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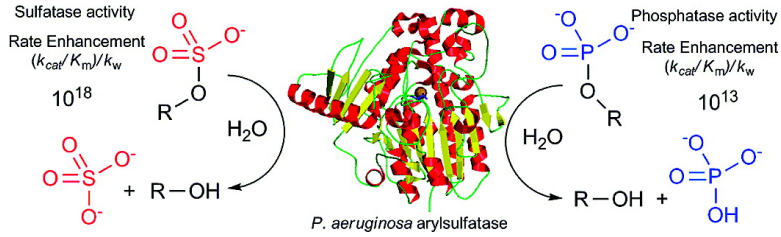

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Efficient Catalytic Promiscuity in an Enzyme Superfamily: An Arylsulfatase Shows a Rate Acceleration of 10^{13} for Phosphate Monoester Hydrolysis

Luis F. Olguin, Sarah E. Askew, AnnMarie C. O'Donoghue,† and Florian Hollfelder*

Department of Biochemistry, University of Cambridge, 80 Tennis Court Road, Cambridge CB2 1GA, United Kingdom

Received June 23, 2008; E-mail: fh111@cam.ac.uk

Abstract: We report a second catalytic activity of *Pseudomonas aeruginosa* arylsulfatase (PAS). Besides hydrolyzing sulfate monoesters, this enzyme catalyzes the hydrolysis of phosphate monoesters with multiple turnovers (>90), a k_{cat} value of 0.023 s^{-1}, a K_M value of 29 μM, and a k_{cat}/K_M ratio of 790 M^{-1} s^{-1} at pH 8.0. This corresponds to a remarkably high rate acceleration of 10^{13} relative to the nonenzymatic hydrolysis [$(k_{cat}/K_M)/k_w$] and a transition-state binding constant (K_{tx}) of 3.4 pM. Promiscuous phosphatase and original sulfatase activities only differ by a factor of 620 (measured by k_{cat}), so the enzyme provides high accelerations for both reactions. The magnitudes and relative similarity of the kinetic parameters suggest that a functional switch from sulfatase to phosphatase activities is feasible, either by gene duplication or by direct evolution via an intermediate enzyme with dual specificity.

Introduction

Most textbooks describe an enzyme as a catalyst with exquisite specificity for just one preferred substrate that is accepted out of the large number of alternative molecules in a cell. However, there is a growing body of evidence suggesting that the rule "one enzyme—one activity" is not absolute. The versatility of some lipases and esterases to process a variety of substrates and the enhancement of these additional activities by directed evolution are industrially important examples in this respect.[1] Even more remarkable than this broad substrate specificity is the ability of one active site to facilitate the turnover of several classes of substrates involving the making or breaking of completely different types of bonds, a phenomenon called *catalytic* promiscuity. Recent reviews list about two dozen examples of such activities,[2−7] but it is conceivable that a much larger number of enzymes with promiscuous activities remain undetected.[7,8]

Jensen[9] and later O'Brien and Herschlag[7] have suggested that catalytic promiscuity plays an important role in protein evolution. Evolution is thought to proceed through a process of gene duplication,[10−14] in which the second gene copy is released from selective pressure and can accumulate mutations that eventually lead to an enzyme with a new function. However, gene duplication is a slow process (estimated to have a time scale of millions of years in eukaryotic organisms).[11] Therefore, it has been suggested that the presence of inherent promiscuous activities allows a more rapid response to an environmental change.[6,7,9,15,16] Directed-evolution and protein-redesign studies have shown that many promiscuous activities can be enhanced by one or a few mutations without abandoning the native original activity, potentially giving an organism dual specificity even before a process of gene duplication occurs.[15,17] Alternatively, it is also possible that evolution occurs directly (i.e., without duplication) through an evolutionary intermediate that has multiple functions at a relevant level (although duplication could still occur later, to provide a handle for more specific regulation).[14a,15] In view of this background, the number of experimentally detected promiscuous activities to date may represent only a small fraction of a more comprehensive system of phylogenetic relationships for catalysis.

In this work, we set out to explore the catalytic promiscuity of *Pseudomonas aeruginosa* arylsulfatase (PAS). PAS is a sulfate monoester hydrolase that uses an unusual amino acid modification to provide an active-site nucleophile. In its active

† Present address: Department of Chemistry, Durham University, South Road, Durham DH1 3LE, United Kingdom.

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site, a cysteine is post-translationally oxidized to a C_α-formylglycine (FGly) whose hydrated form coordinates to a calcium ion. This metal positions and activates the FGly for nucleophilic attack on the sulfur center of the substrate.[18] The oxidation of cysteine (or serine in other sulfatases) is carried out by a range of modification systems,[19−24] although the specific modification machinery for PAS is not known. PAS was found to hydrolyze several aromatic sulfate esters[25] and is overexpressed in response to sulfate starvation conditions;[25,26] also, its gene (*atsA*) is part of an operon coding for sulfate-starvation-induced proteins (a group of proteins that includes other sulfatases, sulfate-binding proteins, and sulfate ester transporters).[26] Although its physiological substrate is not known, these observations suggest that PAS has a genuine physiological function as a sulfatase involved in sulfate scavenging.

PAS has been identified as a member of the alkaline phosphatase (AP) superfamily[27,28] and is classified as a member of this superfamily in the SCOP database.[29] This assignment is based on high structural homology between PAS and the name-giving member of this superfamily, *Escherichia coli* AP. Despite low sequence homology (only 16% identity over 218 residues),[18] the root-mean-square deviation of C_α atoms between PAS and AP is only 3.3 Å over 218 residues.[18] Specifically, sulfatases and AP share a common α/β-fold core structure, conserved metal-binding and active-site residues, and a similarly situated nucleophile.[27,28]

Protein superfamilies have been defined by combinations of criteria, such as sequence and structure[30,31] or common mechanistic steps,[4,32−34] to provide frameworks for understanding the evolutionary development of protein structure and function. There is growing experimental evidence that many enzymes within a superfamily show promiscuous activities toward the natural substrates of other members of the same superfamily.[6,7,16] Defining these promiscuous relationships may provide an additional criterion to facilitate more efficient traversal of the vastness of sequence space (i.e., the enormous combinatorial diversity that defies screening or selection approaches without further information and shortcuts).

