchanged by the enzyme, demonstrating that these enzymes are able to recognize and stabilize the different

Figure 1



Comparisons of the active site architectures of representative members of the alkaline phosphatase superfamily. Shown here are the active sites of **(a)** alkaline phosphatase (1ED9 [10]), **(b)** nucleotide pyrophosphatase/phosphodiesterase (2GSN [12]), **(c)** *Pseudomonas aeruginosa* arylsulfatase (1HDH [54]) and **(d)** a phosphonate monoester hydrolase (2VQR [18]). PDB IDs are shown in parentheses.
Source: This figure was originally presented in Ref. [5]. Reproduced with permission from Ref. [5]. Published by the PCCP Owner Societies.

types of transition states found in mono-ester and diester hydrolysis. This active site plasticity was in turn suggested to be one of the factors underlying the catalytic promiscuity of the AP superfamily, and was interpreted in the context of the ability to bind differently charged substrates in the relevant active sites, as well as the high degree of solvent accessibility of these active sites [20•,23••]. Similar observations were made in the case of *Pseudomonas aeruginosa* arylsulfatase (PAS) [21], where the active site even appears to accommodate the catalytic machinery for multiple mechanisms with different general bases for different substrates. The only deviation appear to be the phosphonate monoester hydrolases from *Rhizobium leguminosarum* and *Burkholderia caryophilli*, where empirical valence bond (EVB) studies suggest almost identical transition states for all substrates irrespective of substrate shape, charge or polarizability [24]. Here, it appears instead that electrostatic flexibility of the active site plays a major role in facilitating the catalysis of different substrates.

In addition, in the case of AP and NPP, there has been significant recent discussion about the distance between the two catalytic metal centers at the respective transition states (Figure 1). Here, computational studies have been contradictory, with some studies suggesting that the two metal ions will move substantially apart during catalysis [19••,28,29], while other computational [20•,23••] and experimental [16] studies have indicated that the metal–metal distances will in fact remain reasonably stable throughout the course of the reaction. We discussed some possible origins of this discrepancy in a recent review [5], and resolving this issue is in particular quite important in light of recent studies arguing that the metal–metal distance in a promiscuous bimetallophosphatase is in fact very important for determining the activity and selectivity [37]. We note, additionally, that there has been recent discussion of the role of metal ions in determining specificity in phosphoryl transfer reactions. For example, a recent study by Herschlag and coworkers compared alkaline phosphatase with the catalytic preferences of three protein tyrosine phosphatases (which do not contain metal ions), and concluded that the positive charge of a metal is not a prerequisite for discriminating between phosphoryl and sulfuryl transfer [38]. However, in a broader context, Tokuriki and coworkers [39], as well as Jonas and Hollfelder [40], have studied the effect of metal substitution on a range of promiscuous metallo-β-lactamases (side activities of which include organophosphatase activity), and a phosphonate monoester hydrolase from the alkaline phosphatase superfamily, respectively, and demonstrated clear metal-dependent specificity patterns. Therefore, while not necessarily playing an important role in discriminating between phosphoryl and sulfuryl transfer, metal ions do appear to play a broader role in determining substrate specificity in (native or promiscuous) metallophosphatases.

Finally, note that there have also been other relevant recent computational studies that we do not discuss here due to space limitations, but instead refer interested readers to Refs. [19••,22,28–30,41]. In addition, for interesting bioinformatics and structural studies of protein evolution in enzyme superfamilies, we refer the readers to, for example, Refs. [42,43] (among others).

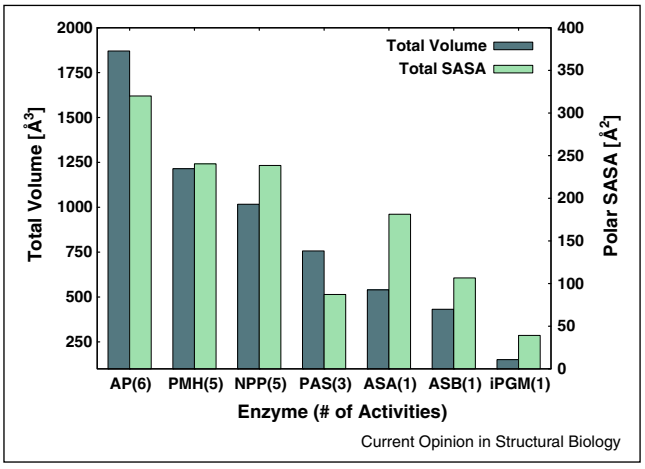
Electrostatic flexibility and catalytic promiscuity in this superfamily

As can be seen from recent computational studies, none of the 'usual culprits' for the promiscuity of these enzymes appear to play a prominent role in facilitating the promiscuity for these particular enzymes. That is, for example, that while conformational diversity has been proposed to play an important role in promiscuity [44], and despite the *active site* plasticity discussed in Refs. [20•,23••], the members of the alkaline phosphatase superfamily are large, rigid enzymes that bind their substrates in similar positions. The nature of the catalytic metal center plays a role in determining whether the nucleophile is preferentially an alcohol or alkoxide; however, in the examples where the nucleophile is an alkoxide, there is little change in transition state for the different substrates [20•,23••,24], and in the examples where the nucleophile is an alcohol, the transition state and preferred mechanism changes radically with the specific substrate and reaction, but there is no correlation between transition state size and observed catalytic efficiency [21,22] (where transition state size is defined as the sum of the P(S)–O distances to the incoming nucleophile and departing leaving group). What, then, is the origin of the observed promiscuity among these enzymes?

