

Transition State Interactions in a Promiscuous Enzyme: Sulfate and Phosphate Monoester Hydrolysis by *Pseudomonas aeruginosa* Arylsulfatase

*Bert van Loo^{†§}, Ryan Berry[‡], Usa Boonyuen^{†,1}, Mark F. Mohamed[†], Marko Golcnik^{†,1}, Alvan C.
Hengge^{*‡} and Florian Hollfelder^{*†}*

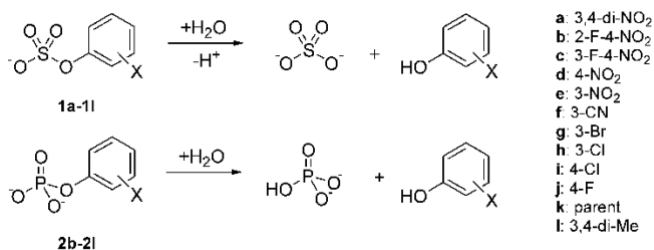
[†]Department of Biochemistry, University of Cambridge, Cambridge, United Kingdom.

[‡]Department of Chemistry and Biochemistry, Utah State University, Logan, Utah 84322, United States.

ABSTRACT

Pseudomonas aeruginosa arylsulfatase (PAS) hydrolyses sulfate and, promiscuously, phosphate monoesters. Enzyme-catalyzed sulfate transfer is crucial to a wide variety of biological processes, but detailed studies of the mechanistic contributions to its catalysis are lacking. We present linear free energy relationships (LFERs) and kinetic isotope effects (KIEs) of PAS and analyses of active site mutants that suggest a key role for leaving group (LG) stabilization. In LFERs PAS^{WT} has a much less negative Brønsted coefficient ($\beta_{\text{leaving group}}^{\text{obs-Enz}} = -0.33$) than the uncatalyzed reaction ($\beta_{\text{leaving group}}^{\text{obs}} = -1.81$). This situation is diminished when cationic active site groups are exchanged for alanine. The considerable degree of bond breaking during the TS is evidenced by an $^{18}\text{O}_{\text{bridge}}$ KIE of 1.0088. LFER and KIE data for several active site mutants point to leaving group stabilization by active-site K375, in cooperation with H211. ^{15}N KIEs and the increased sensitivity to leaving group ability of the sulfatase activity in neat D₂O ($\Delta\beta_{\text{leaving group}}^{\text{H-D}} = +0.06$) suggest that the mechanism for S-O_{bridge} bond fission shifts, with decreasing leaving group ability, from charge compensation via Lewis acid interactions towards direct proton donation. $^{18}\text{O}_{\text{nonbridge}}$ KIEs indicate that the TS for PAS-catalyzed sulfate monoester hydrolysis has a significantly more associative character compared to the uncatalyzed reaction, while PAS-catalyzed phosphate monoester hydrolysis does not show this shift. This difference in enzyme-catalyzed TSs appears to be the major factor favoring specificity toward sulfate over phosphate esters by this promiscuous hydrolase, since other features are either too similar (uncatalyzed TS) or inherently favor phosphate (charge).

Arylsulfatases catalyze the *in vivo* hydrolysis of sulfate monoesters, producing inorganic sulfate, typically removing it from a sugar or a steroid hormone. Sulfatases are highly proficient enzymes, with catalytic proficiencies ($(k_{\text{cat}}/K_M)/k_{\text{uncat}}$) well above 10^{13} - 10^{17} M^{-1} for the model substrate 4-nitrophenyl sulfate **1d** (Scheme 1).¹⁻⁴ Despite their occurrence in eukaryotes and prokaryotes, relevance for a variety of key processes (e.g. development,⁵⁻¹¹ germination,¹² resistance against toxic defense molecules,^{13, 14} mucin desulfatation,¹⁵⁻¹⁷ or degradation of mucopolysaccharides^{18, 19}) and the danger of various diseases as a result of their malfunction (e.g. lysosomal disorders^{18, 20}), their mechanism has not been studied in the same detail as that of the related phosphatases.