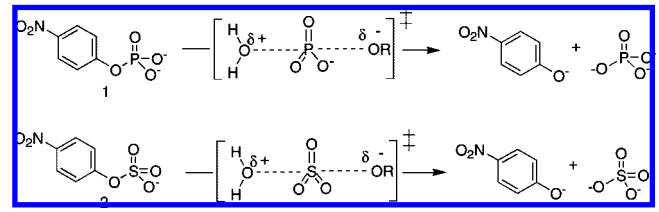

Figure 1. PAS catalyzes hydrolysis of sulfate and phosphate monoesters. In solution, these reactions share similar dissociative transition states. Compound **1** is 4-nitrophenyl phosphate (PNPP), and compound **2** is 4-nitrophenyl sulfate (PNPS).

The assignment of this class of sulfatases to the AP superfamily on the basis of catalytic as well as structural features prompts the question of whether PAS may promote other types of reactions in this superfamily[35] despite differences in the reaction centers of the substrates. Catalytic promiscuity within this superfamily has been observed previously in *E. coli* AP, which exhibits promiscuous sulfatase activity with a substantial rate acceleration [$(k_{cat}/K_M)/k_w \approx 10^9$].[35] Sulfate and phosphate monoesters share tetrahedral geometry with comparable bond angles and lengths.[36] However, the amount and distribution of charge[37] is different: while sulfate monoesters bear one negative charge, phosphate monoesters have two negative charges near pH 7.0 and above. On the other hand, the uncatalyzed hydrolyses of sulfate and phosphate monoesters occur with similar rate constants [k_{uncat}(PNPP)/k_{uncat}(PNPS) $\approx$ 4][38] and proceed through dissociative transition states (Figure 1). In each case, there is a small extent of bond making to the incoming nucleophile and a large extent of bond breaking to the leaving group.[39−43] This observation suggests that the similarity of the substrate structures and transition states involved can also be used as a criterion to predict efficient catalysis and define a mechanism-based superfamily.[35,36,44]

Previous qualitative evidence suggests that sulfatases can also hydrolyze phosphate esters. For example, a preparation of human arylsulfatase B had phosphatase activity, although an impurity as a possible source of this activity was not ruled out.[45] On the basis of this observation, it has been suggested that sulfatases might be promiscuous phosphatases.[35] More recently, the crystal structure of human arylsulfatase A showed a covalently bound

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Table 1. Summary of Kinetic Data for the Native and Promiscuous Activities of Wild-Type and Mutant PAS at pH 8.0[a]

	native sulfatase activity[b]			promiscuous phosphatase activity[c]		
	K_M (μM)	k_{cat} (s^{-1})	k_{cat}/K_M (M^{-1} s^{-1})	K_M (μM)	k_{cat} (s^{-1})	k_{cat}/K_M (M^{-1} s^{-1})
wild-type	0.29 ± 0.03	14.2 ± 0.6	$(4.9 \pm 0.8) \times 10^7$	29.1 ± 2.0	0.023 ± 0.0006	790 ± 58
C51S	0.25 ± 0.06	$(5.4 \pm 0.2) \times 10^{-3}$	$(2.1 \pm 0.5) \times 10^4$	75.2 ± 5.8	$(9.5 \pm 0.3) \times 10^{-6}$	0.13 ± 0.01
C51A	0.40 ± 0.05	$(6.1 \pm 1.3) \times 10^{-5}$	$(1.5 \pm 0.4) \times 10^2$	—[d]	—[d]	—[d]

[a] Conditions: 100 mM Tris, pH 8.0, 25 °C. [b] For the reaction with PNPS. [c] For the reaction with PNPP. [d] The detection limit for k_{cat}/K_M was a factor of $\sim 2 \times 10^5$ less than the wild-type activity, providing an upper limit of 4×10^{-4} M^{-1} s^{-1} for k_{cat}/K_M for this mutant. Background activity and loss of enzyme activity over longer time periods (>2 days) precluded reliable measurements.

phosphate attached to the active site FGly after incubation with a phosphate monoester, pointing to at least a single turnover.[46]

We now provide further, quantitative evidence for the postulated evolutionary relationship among the proteins of the AP superfamily[35] and show that PAS is an efficient phosphatase that produces a rate acceleration of 10^{13}. The relative magnitudes of the promiscuous and native activities are remarkable. While the k_{cat}/K_M values for the native and promiscuous activities of AP differ by 9 orders of magnitude, they are in a much narrower range for PAS: the k_{cat}/K_M values are within a factor of 10^4, and the k_{cat} values are 620-fold different, facilitating the acquisition of a new function by provision of a head-start activity.

Results and Discussion

PAS Is a Proficient Promiscuous Phosphatase. We observe a strong promiscuous phosphatase activity in a highly purified sample of PAS. Figure 2 shows a time course of the promiscuous activity, which exhibits more than 90 turnovers per active site, and a comparison with the uncatalyzed background reaction. The promiscuous phosphatase reaction of PAS follows saturation kinetics described by a K_M value of 29.1 μM (100-fold larger than that for the sulfate substrate) and a k_{cat} value of 0.023 s^{-1} (620-fold smaller than that for the sulfate substrate) at pH 8.0.

A number of observations (outlined in the following paragraphs) collectively suggest that the catalysis is not due to a contaminant but is a genuine activity of PAS. These observations include copurification of native and promiscuous activities on three columns, coincidence of the two activities on a native gel, decrease of native and promiscuous activities in active-site mutants, and competitive inhibition of the native sulfatase activity by the promiscuous phosphate substrate.

