Catalytic Promiscuity

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Efficient Catalytic Promiscuity for Chemically Distinct Reactions**

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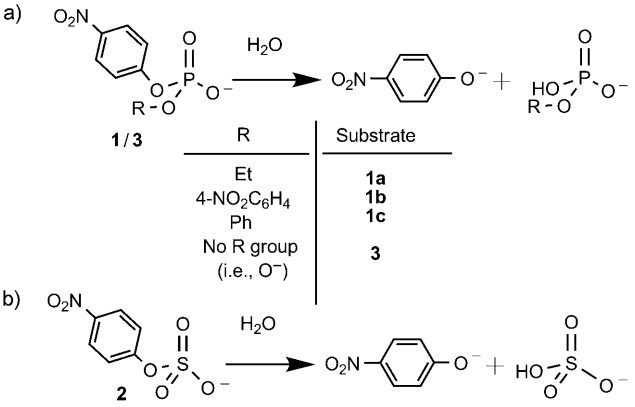
The extraordinary rate enhancements achieved by enzymes, with values up to 10^{26} reported for $(k_{cat}/K_m)/k_2$,[1] are usually associated with exquisite specificity for one transition state in textbooks.[2] In this context, it is remarkable that some enzymes are able to combine efficient catalysis of their native function with the ability to accelerate chemically distinct reactions involving formation and cleavage of different bonds. This phenomenon is known as catalytic promiscuity.[3–6] It is proposed to play a role in the divergent evolution of enzymes by providing a head start in activity and a possible selective advantage to a duplicated gene.[6–9] In most cases, catalytic proficiencies[10] for the promiscuous activities are much lower than that for the parent reaction, not exceeding $(k_{cat}/K_m)/k_2$ values of 10^{13}–10^{15} and typically much less than that.[6,11] Herein we report a promiscuous phosphodiesterase activity for *Pseudomonas aeruginosa* arylsulfatase (PAS) with a remarkably high proficiency of 10^{18}. Several experimental observations suggest that PAS acts as a sulfatase in vivo: it hydrolyzes several aromatic sulfate esters,[12] its expression is induced under sulfate starvation conditions and repressed in the presence of inorganic sulfate,[12,13] and its gene (*atsA*) is part of a gene cluster that also encodes a sulfate ester transport system.[13] A promiscuous phosphate monoesterase activity has been reported for this enzyme,[14] but this reaction is catalyzed much less efficiently than sulfate ester hydrolysis. The promiscuous diesterase activity described herein surpasses all other reported promiscuous activities, and is comparable to the proficiency of the native sulfatase activity (4×10^{18})[14] of PAS, thus challenging the idea that efficient catalysis requires specialization.

PAS expressed in *E. coli* was purified in three steps as described previously,[14] and appears as a single band on a SDS-PAGE gel. The purified enzyme hydrolyzes phosphate diesters **1** (Scheme 1) to produce 4-nitrophenolate; the yellow color thus enables the progress of the reaction to be followed spectrophotometrically. For the reaction of diester **1b** at pH 8.0, we observed saturation kinetics with Michaelis–Menten parameters $k_{cat}=0.55\ s^{-1}$ and $K_m=2.2\ \mu M$, and multiple turnovers per active site (Figure 1). The kinetic parameters for all reactions catalyzed by PAS are shown in Table 1.



Scheme 1. PAS hydrolyzes a) phosphate diesters **1**, phosphate monoester **3**, and b) sulfate monoester **2**.