Scheme 1. General reaction scheme for the PAS-catalyzed hydrolysis of aryl sulfate monoesters **1a-1l** and aryl phosphate monoesters **2b-2l**.

The majority of sulfatases known to date are members of the alkaline phosphatase (AP) superfamily. The mechanism of transition state (TS) stabilization during enzyme-catalyzed substrate hydrolysis of one member of this superfamily, *Escherichia coli* alkaline phosphatase (*EcAP*), has been subject of a large number of in-depth studies involving the experimental tools of linear free energy relationships (LFERs), kinetic isotope effects (KIEs), mutant studies, and

structural analysis by X-ray crystallography.²¹⁻³⁷ In addition computational simulations have been employed to pin-point transition state interactions.^{27, 28, 38-43}

Sulfatase members of the AP superfamily have been studied less extensively. Catalytically important residues have been shown to be conserved among the arylsulfatases^{2, 4, 44-48} and mutant studies (e.g. of human arylsulfatase A,⁴⁹ choline sulfatase,⁴ and the closely related phosphonate monoester hydrolase⁵⁰) have suggested that many of these conserved residues are indeed involved in the catalytic pathway in the enzyme active site. LFERs and KIEs have been measured for a number of phosphatases,^{21-24, 36, 51-59} but only one such study is available for sulfatases.⁶⁰

In addition to the family relationship, members of the AP superfamily are also typically catalytically promiscuous,⁶¹⁻⁶³ i.e. they catalyze multiple, chemically distinct reactions with large rate accelerations.^{64, 65} Within the superfamily reciprocal relationships of crosswise catalytic promiscuity are observed, i.e. the promiscuous activity of one member is the native function of another, and *vice versa*.^{64, 65} Given the postulated role of promiscuity in evolution by gene duplication,^{61, 66} functional crossover defines such functional relationships as pathways for respecialization or repurposing in enzyme superfamilies,⁶⁷ which was experimentally demonstrated recently by a >10⁷-fold specificity switch of a sulfatase to enhance phosphonate hydrolase activity.⁶⁸ As catalysis for the activity under selective pressure must be maintained at a relevant level during evolution, the question of how mechanistic features of these enzymes can be effective for catalysis of different reactions arises.

In this work we have studied the reactions catalyzed by the promiscuous arylsulfatase from *Pseudomonas aeruginosa* (PAS) using LFERs and KIEs. PAS has a wide active site opening⁴⁴ and – in contrast to sulfatases with high specificity for a particular leaving group (such as a sugar moiety^{69, 70} or choline⁴) – accepts a range of aromatic substrates: so that the construction of LFERs

based on series of aryl sulfates of different reactivity is possible with minimal interference from unique binding effects. PAS operates at high rates (k_{cat}/K_M $4.9 \times 10^7 \text{ s}^{-1}\text{M}^{-1}$), even for the hydrolysis of the non-natural substrate 4-nitrophenyl sulfate **1d**.¹ In addition, PAS promiscuously catalyzes the hydrolyses of phosphate mono-,¹ and diesters⁶³ as well as phosphonates,⁷¹ thus covering mechanistically distinct hydrolase reactions.⁷² However, despite its catalytic promiscuity, PAS is a genuine sulfatase: it is typically expressed under sulfate starvation conditions, part of an operon coding for sulfate-processing enzymes, and thought to act as a sulfate scavenger.^{73, 74}