1. Copurification of Sulfatase and Phosphatase Activities. We purified PAS using three different types of columns: anion-exchange, hydrophobic-interaction, and size-exclusion chromatography. The fractions of the respective elutions were assayed for phosphatase and sulfatase activities. For each column purification, the two activities were found to elute simultaneously, and the ratio of the two activities remained constant (see Figure 3 and Figures S1 and S2 in the Supporting Information). If a protein other than PAS were responsible for the promiscuous activity, it would be expected not to coelute with PAS every time, because it would be unlikely to interact in equal measure with three column materials that employ different types of interactions with proteins.[47] If PAS had attached a contaminant through specific interactions, it would not be expected to yield a single band on an SDS−PAGE gel.

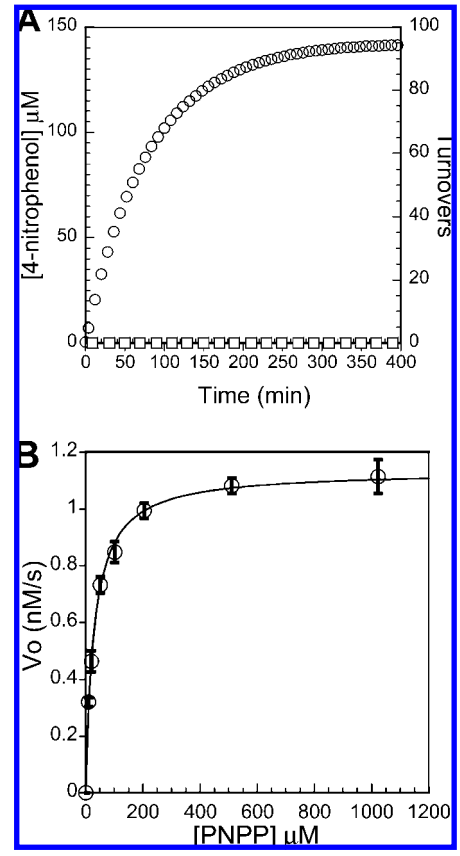

Figure 2. PAS is a promiscuous phosphatase. (A) Time course for the reaction of PAS with PNPP (○) under conditions ([PNPP] = 140 μM, [PAS] = 1.5 μM) at which multiple turnovers of substrate are observed (>90 per active site) compared with the uncatalyzed background reaction (□). (B) Michaelis−Menten representation of initial-rate data, demonstrating saturation kinetics ([PAS] = 1.15 nM). Kinetic data are shown in Table 1. Conditions: [Tris-HCl] = 100 mM, pH 8.0, T = 25 °C.

2. Analysis by Native Gel. In common with the SDS−PAGE gels described above, a native gel of highly purified PAS shows only one band, indicating high purity (see Figure 4A). Figure 4B,C shows incubations of native gels of PAS with phosphatase and sulfatase substrates (indoxyl phosphate and sulfate, respectively) that produce an insoluble, colored product, allowing a qualitative activity assay. Only one colored band is visible in each case, and the two spots appear at the same running distance, showing that the phosphatase and sulfatase activities are caused by the same protein. The running distance is also identical to

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(47) *E. coli* is not known to have clearly identified sulfatases but has putative sulfatase genes identified by homology.[49,97,98] In the absence of the plasmid pME4322, no sulfatase activity was observed in lysates of the *E. coli* strain BL21(DE3) used for expression. We also observed that crude lysates of *E. coli* BL21(DE3) overexpressing PAS showed a 15-fold increase in their phosphatase activity compared with cells treated the same way but harboring an "empty" pET vector lacking the PAS gene. The observed increase suggests that overexpression of PAS is responsible for the phosphatase activity.

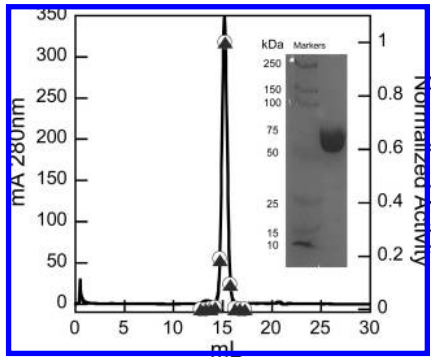

Figure 3. Copurification of native sulfatase and promiscuous phosphatase activities of PAS. The chromatogram of the purification of PAS on a gel-filtration column (Superdex 200 HR 10/30) shows that the protein eluted as a single symmetrical peak and that sulfatase (○) and phosphatase (▲) activities coeluted in the same fractions. The inset shows an SDS−PAGE gel of the protein recovered from the gel-filtration column, with a band corresponding to the expected molecular mass of PAS (60 kDa).[25] The two other column purifications showed similar patterns of coelution and are displayed in Figures S1 and S2 in the Supporting Information.

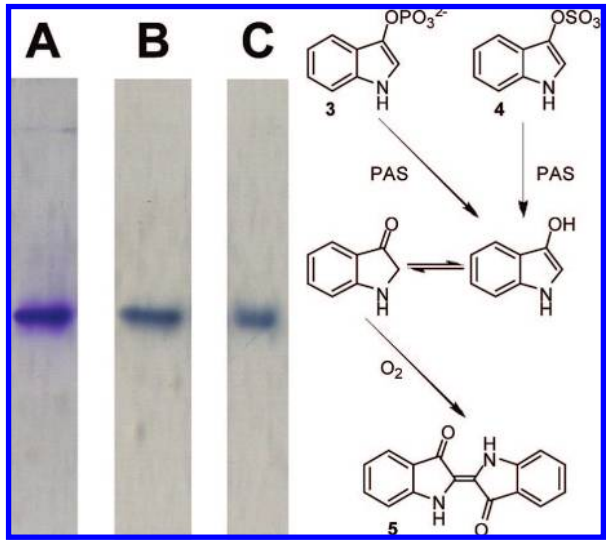

Figure 4. Native and promiscuous activities coincide in a native PAGE gel (pH 8.5). The native PAGE gel was cut into three pieces and stained with (A) Coomassie Blue stain, (B) a sulfatase substrate, and (C) with a phosphatase substrate. The assay reactions in (B) and (C) rely on the formation of an insoluble indigo blue dye (**5**) formed after hydrolysis of indoxyl sulfate (**4**) or indoxyl phosphate (**3**).[48]

that of the spot in Figure 4A, indicating that the PAS protein that brought about these spots, catalyzes both reactions.