Several experimental methods were used to confirm phosphate diester hydrolysis as a genuine activity of PAS, rather than the result of a contaminating enzyme. During purification of PAS, the diesterase activity co-elutes with the native sulfatase activity and previously observed promiscuous phosphate monoesterase activity[14] from three different columns with a constant ratio between the three activities

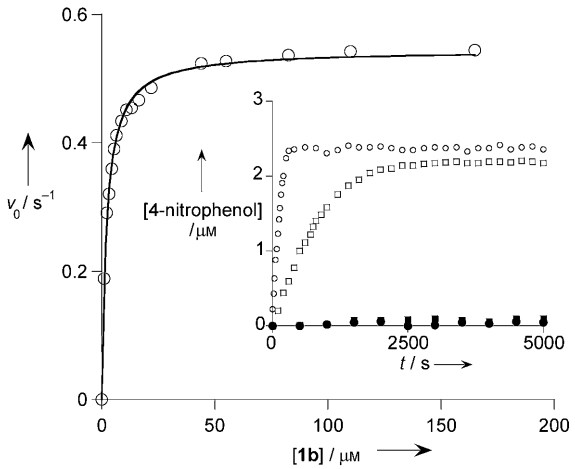


Figure 1. Promiscuous phosphodiesterase activity of PAS. A typical Michaelis–Menten plot of the initial rate v_0 for phosphate diester **1b** ([PAS] = 15 nM; [**1b**] = 1.1–165 μM). Inset: Time course of the reactions of PAS with phosphate diester **1b** (□, [PAS] = 10 nM, [**1b**] = 2 μM) with more than 200 reaction cycles per active site. For comparison, the reaction of sulfate monoester **2** (○, [PAS] = 0.5 nM, [**2**] = 2.3 μM) and the corresponding uncatalyzed reactions (■,●) are shown. Conditions: 25 °C, 0.1 M Tris-HCl (pH 8.0), 0.5 mg mL^{-1} BSA, 0.5 M NaCl. Tris = tris(hydroxymethyl)aminomethane.

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Table 1: Kinetic parameters for the PAS-catalyzed hydrolysis of phosphodiesters **1**, sulfate monoester **2**, and phosphate monoester **3**.[a]

Substrate	K_m [μM]	k_{cat} [s^{-1}]	k_{cat}/K_m [$M^{-1}s^{-1}$]	$(k_{cat}/K_m)/k_2$[b]
1a	617 ± 61	0.073 ± 0.004	119 ± 7	6.5×10^{15}
1b	2.2 ± 0.3	0.55 ± 0.02	(2.5±0.3) × 10^5	1.3×10^{18}
1c	25 ± 2	0.19 ± 0.01	(7.6±0.5) × 10^3	
2[c]	0.29 ± 0.03	14.2 ± 0.6	(4.9±0.8) × 10^7	4.3×10^{18}
3[c]	29.1 ± 2.0	0.023 ± 0.001	790 ± 58	1.6×10^{13}

[a] Reaction conditions: 25 °C, 0.1 M Tris-HCl (pH 8.0), 0.5 mg mL^{-1} bovine serum albumin (BSA),[15] 0.5 M NaCl (for **1a–c**). [b] For hydrolytic reactions, the second-order rate constant k_2 for the uncatalyzed reaction is taken as $k_{uncat}/55$ M. Values for k_{uncat}/s^{-1} at 25 °C, pH 8.0:[11,14] 1.0×10^{-12} (**1a**), 1.1×10^{-11} (**1b**), 6.2×10^{-10} (**2**), 2.7×10^{-9} (**3**). [c] Data for sulfate (**2**) and phosphate (**3**) monoesters[14] are included for comparison.

(Supporting Information, Figure S1). A phosphate diester substrate acts as a competitive inhibitor of the native sulfatase activity of PAS (Supporting Information, Figures S2 and S3), indicating that both reactions occur in the same active site. Furthermore, a mutant, C51S, lacking the wild-type nucleophile (see Scheme 2) has a reduced k_{cat} value for the reactions with both phosphate diester **1b** and sulfate monoester **2**[14] (by more than 10^4 and 10^3 respectively; Table 2).[16]

Table 2: Kinetic parameters for the C51S mutant of PAS.[a]

Substrate	K_m [μM]	k_{cat} [s^{-1}]	k_{cat}/K_m [$M^{-1}s^{-1}$]
1b	3.7 ± 0.3	(<5.5±0.1) × 10^{-5}	< 15 ± 1
2[b]	0.25 ± 0.06	(5.4±0.2) × 10^{-3}	(2.1±0.5) × 10^4