The importance of electrostatics in enzyme catalysis has been well-established [45,46], and in the case of the members of the alkaline phosphatase superfamily (and related phosphatases) can be further observed from the dependence of the selectivity patterns on substrate charge [24], and from studies of metal-fluoride transition state analogues (TSAs) for phosphate substrates, which demonstrate that when binding different TSAs, these enzymes would rather sacrifice shape complementarity in the binding pocket than anionic charge in their binding preferences [47]. In addition, when comparing the different factors contributing to the promiscuity of phosphonate monoester hydrolases, we observed that despite the minimal differences in actual transition state geometries for the different substrates, there is a subtle preference for accommodating substrates that minimize the buildup of negative charge at the transition state [24], providing further support for the importance of electrostatics and charge discrimination in determining the selectivity.

To further explore this, we recently performed a comparative analysis of the structural and physical properties of a number of members of the alkaline superfamily [24], correlating these properties to the number of known catalytic activities according to the BRENDA database [48] and information in the literature, as shown in Figure 2. Many of these enzymes have been reviewed in Ref. [4], and were selected for comparison here based both on the availability of experimental data and the fact that they provide comparative examples of both very promiscuous and very specific enzymes. From this figure, it can be seen that there seems to be a direct correlation between active site volume, polar solvent accessible surface area (SASA) and the number of known catalytic activities, with enzymes with larger active sites and polar SASAs in general having more known catalytic activities. This is in part because having a large active site volume allows the enzyme to accommodate substrates of a broader range of shapes and sizes, or allow the same substrate to (in principle) bind in multiple conformations, thus optimizing the number of productive binding conformations. A large active site volume is, in and of itself, insufficient for promiscuity if the minimal number of

Figure 2



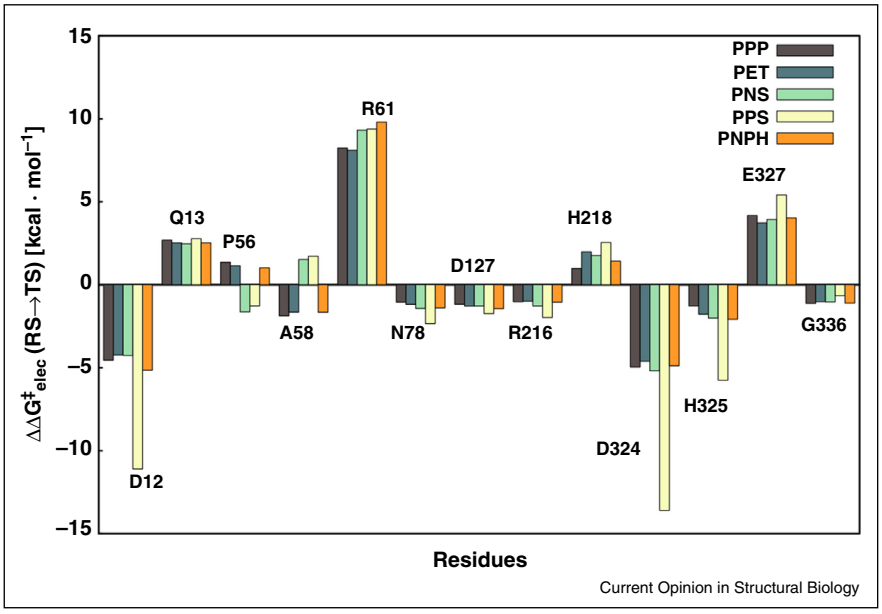
Correlation between the total active site volume and corresponding polar solvent accessible surface area (SASA) and number of known catalytic activities for a number of members of the alkaline phosphatase superfamily. Shown here are *Escherichia coli* alkaline phosphatase (AP), *Burkholderia caryophili* phosphonate monoester hydrolase (PMH), *Xanthomonas axonopodis* nucleotide pyrophosphatase/phosphodiesterase (NPP), *Pseudomonas aeruginosa* arylsulfatase (PAS), *Homo sapiens* lysozomal arylsulfatase A (ASA), *Homo sapiens* lysozomal arylsulfatase B (ASB) and *Bacillus stearothermophilus* phosphoglycerate mutase (*i*PGM).
Source: This figure and its associated data were originally presented in Ref. [24]. Reproduced with permission from Ref. [24] (http://pubs.acs.org/doi/pdf/10.1021/jacs.5b03945).

interactions for efficient transition state stabilization is not met (in particular as a large binding pocket would also increase the probability of a larger number of *non-productive* binding events). However, when a large binding pocket is combined with a large polar surface (which allows for both electrostatic interactions and also other non-covalent interactions that can facilitate binding and catalysis such as hydrophobic contacts and hydrogen bonding interactions), this allows the enzyme to adapt its active site electrostatics in order to create an optimal electrostatic environment for facilitating turnover of the given substrate.