3. Active-Site Mutants. PAS has a unique post-translational modification in which the active-site Cys51 is oxidized to FGly. *E. coli* can efficiently mature this residue to give complete oxidation to formylglycine.[49,50] We tested the presence of unmodified cysteines by mixing a denatured sample of PAS with 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB). Only 4.3% of the unique Cys residues (Cys51) in a PAS sample were found to be unmodified (see Figure S4 in the Supporting Information), which is within experimental error of full modification. The

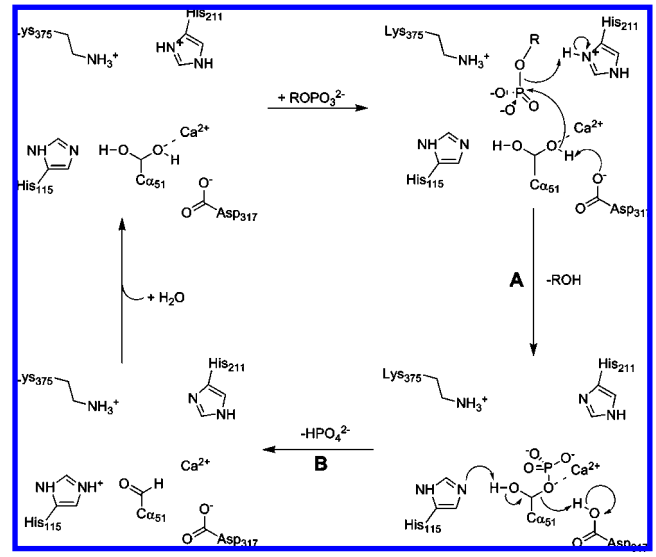

Figure 5. Proposed mechanism for the promiscuous phosphatase reaction of PAS (modified from ref 18), which involves (A) attack of the hydrate of a metal-coordinated formylglycine as the nucleophile followed by (B) breakdown of the intermediate by hemiacetal cleavage. It should be noted that the step (B) of the reaction would involve essentially identical C_β−O bond-breaking events (albeit with different leaving groups) for the native and promiscuous reactions.

hydrated form of the formylglycine residue is believed to act as the catalytic nucleophile in the arylsulfatase reaction, forming a reaction intermediate.[18,51] Formation of the intermediate has been proposed to be followed by cleavage of the C_β−O bond of the hemiacetal (rather than the S−O or P−O bond) (Figure 5).[18]

We mutated the active-site nucleophile Cys51 in PAS to serine and alanine. Neither mutant can be oxidized to FGly.[49] Analyzing these mutants for sulfatase catalysis, we observed similar K_M values in the low μM range but reductions in k_{cat}/K_M by factors of 2,330 in C51S and 327,000 in C51A (Table 1). The reduction in catalysis in the alanine mutant is comparable to an analogous mutation in AP in which deletion of the nucleophile in the S102A mutant led to a 10^5-fold reduction in k_{cat}/K_M.[52] For the promiscuous phosphatase catalysis by C51S, the K_M value is double that of the wild-type enzyme but is still in the μM range. The k_{cat}/K_M value is reduced 6080-fold in C51S. The C51A mutation reduced the activity below the sensitivity of our assay. C51S exhibits more than 40 turnovers for the native sulfatase activity but shows less than a single turnover as a promiscuous phosphatase. The latter experiments took days and were accompanied by activity loss of the enzyme, limiting the dynamic range of our experiments.

The observation that both the native and promiscuous reactions are slowed down by similar factors rules out the possibility that a contaminant would have copurified with PAS. A hypothetical contaminant would not be affected by a mutation in PAS and thus would not give rise to such similarly large decreases in the two activities. This observation is also consistent with a crucial role of the formylglycine nucleophile (generated from Cys51) in both reactions.

4. Inhibition. Since the k_{cat} values for the native and promiscuous substrates differ by a factor of 620, the promiscu-

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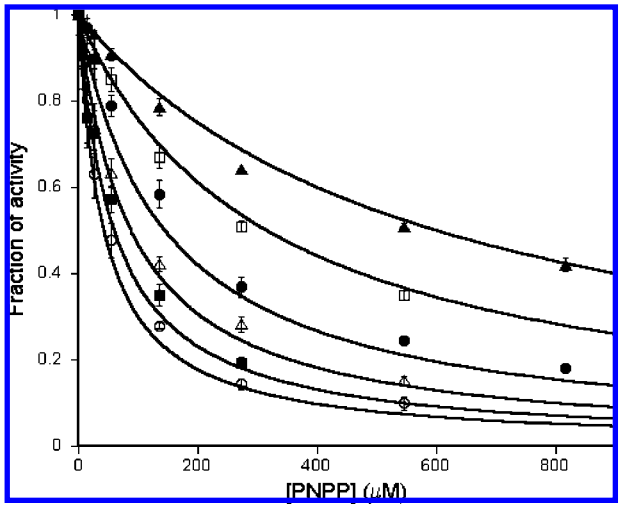

Figure 6. Catalysis of PNPS hydrolysis by PAS is competitively inhibited by PNPP. The fraction of activity is plotted as a function of PNPP concentration at different fixed concentrations of PNPS. A K_I value of 29.2 ± 1.3 μM was obtained by simultaneously fitting all of the data to a competitive inhibition model. This value is in good agreement with the K_M value of 29.1 ± 2.0 μM measured for PNPP hydrolysis (Figure 2). Conditions: [Tris/HCl] = 100 mM, pH 8.0, 25 °C. Different fixed amounts of PNPS [(○) 0.13, (■) 0.32, (△) 0.64, (●) 1.27, (□) 3.18, and (▲) 6.36 μM] were hydrolyzed in the presence of increasing amounts of PNPP. The solid lines represent the best fit of the experimental data to a competitive inhibition model.