Based on the X-ray structure of PAS⁴⁴ (and those of the human arylsulfatases A^{47, 75} (hASA) and B⁴⁵ (hASB) combined with mutant data for hASA⁴⁹) a double displacement catalytic pathway was proposed, in which a post-translationally modified cysteine,⁷⁶ formylglycine fGly51, performs a nucleophilic attack on the sulfur center (Figure 1b). The covalent hemiacetal intermediate is broken down with assistance of H115 acting as a general base (step 2 in Figure 1b). Finally the aldehyde form of the fGly nucleophile is again hydrated, regenerating the enzyme for the next round of catalysis. Mutant data for several of the analogous active site residues in hASA show that a single mutation of one of the residues likely to be involved in charge compensation during the TS results in lowered but still detectable levels of activity.⁴⁹ During the hydrolysis of sulfates, negative charge is expected to build up in the TS on the sulfuryl group and on the leaving group.^{3, 77} TS stabilization can be achieved by offsetting this charge build-up: using positively charged functionalities such as metal ions, or by using hydrogen bonding or electrostatic interaction with positively charged amino acid side chains. The latter possibly may involve proton transfer to the leaving group oxygen. The active site of PAS contains a number of residues that could neutralize charge build-up on oxygen atoms during the transition state or affect the pK_a of the formylglycine (fGly51) nucleophile (Figure 1).

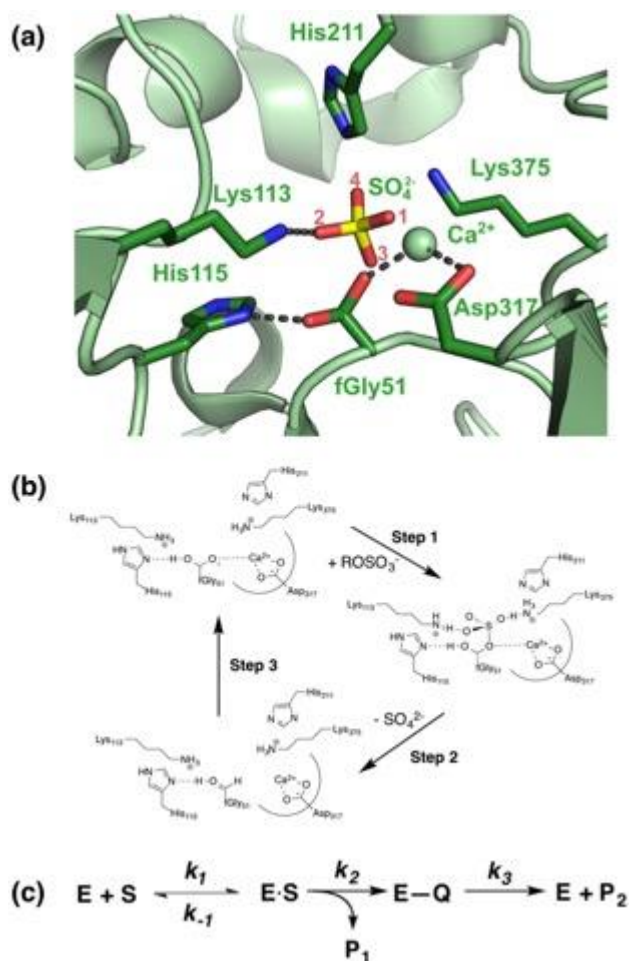


Figure 1. Active site and reaction mechanism of PAS. (a) 3D representation of the active site of PAS with a bound sulfate ion.⁴⁴ The assignment of the analogous non-bridging (O₁-O₃) and bridging (O₄) oxygens in the sulfate ester substrate were based on positions of the active site residues in the X-ray structure of human arylsulfatase A C69A⁷⁵ with *p*-nitrocatechol sulfate bound in the active site. (b) Proposed catalytic pathway for PAS-catalyzed sulfate monoester hydrolysis. Upon binding of the sulfate monoester substrate, the hydrated fGly51 performs a nucleophilic attack on the sulfur atom and the bond to the alcohol leaving group (ROH) is broken (S-O_{bridge} bond fission) (step 1). The covalent intermediate is broken down by base-catalyzed hemiacetal cleavage in which inorganic sulfate acts as the leaving group (step 2). The enzyme is subsequently