[a] Reaction conditions: 25 °C, 0.1 M Tris-HCl (pH 8.0), 0.5 mg mL^{-1} BSA, 0.5 M NaCl (for **1b**). [b] Data for sulfate monoester **2** included for comparison.[14]

Catalytic promiscuity is a familiar feature in PAS, which also hydrolyzes phosphate monoesters, such as **3**, but much less efficiently than sulfate monoesters, such as **2**.[14] The catalytic proficiencies $(k_{cat}/K_m)/k_2$ of the enzymatic reactions for substrate **3** is 1.6×10^{13} and 4.3×10^{18} for **2** (Table 1). However, the phosphodiesterase activity (**1b**) of PAS has an exceptionally high catalytic proficiency of 1.3×10^{18}. Promiscuous cyclic phosphodiesterase activity has been previously observed for a sulfatase, but with lower efficiency.[17] This value surpasses the rate accelerations observed for the native reactions of many enzymes, and to our knowledge is greater than the proficiency of any previously reported promiscuous enzyme activity.[6,11] In comparison, two structurally and evolutionarily related enzymes that belong to the same superfamily as PAS catalyze the same three hydrolytic reactions but show greater discrimination between the native and promiscuous activities. *E. coli* alkaline phosphatase (AP) is a native phosphate monoesterase that also hydrolyzes sulfate monoesters and phosphate diesters,[18,19] but the proficiencies for the promiscuous reactions are at least 10^6-fold lower than for the native phosphatase activity ($(k_{cat}/K_m)/k_2 = 7\times10^{17}$). *Xanthomonas axonopodis* nucleotide phosphodiesterase/pyrophosphatase (NPP) preferentially hydrolyzes phosphate diesters ($(k_{cat}/K_m)/k_2 = 8\times10^{15}$) with lower sulfate and phosphate monoesterase activities (proficiencies less than 10^{11}).[20,21]

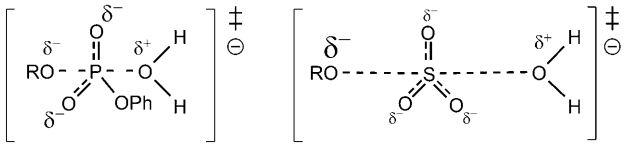
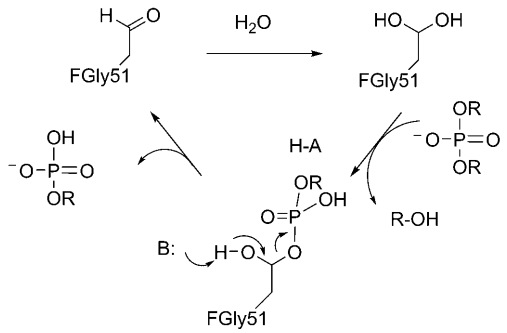


Figure 2. Transition states during phosphodiester (associative; left) and sulfate monoester hydrolysis (dissociative; right).

It is remarkable that PAS can achieve large and comparable rate enhancements for two reactions with distinct features. Both catalyzed reactions are hydrolytic, but the intrinsic chemistry, which is defined by bond-making and bond-breaking, involves mechanistic alternatives. In solution, the hydrolysis of phosphodiesters **1** occurs via a transition state that is more associative in nature, with little negative charge development on the leaving group.[22–24] By contrast, sulfate and phosphate monoesters proceed through dissociative transition states, with significant negative charge on the leaving group (Figure 2).[22,25,26] PAS displays greater selectivity for substrate structure than for the transition-state characteristics of the background reactions: we observed a 10^3-fold variation in k_{cat}/K_m for diesters **1a–c**, with an apparent preference for diesters with two aromatic rings, yet the values for diester **1b** and sulfate **2** differ by just 100-fold. Substrate charge appears to have a large influence on reactivity; the increase in charge on phosphate monoester **3** relative to sulfate **2** is penalized by a 6×10^4-fold decrease in k_{cat}/K_m, despite both reactions occurring via a dissociative transition state in solution. The nature of the transition states for PAS-catalyzed reactions remain to be determined. AP,[27,28] many other phosphatases,[22,29] and a sulfatase[30] use similar mechanisms to those in solution, but an example of a promiscuous enzyme that hydrolyzes phosphate and phosphonate monoesters with transition states that are closer to one another than those of the non-enzymatic reaction was recently reported.[31]