ous substrate should not react on the time scale of the sulfatase reaction. We were therefore able to probe the ability of a phosphate monoester (i.e., the promiscuous substrate) to act as a competitive inhibitor of the native reaction.[53] Inhibition was indeed observed, as shown in Figure 6. The data were fitted by nonlinear regression analysis to a competitive inhibition model.[54] The value of K_I obtained for PNPP was 29.2 ± 1.3 μM, which is in close agreement with the K_M value measured for PNPP hydrolysis (29.1 ± 2.0 μM). The competitive inhibition pattern with almost identical K_M and K_I values for the phosphate monoester is consistent with catalysis of both the native and promiscuous activities occurring in the same active site.[53]

5. pH Rate Profiles. Identical pH dependences of native and promiscuous activities has previously been used as a criterion indicating that both reactions are carried out by the same active site. Figure 7 shows the pH profiles of k_{cat}/K_M for the sulfatase and phosphatase activities of PAS between pH 7.2 and 10, which are in qualitative but not quantitative agreement. Over the measured pH range, the k_{cat}/K_M values for the sulfatase and phosphatase activities have their maximum values at pH 7.2 and then decline as the pH increases (Figure 7). Reliable data above pH 10 could not be collected because the enzyme lost activity in less time than that required to conduct an assay. Below pH 7, the K_M for PNPS was less than 0.1 μM; thus, the errors in absorbance at concentrations around K_M precluded accurate measurements, and the limited range at which data could be obtained necessarily limits the reliability of the mechanistic implications. However, the apparent pK_a values in the k_{cat}/K_M pH profiles for the sulfatase and phosphatase reactions (8.3 ± 0.1 and 6.3 ± 0.2, respectively) differ by 2 pH units. The dissimilarity of the k_{cat}/K_M pH profiles points

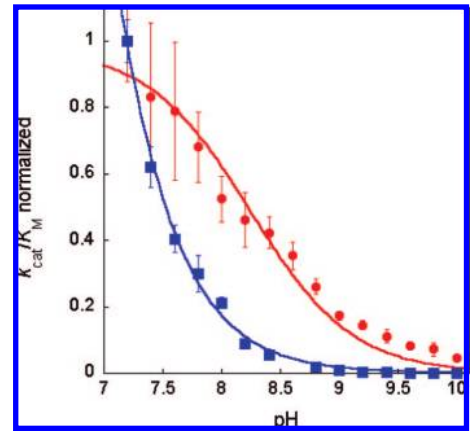

Figure 7. pH dependence of k_{cat}/K_M for native sulfatase activity toward PNPS (red ●) and promiscuous phosphatase activity toward PNPP (blue ■). The shapes of the two activity profiles imply that negatively charged substrates preferentially bind to protonated residues in the active site. The larger slope in the profile of the phosphatase activity could represent the involvement of two residues or the protonation of the PNPP substrate. Both k_{cat}/K_M pH profiles were fit to the following equation:[56] $y = (k_{cat}/K_{M,max})/[1 + 10^{pH-pK_a}]$.

toward further kinetic complexity that affects catalysis of sulfate and phosphate hydrolysis differently. This difference could represent the protonation of different residues in the free enzyme (pK_E) involved in the binding of a substrate carrying one (sulfate ester) or two (phosphate ester) negative charges. In the case of the phosphatase activity, the slope of log(k_{cat}/K_M) for the pH profile is greater than 1, which could represent ionization of two enzymatic groups or ionization of one enzymatic group and protonation of the dianion of PNPP (pK_a = 4.79).[55] However, these scenarios are merely plausible and not conclusive.

Rate Accelerations. A comparison of the k_{cat} value with the first-order rate constant of the corresponding uncatalyzed reaction in water, k_{uncat}, gives the first-order rate enhancement, k_{cat}/k_{uncat}. The value of k_{cat}/k_{uncat} for the promiscuous phosphatase activity is 8.4×10^6 (Table 2). This is an unusually large value for a promiscuous activity, second only to the promiscuous phosphodiesterase activity of a phosphotriesterase ($k_{cat}/k_{uncat} \approx 6 \times 10^8$).[7,57] Compared with the rate enhancement of PAS for its native sulfate-transfer activity ($k_{cat}/k_{uncat} = 2.3 \times 10^{10}$), this value is smaller by only 3 orders of magnitude.

The *catalytic proficiency* is defined as the ratio of k_{cat}/K_M to k_{uncat} and gives information about the amount of transition-state stabilization provided by a catalyst, relating to free rather than bound substrate and enzyme.[59] For PAS, the promiscuous activity achieves a catalytic proficiency of $\sim 10^{11}$ M^{-1}, which can be compared with $\sim 10^{16}$ for the native activity. The inverse of the catalytic proficiency is the transition-state binding constant, K_{tx}, which we calculate as 3.4 pM for the promiscuous activity. The catalytic proficiency of PAS is higher than that obtained for the promiscuous activities of carbonic anhydrase[60,61] (3.6×10^9 and 1×10^8 M^{-1} for its esterase and phosphate

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Table 2. Values of First-Order Rate Enhancement (k_{cat}/k_{uncat}), Catalytic Proficiency [$(k_{cat}/K_M)/k_{uncat}$], and Second-Order Rate Enhancement [$(k_{cat}/K_M)/k_w$] for the Native and Promiscuous Activities of Wild-Type PAS at pH 8.0[a]

activity	k_{cat}/k_{uncat}	$(k_{cat}/K_M)/k_{uncat}$ (M^{-1})[b]	$(k_{cat}/K_M)/k_w$ [c]	K_{tx} (M)[d]	$\Delta\Delta G^{\ddagger}$ (kcal/mol)[e]
sulfatase	2.3×10^{10} [f]	7.9×10^{16} [f]	4.3×10^{18}	1.3×10^{-17}	7.4
phosphatase	8.4×10^{6} [g]	2.9×10^{11} [g]	1.6×10^{13} [h]	3.4×10^{-12}	