regenerated by hydration of the formylglycine aldehyde (step 3). (c) Schematic representation of the steps in panel b. k_1 is the rate of formation of the enzyme-substrate (E.S) complex from free enzyme (E) and substrate (S) and k_{-1} represents the dissociation rate of the ES-complex. k_2 is the rate constant for the nucleophilic attack of the hydrated formylglycine and subsequent S-O_{bridge} bond fission (step 1), and k_3 that of hemiacetal cleavage (step 2). The rehydration of the fGly residue (step 3) is expected to be several orders faster than hemiacetal cleavage, and thus was not considered for the interpretation of pre-steady state kinetics. Product P₁ is the phenolate leaving group expelled from the substrate in step 1; product P₂ is inorganic sulfate.

Here we provide a detailed quantitative examination of the interaction of active site residues with the TS in the arylsulfatase-catalyzed reaction. We use LFER and KIE data to compare the nature of the TS for the native PAS-catalyzed sulfate monoester hydrolysis with the uncatalyzed reaction, and show that, while both are dissociative, the TS of the enzyme-catalyzed reaction has somewhat more associative character. The latter difference is absent for phosphate monoester hydrolysis. Pre-steady-state kinetic data provide information about the rate-limiting step in the overall kinetic mechanism. Finally, changes in LFERs and KIEs as a result of alanine scanning mutagenesis of PAS active site residues suggest that K375 serves as the general acid that minimizes leaving group charge change in the TS and provides leaving group stabilization.

MATERIALS AND METHODS

Sulfate monoester compounds for linear free energy relationships (LFERs) and kinetic isotope effect studies. Sulfate monoester **1d** and phosphate monoesters **2d** and **2k** (Scheme 1) were purchased from Sigma. Sulfate esters were synthesized from the respective phenol and

chlorosulfonic acid, phosphate monoesters from phosphoryl chloride and the respective phenol. Detailed procedures are given in the Supplementary Information (SI). The isotopically labeled forms of 4-nitrophenyl sulfate (**1d**)⁷⁸ and phosphate (**2d**)⁵³ for measurement of kinetic isotope effects were synthesized as described previously.

Protein production and purification. Expression of recombinant *Pseudomonas aeruginosa* Arylsulfatase (PAS, Uniprot ID K9NCQ5) from plasmid pME4322⁷⁹ and derived mutants was done in *E. coli* BL21 (DE3) growing in LB or 2YT-medium containing 30 mg mL⁻¹ kanamycin. The cells were grown at 37 °C until an OD₆₀₀ of around 0.6-0.8 was reached. The culture was cooled to 30 (WT) or 20 °C (Mutants), IPTG was added up to 0.75 mM and the culture was grown for 4 hours at 30 °C (WT) or overnight at 20 °C (Mutants).

Cells expressing PAS were harvested by centrifugation and resuspended in 50 mM Tris-HCl pH 8.0. One tablet of Complete[®] EDTA-Free protease inhibitor cocktail (Roche) per 12 g of wet cell pellet was added to the suspension and the cells were lysed either by using an emulsiflex-C5 homogenizer (Avestin) or by sonication. Cell-free extract (CFE) was obtained by centrifugation of the crude cell lysate at 30,000×g for 90 min. The PAS variants were purified from CFE by subsequent anion exchange (Q-sepharose), hydrophobic interaction (Phenyl sepharose), and size exclusion (Superdex 200) chromatography. All steps were performed in 50 mM Tris-HCl pH 8.0 with the appropriate additive for each step. The anion exchange chromatography was performed as described before.¹ Protein containing fractions that eluted from the anion exchange column were pooled and the combined fractions were brought to 200 mM (NH₄)₂SO₄ by adding the appropriate volume of 50 mM Tris-HCl pH 8.0 + 2 M (NH₄)₂SO₄ and subsequently loaded onto a phenyl sepharose hydrophobic interaction column. The column was washed with two column volumes (CV) of 50 mM Tris-HCl pH 8.0 + 200 mM (NH₄)₂SO₄. Protein was eluted from the column with