While such large binding pockets with highly polar active sites can explain why these enzymes can accommodate multiple substrates, it still does not explain where the selectivity *between* these substrates comes from, or how some members of the superfamily can be simultaneously extremely promiscuous, catalyzing multiple, chemically distinct substrates with low selectivity, while remaining proficient enzymes towards their native substrate(s) [15,18,49]. That is, while the differences in specificity patterns *between* different alkaline phosphatases can be linked to differences in metal and nucleophile preferences, including whether the active site is mono-nuclear or dinuclear, as well as positioning of key catalytic residues (for a structural comparison, see e.g. Figure 1), it is harder to use such structural comparisons to explain the specificity patterns and promiscuity of *individual* members of the superfamily. Experimental studies of the promiscuity of serum paraoxonase 1 suggested the presence of catalytic backups in the active site, such that the same catalytic task can be fulfilled by multiple residues, and, simultaneously, the same residue can fulfill multiple catalytic tasks [50•]. Recent work on alkaline phosphatase suggested similar cooperativity between active site residues [24] (cooperativity in this context refers to the effect of two or more mutations simultaneously being different from the sum effect of the individual mutations). We have quantified and examined the breakdowns of the electrostatic contributions of individual amino acids to the activation barriers for the hydrolysis of multiple substrates by PAS [21] and different PMHs [24], and a representative overlay of these contributions for different substrates in *R*/PMH can be seen in Figure 3. These are distinct calculations, with distinct transition states, different charge distributions, and each calculation has no knowledge of the other substrates; yet, from these figures, it can be seen that exactly the same residues are flagged for each enzyme, albeit with quantitatively different contributions in line with the differing electrostatic needs of each substrate for transition state stabilization. A similar trend also emerges when considering mutant forms of the PMHs [24], and such observations have also been indirectly alluded to in other recent works [50•,51•]. This is a different version of the conformational diversity hypothesis [44] where the diversity is not driven by large global differences of enzyme structure or substrates binding positions, but rather the flexibility and more importantly cooperativity of the active site environment that can apparently easily adapt itself to the needs of stabilizing multiple, chemically distinct transition states.

This then leads to the most crucial insight obtained from the computational work. That is, as shown here, molecular simulations can map structure–function–energetics relationships, provide microscopic insights into the structure and electron distribution of transition states and the interactions stabilizing them, and model dynamic changes along reaction trajectories. When this information is put together with the large active site volumes and highly polar active sites of these enzymes, it becomes increasingly clear that one does not only need to take into account the number of interactions that are *available* for transition state stabilization, but also, more importantly, the number of interactions that are actually *needed* for transition state stabilization. As long as the number of available interactions exceeds the minimum number of necessary interactions, these enzymes are apparently capable of being promiscuous, which in turn will allow them to accommodate a broad range of substrates and thus to rapidly evolve as a response to external environmental change [24].

Figure 3



Electrostatic contributions of individual amino acids to the calculated activation barriers for the hydrolysis of phenyl *p*-nitrophenyl phosphonate (PPP), ethyl *p*-nitrophenyl phosphate (PET), *p*-nitrophenyl sulfate (PNS), phenyl *p*-nitrophenyl sulfonate (PPS) and the protonated *p*-nitrophenyl phosphate monoanion (PNPH). The contributions were calculated using the linear response approximation, as described in Ref. [24].
Source: This figure was originally presented in Ref. [24]. Reproduced with permission from Ref. [24] (http://pubs.acs.org/doi/pdf/10.1021/jacs.5b03945).

Overview and conclusions

In this review, we have discussed recent computational contributions to our understanding of the origin of catalytic promiscuity in the alkaline phosphatase superfamily and its implications for the evolution of new enzyme functions. The studies outlined here demonstrate that, like other phosphatases [52,53], members of the AP superfamily possess electrostatically flexible and cooperative active sites that are able to facilitate the hydrolysis of multiple, chemically distinct substrates, and the discrimination between different transition states is primarily based on the charge distribution [20•,21,23••,24]. In addition, despite their overall rigid scaffolds, these enzymes have large active site volumes with large polar surfaces, allowing them to obtain the optimal electrostatic environment for accommodating the catalytic requirements of a broad range of substrates. This underlines the general importance of electrostatics in enzyme catalysis [46], and points to electrostatic *flexibilty* and *cooperativity* as the key driving force for the selectivity and thus ultimately functional evolution of the promiscuous activities in the AP superfamily [24]. Finally, these recent studies demonstrate how theory can provide valuable insight into the basis of the enzyme specificity and promiscuity, and thus help elucidate the structure–function relationship in enzyme superfamilies, as well as explaining the features governing the functional evolution of the superfamily members. Such findings not only help us understand enzyme's functional evolution at the molecular level, but also provide a training ground that can be applied to the design of the artificial enzymes.

Conflict of interest

Nothing declared.

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- • of special interest
- •• of outstanding interest

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