[a] For the reaction of PNPS (native) and PNPP (promiscuous). [b] The catalytic proficiency is defined as $(k_{cat}/K_M)/k_{uncat}$.[58] [c] For hydrolytic reactions, $k_w = k_{uncat}/(55\ M)$.[35] [d] K_{tx}, the transition-state binding constant, was calculated as the inverse of the catalytic proficiency.[58] [e] $\Delta\Delta G^{\ddagger}$ is the difference in transition-state stabilization for sulfate monoester and phosphate monoester by PAS and was calculated using the following equation:[35] $\Delta\Delta G^{\ddagger} = -RT \ln\{[(k_{cat}/K_M)/k_w \text{ for PNPS}]/[(k_{cat}/K_M)/k_w \text{ for PNPP}]\} = (0.001986 \text{ kcal mol}^{-1} \text{ K}^{-1})(298 \text{ K}) \ln[(4.3 \times 10^{18})/(1.6 \times 10^{13})] = 7.4$ kcal/mol. [f] Using $k_{uncat} = 6.2 \times 10^{-10}\ s^{-1}$ for PNPS at 25 °C.[39] This value was obtained by correcting the original rate constant at 35 °C to a value at 25 °C using the following equations: $\log(k_2/k_1) = (E_a/2.303R)(T_2 - T_1)/(T_1T_2)$ and $\Delta H^{\ddagger} = E_a - RT$. [g] Using $k_{uncat} = 2.7 \times 10^{-9}\ s^{-1}$ for PNPP at 25 °C. This value was obtained by correcting the original rate constant at 39 °C[42] to a value at 25 °C using the same equations as for PNPS. [h] The k_{cat}/K_M values were larger at lower pH, corresponding to a larger rate acceleration. However, the large errors in absorbance ascribed to the small values of K_M at lower pH precluded more accurate measurements.

monoesterase activities, respectively)[62] and phosphotriesterase[57] (1.6×10^{10} for its phosphodiesterase activity).[63] Finally, the second-order rate acceleration for the promiscuous activity of PAS relative to the nonenzymatic attack by water for the same reaction [i.e., the quantity $(k_{cat}/K_M)/k_w$, where $k_w = k_{uncat}/(55\ M)$] has a value of 1.6×10^{13} (see Table 2). This benchmark has been used for the promiscuous sulfatase activity of AP, which gave a value of 10^9.[35]

The second-order rate acceleration exhibited by PAS for its promiscuous activity is larger than the corresponding values for many enzymes for their cognate reactions (3×10^{10} to 7.8×10^{26}).[58,64]

Implications and Conclusions

Relevance for Evolution of Function. Hydrolysis of monophosphates by AP proceeds with a k_{cat}/K_M value of $3 \times 10^7\ M^{-1}\ s^{-1}$.[35] In contrast, its promiscuous activities are smaller by factors of 10^8 for diesterases ($5 \times 10^{-2}\ M^{-1}\ s^{-1}$)[44] and 10^9 for sulfatase ($1 \times 10^{-2}\ M^{-1}\ s^{-1}$)[35] with respect to the native reactions. The comparison with PAS is striking: k_{cat}/K_M for the promiscuous phosphatase activity of PAS is a factor of $\sim 6 \times 10^4$ less than its native activity, and the difference between the turnover numbers (k_{cat}) for the native sulfatase and promiscuous phosphatase activities is a factor of only 620. Both comparisons must be considered to reflect relatively small differences between chemically distinct promiscuous reactions catalyzed with high rate accelerations.

The high level of the promiscuous activity and the relative similarity of the kinetic parameters of the two reactions could potentially facilitate an evolutionary transition between sulfate and phosphate transfer. It is difficult to predict a priori whether the level of this activity is immediately significant, but activities with relatively small k_{cat}/K_M values (as small as $\sim 0.01\ M^{-1}\ s^{-1}$) have been shown to provide a significant evolutionary advantage to an auxotrophic *E. coli* strain.[65] In that case, the promiscuous activity of the rescuing enzyme was more than 10^7-fold smaller (as measured by k_{cat}/K_M) than that of the naturally evolved enzyme that had been removed in the auxotroph.[65] The k_{cat}/K_M value for the promiscuous phosphatase reaction of PAS is only $\sim 4 \times 10^4$ smaller than the native phosphatase activity of, e.g., AP[44] (as an example for a relevant phosphatase), and thus it is conceivable that it can potentially carry out a useful function for an organism. Similar examples in this respect are the roles played by the glucokinase activities of four uncharacterized proteins[66,67] and the phosphite-dependent hydrogenase activity of AP.[68]

It is clear that PAS is an efficient catalyst for both reactions, as the catalysis for both the native and promiscuous reactions is substantial. The rate accelerations of the promiscuous activity listed in Table 2 fall in the lower range of enzymatic rate accelerations but are well above those exhibited by enzyme models.[69] The observation that the thermodynamic difference between the catalysis of the two reactions ($\Delta\Delta G^{\ddagger} = 7.4$ kcal mol^{-1}) is small compared to the overall transition-state stabilization ($\Delta G^{\ddagger} = 25.4$ kcal mol^{-1} for sulfate transfer and 18.0 kcal mol^{-1} for phosphate transfer by PAS)[70] is also relevant for the question of whether evolutionary specialization is necessary, as catalysts with higher efficiency are evolved. It has been assumed that negative trade-offs between different functions are unavoidable as evolutionary specialization proceeds.[71,72] Aharoni et al.[15] have shown that such specialization is not absolutely necessary and that coevolution of activities can be observed to some extent, at least for thermodynamically less demanding reactions. The example of PAS, an enzyme that also has to be considered as the product of an evolutionary process, shows that retention of two chemically distinct activities is also possible for more difficult reactions that require more substantial transition-state stabilization.

Phosphate and sulfate monoesters have similar bond lengths, geometries, and transition-state charge changes but differ in substrate charge and the identity of atoms undergoing bonding changes. PAS remarkably accepts these substrates with little discrimination (7.4 kcal/mol), while in AP the same substrate pair is differentiated by 12 kcal/mol.[35] It will be interesting to

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(63) This value was calculated using the first-order rate constant for the hydrolysis of ethyl-4-nitrophenyl phosphate ($k_{uncat} = 1 \times 10^{-10}\ s^{-1}$)[57] and the k_{cat}/K_M value for the promiscuous phosphodiesterase activity ($1.6\ M^{-1}\ s^{-1}$).[57]
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explore the detailed factors contributing to the different degrees of selectivity in the AP superfamily.