Efficient hydrolysis of phosphate diesters by PAS may result from active-site groups that could carry out similar roles during catalysis of both native and promiscuous reactions. Specifically, the proposed double displacement mechanism for sulfate hydrolysis[32] involves Lewis acid catalysis by a calcium ion, a reactive nucleophile, and efficient substrate binding and general acid catalysis by several cationic active site groups (Supporting Information, Figure S4). The decreased activity of the C51S mutant confirms the key role of the formylglycine (FGly) nucleophile, which is formed by post-translational modification of cysteine 51,[33] in both reactions. This nucleophile can be regenerated by hemiacetal cleavage, enabling multiple turnovers per active site (Scheme 2 and inset in Figure 1).

The substantial rate accelerations show that PAS is a highly efficient catalyst for both sulfate monoester and phosphate diester hydrolysis. This efficiency is also reflected in the tight transition state binding ($K_{tx} = 1.3\times10^{-17}$ M and 4.2×10^{-17} M for substrates **1b** and **2**, respectively). This



Scheme 2. Role of the key nucleophilic residue FGly51 for the multiple turnover of the phosphodiesterase activity of PAS. The intermediate is broken down by base catalysis, leading to C–O bond cleavage. This mechanism is available regardless of whether a phosphate or sulfate intermediate is generated after reaction with the FGly nucleophile, resulting in a common intermediate breakdown step for the three reactions catalyzed by PAS. The metal ion and the residues acting as general acid and base catalysts are not shown for clarity (see Supporting Information, Figure S4).

combination of considerable catalytic proficiency and a lack of specificity is remarkable. Presumably there has been little selective pressure against the secondary activity for this enzyme during evolution, which may be an advantage for the organism. The recycling of catalytic functionality for three reactions indicates that, despite different transition-state structures, substrate charges, and sizes, evolutionary crossover between such reactions might be achieved with relative ease. This observation also suggests that the ability to catalyze a second reaction can not only occur in early stages of functional adaptation with low rate accelerations,[34] but also in catalysts of high proficiency. A catalyst with multiple head-start activities (in this case with k_{cat}/K_m values differing only 200-fold for two and 6×10^4-fold for three reactions) may provide a good starting point for future evolutionary adaptation: versatile catalysts, such as PAS, could be useful cellular tools as generalist scavengers that can respond quickly to a range of environmental influences.

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[15] BSA was added to stabilize PAS at low concentrations, as described previously.[14] Although it does not itself catalyze the reaction, it may slightly increase the K_m: in the absence of BSA the K_m for **1b** was approximately 20 μM, but rapid loss of enzyme activity prevented accurate determination of kinetic parameters under these conditions.
[16] The stated diesterase activity of the C51S mutant represents an upper limit owing to the possibility of small amounts of a contaminating phosphodiesterase in the enzyme preparation. Low levels of phosphodiesterase contamination of a comparable activity were seen for a C51A mutant preparation. However, the large decrease in activity on mutation of the catalytic nucleophile confirms that PAS is indeed responsible for the observed phosphate diester hydrolysis. The presence of a possible contamination would not affect the analysis of the diesterase activity of wild-type PAS, as the activity is negligible (less than 1/10000 of the k_{cat}/K_m value of wild-type PAS).
[17] Ox liver sulfatase hydrolyzes cAMP with k_{cat}/K_m values 10^3–10^4 fold lower than the measured sulfatase activity[35] and a proficiency $(k_{cat}/K_m)/k_2 = 1\times10^{16}$ calculated by O'Brien and Herschlag[6] using an estimated value for the uncatalyzed hydrolysis of cAMP.[36] However, this value relies on a rather long extrapolation.
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