Molecules with multiple roles are rare in nature, and the need for tight control over cellular function is thought to bias against unselective catalysts.[73,74] However, the relative lack of selectivity in PAS could make it a useful scavenging enzyme, enabling an organism to be readily adaptable by hydrolyzing a wide range of compounds.

General Criteria for Catalytic Promiscuity. Our observations suggest that the promiscuous activity of PAS can be explained by a number of perhaps more general criteria:

(i) Native and promiscuous reactions share key features. In this case, the reactions share a trigonal-bipyramidal geometry at the reaction center. In solution, the two types of reactions proceed through similar dissociative mechanisms (characterized by a small degree of bond making and a large amount of bond breaking).[39,42,43] If, to a first approximation, the transition states of the catalyzed reactions are presumed to be of similar size and geometry and differ only by one extra negative charge in phosphate monoester dianion,[36,41,75] then the high-energy species arising during the course of the reaction can be stabilized by similar interactions.

However, the requirement that the transition states have similar natures (i.e., dissociative or associative) is not absolute. AP achieves a higher rate acceleration for phosphate diester hydrolysis (with an associative transition state) than for sulfate ester hydrolysis.[76] Some DNases are phosphomonoesterases in addition to having endo/exonuclease activity.[77] Likewise, purple acid phosphomonoesterase acts on phosphate mono- and diester substrates with similar k_{cat} values.[78]

Alternatively, electrostatic interactions between the substrate and the metal ion may differentiate between phosphate and sulfate esters that carry different total charges, leading to differences in their rate enhancements, as has been found for AP.[36,37]

(ii) The catalytic motifs employed by PAS to promote sulfate monoester hydrolysis are common to many other enzymes that catalyze hydrolysis reactions. For example, provision of a reactive metal ion has been shown in numerous model systems[79−82] to accelerate hydrolytic reactions by making a highly reactive nucleophile available for the reaction. Notably, several highly promiscuous enzymes (such as AP,[7,35,44,83] serum paroxonases,[15,84,85] phosphotriesterase,[57,86] and carbonic anhydrase[60,87]) contain metal ions in their active sites. The availability of a nucleophile activated by a metal ion invariably accelerates hydrolytic reactions as long as the substrate is brought into proximity, thus reducing the problem of catalysis to some extent to a problem of binding (i.e., orientation and positioning of the substrate with respect to the reactive nucleophile).

The members of the AP superfamily for which promiscuity has been detected[35,44,55] are metalloenzymes that share active-site features in addition to substantial structural homology.[28,45,55,88] In a similar core domain,[28] the active site of PAS possesses a Ca^{2+} ion coordinated by three conserved aspartic acids and one asparagine.[18] In contrast, the active site of AP contains a Mg^{2+} and two Zn^{2+} ions, but the Zn^{2+}-chelating residues Asp51 and Asp369 in AP are superimposable with Asp13 and Asp317 that chelate the Ca^{2+} atom in PAS. In both cases, metal ions interact strongly with the negatively charged substrates and position the catalytic nucleophile (Ser102 or FGly51) for attack.[18,52,88,89] In the position of the other two phosphatase metals, the sulfatases have two conserved lysines, providing alternative Lewis acid catalysts.[88] In both enzymes, the catalysis involves a covalent adduct: in the case of AP, a serine−phosphate intermediate is broken down by cleavage of the P−O bond, predicting retention of configuration (i.e., double inversion); in the case of PAS, the FGly−sulfate adduct is instead broken down by cleavage by the C_β−O bond (resulting in single inversion).[18,51] The catalytic mechanism of PAS has been postulated to involve general acid−base catalysis,[18,56] while in AP, no candidate for a general base has been identified. Despite small differences, active-site functionality appears to be largely congruent in AP and PAS.

Other structural features that contribute to catalysis may be present in PAS and AP. In the case of AP, the tolerance for a wide variety of substrates[35,44,76,90,91] can be explained by its relatively accessible binding site, which can accommodate substrates with different steric demands by possibly allowing different binding modes in a large binding pocket. The binding pocket for PAS is not as wide open as that of AP but is still large enough to readily accommodate different binding modes for sulfate and phosphate esters. In addition, we have used relatively reactive substrates, although less reactive phenyl phosphates are also accepted by PAS.[92]

(iii) It is possible that the unique reaction mechanism of PAS may be set up for particularly efficient promiscuity. First, the enzyme takes advantage of nucleophilic catalysis by the unusual *geminal diol* nucleophile instead of the much more common side chains of serine, threonine, tyrosine, or unmodified cysteine. It may be thermodynamically advantageous to utilize this mechanism for sulfate-transfer hydrolysis, because a sulfate intermediate would be harder to hydrolyze via cleavage of the S−O bond. Other sulfatases without a formylglycine also prefer to break the C−O bonds in sulfate esters instead of the stronger S−O bonds.[93−95] If the proposed two-step reaction catalyzed by PAS is correct, the breakdown of the intermediate involves

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the same C_β−O bond (rather than S−O or P−O) for both the native and promiscuous reactions. Chemically, the reaction of the second step (Figure 5, step B) then differs only in the leaving group (phosphate or sulfate), but the requirement for general acid−base catalysis of hemiacetal cleavage is identical. Thus, the unusual nucleophile unifies the second step of the catalytic cycle.

This mechanistic feature together with the commensurate modification of activity in the C51S and C51A mutants discussed above suggests that at least the catalytic effects associated with the nucleophile in the PAS active site promote both activities to the same extent. This would mean that at least one catalytic feature is intrinsically promiscuous, and given how highly cooperative the interactions in enzyme active sites are, the same may be true for the other catalytic effects in operation in PAS. In addition, it has been proposed that general acid−general base catalysis occurs in the sulfuryl transfer from substrate to enzyme in PAS (His211 and Asp317)[18] and in the *Aerobacter aerogenes* sulfatase.[56] This could potentially enhance the rate of the first step of the native and promiscuous reactions.

It will be interesting to test other sulfatases following the same mechanism for their ability to catalyze phosphate transfer as efficiently as PAS or to trace whether differentiation between the two activities has taken place. It may be that for a further increase in efficiency, more pronounced specialization is necessary. However, PAS is an example where two activities are catalyzed at a relatively high level, suggesting that the trade-off between efficiency and specificity can be relaxed to create an efficient yet multispecific enzyme.

An extrapolation from this single example suggests that efficient promiscuity is most likely to be found when the geometries of the transition states are similar, the substrates have similar steric demands, the promiscuous reaction is similarly or less thermodynamically demanding than the native one, and (in a multistep reaction) some of the steps of the promiscuous reaction are identical with steps in the native reaction. Further work must be done to rank and quantify the contributions of these criteria or ascertain their generality, but they may provide a basis for systematic analysis and exploration of the ability of existing protein structures to catalyze a wider range of reactions than currently known.

Materials and Methods

Materials. The plasmid pME4322 was a gift from Dr. Michael Kertesz.[49] The sodium salt of 4-nitrophenyl phosphate hexahydrate **1**, the potassium salt of 4-nitrophenyl sulfate **2**, indoxyl phosphate **3**, and indoxyl sulfate **4** were purchased from Sigma.

Overproduction and Purification of PAS and Its Mutants. Overproduction of the wild-type sulfatase proteins was carried out in *E. coli* BL21(DE3) harboring the plasmid pME4322 following the procedure described in ref 49. The protein was purified in the same way as described previously,[49] except that an additional step of gel filtration was performed using a Superdex 200 HR 10/30 column. The copurification data described in the Results and Discussion were identical for virginal and reused columns, ruling out possible "leaching" of phosphatases from the columns over time as the source of the observed promiscuous activity. Enzyme-containing fractions were concentrated and stored at −20 °C. Typical yields of purified protein were in the range 18−23 mg of protein/L of culture. The extinction coefficient of the protein at 280 nm was determined according to a reported procedure,[96] which gave an ε_{280} value of 100 400 M^{-1} cm^{-1}.

The cysteine mutants C51S and C51A were prepared using the QuikChange XL Site-Directed Mutagenesis Kit (Stratagene) with pairs of complementary mutagenic primers containing the desired mutation (C51Sfwd, 5′-cac cgc ctc gac c**tc g**tc gcc gac ccg ctc-3′; C51Srev, 5′-gag cgg gtc ggc ga**c ga**g gtc gag gcg gtg −3′; C51Afwd, 5′-aca ccg cct cga cc**g cc**t cgc cga ccc gct-3′; and C51Arev, 5′-agc ggg tcg gcg a**gg c**gg tcg agg cgg tgt-3′). The overexpression conditions for these mutants were modified to 30 °C and 0.5 mM of IPTG. Previous reports had mentioned that the C51A mutant was degraded by the cells on overexpression,[49] but the reduction in temperature and IPTG allowed us to produce 16 mg of purified protein/L of culture.

Kinetic Measurements. Reactions were followed by measuring the absorbance of the 4-nitrophenolate product at 405 nm in a Spectramax plate reader (Molecular Devices) at 25 °C. All of the reactions were performed in buffered solutions (100 mM Tris-HCl, pH 8.0). The experiments were performed in triplicate and carried out in a final volume of 220 μL in 96-well plates (Nunc). The extinction coefficient for 4-nitrophenolate at pH 8.0 was determined with a standard curve of known concentrations using a volume of 220 μL. Initial rates (less than 5−20% of the reaction) were calculated from the linear part of the time course and then used to construct Michaelis−Menten plots. Values for V_{max} and K_M were obtained by fitting the data to the Michaelis−Menten equation using a nonlinear least-squares fit (Kaleidagraph, Synergy Software). Values of k_{cat} were obtained by dividing V_{max} by the corresponding enzyme concentration. Enzyme concentration ranges of 0.1−0.5 and 20−50 nM were used in the measurements of the wild-type sulfatase and promiscuous phosphatase activities, respectively. The activities of the mutants C51S and C51A were measured in 1.0 cm path length quartz cuvettes using the same equipment. The concentration of enzyme in these assays was increased ∼100 and 1,000-fold for the C51S and C51A mutants, respectively. The substrate concentration was typically varied from $0.2K_M$ to $10K_M$, provided that the limits of detection and solubility permitted it. Higher substrate concentrations led to substrate inhibition. Dilutions of the enzyme at concentrations lower than 500 nM led to rapid loss of activity. Addition of 500 μg/mL bovine serum albumin (BSA) stabilized the protein at low concentrations (<500 nM). BSA itself did not accelerate the sulfatase or phosphatase reaction. PAS copurified with Ca^{2+} in its active site,[18,49] and this metal was not added to the kinetic assays. Addition of further Ca^{2+} did not enhance the enzymatic reaction, suggesting that the enzyme was saturated. The pH profiles were constructed by measuring the steady-state kinetic parameters over a pH range of 7.2 to 10 using the following buffers: Tris (100 mM, pH 7−9) and CHES (100 mM, pH 9−10).

For the inhibition experiments, the data were fit by nonlinear regression analysis to a competitive inhibition model[54] using Kaleidagraph. Alternative inhibition models did not converge with the experimental data.

Native PAGE Gel. A native PAGE gel at pH 8.5 was loaded with 4 μg of purified PAS on each lane and run at 100 V. Next, the gel was divided into three portions; one was stained with Coomassie Blue solution, another incubated in 7 mL of a solution containing indoxyl sulfate (30 μg/mL) in 50 mM Tris (pH 8.0) for 60 min, and the third incubated with indoxyl phosphate (400 μg/mL) in 50 mM Tris (pH 8.0) for 6 h.

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Supporting Information Available: Chromatograms of the purifications of PAS (Figures S1 and S2), determination of unmodified cysteines in PAS (Figure S3), wavelength scans of product formation (Figure S4), and pH$-K_M$ and pH$-k_{cat}$ profiles for sulfatase and phosphates activities (Figure S5). This material is available free of charge via the Internet at http://pubs.acs.org.

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