a gradient of 200-0 mM (NH₄)₂SO₄ in 50 mM Tris-HCl over 5 CV followed by a further 5 CV with 50 mM Tris-HCl pH 8.0. Fractions containing active protein were pooled and concentrated into 50 mM Tris-HCl pH 8.0 to ~10-15 mg mL⁻¹ protein. The concentrated protein was loaded onto a Superdex 200 prep grade gel filtration column. PAS eluted at the expected elution volume of monomeric PAS. Protein containing fractions were pooled and concentrated to 100-350 μM in 50 mM Tris-HCl and aliquoted in appropriate portions, flash frozen in liquid N₂ and stored at -20 °C. Protein concentrations were calculated based on the molar extinction coefficient at λ = 280 nm, ε₂₈₀ = 102790 M⁻¹ cm⁻¹, calculated from the amino acid sequences using ProtParam (<http://expasy.org/tools/protparam.html>).

Construction of mutants. All mutants of PAS, except for mutant C51S, which was constructed previously,¹ were made by site-directed mutagenesis according to the QuikChange protocol (Agilent), using primers listed in Table S16 (SI) and the appropriate template plasmid.

Enzyme kinetics. All data for steady state enzyme kinetics were recorded at 25 °C in 100 mM Tris-HCl pH 8.0 supplemented with 500 mM NaCl or as indicated. Observed initial rates (V_{obs}) were determined by following an increase in absorbance at a fixed wavelength (ranging from 270 to 400 nm depending on the substrate) as a result of product formation over time in microtiterplates (Spectramax Plus, Molecular Devices) or quartz cuvettes (Varian 100 Bio). Catalytic parameters k_{cat} , K_M and/or k_{cat}/K_M were obtained by fitting the dependency of V_{obs} on substrate concentration ([S]) at a fixed enzyme concentration ([Enz]) (equation 2).

$$V_{obs} = \frac{k_{cat} \times [Enz] \times [S]}{K_M + [S]} \quad \text{eq. 2}$$

The dependency of the various kinetic parameters (k_{cat} , $1/K_M$ and k_{cat}/K_M , represented by K in equation 3) on leaving group ability (as represented by their $\text{p}K_a$ value) was fitted to equation 3 to obtain the observed Brønsted constants for leaving group dependence ($\beta_{\text{leaving group}}^{\text{obs}}$).

$$\log[K] = \beta_{\text{leaving group}}^{\text{obs}} \times \text{p}K_a^{\text{leaving group}} + C \quad \text{eq. 3}$$

Stopped-flow kinetics. Fast kinetics for PAS WT-catalyzed hydrolysis of sulfate monoester **1d** were recorded using a SX20 stopped-flow setup (Applied Photophysics). No unambiguous burst phase could be detected with PAS concentrations between in 1 and 8 μM and 1 mM sulfate monoester **1d**, with 100 mM Tris-HCl pH 8.0, containing 500 mM NaCl at 20 °C. Data are shown in Figure S3.

Kinetic isotope effects (KIEs). Natural abundance **1d** or **2d** was used for measurements of $^{15}(\text{V}/K)$. The ^{18}O KIEs $^{18}(\text{V}/K)_{\text{bridge}}$ and $^{18}(\text{V}/K)_{\text{nonbridge}}$ were measured by the remote label method, using the nitrogen atom in *p*-nitrophenol as a reporter for isotopic fractionation in labeled bridging or nonbridging oxygen positions.⁸⁰ The particular isotopic isomers used are shown in the SI. Isotope effect experiments used 100 μmoles of substrate, at 25 °C in 50 mM Tris buffer, pH 8.0. The substrate concentration was 19 mM and the reactions were started by addition of wild-type or mutant enzyme, 1 μM for substrate **1d**, and 725 μM for substrate **2d**. After reactions reached completion levels between 40% and 60% they were stopped by titration to pH 3 with HCl. Protocols for isolation of *p*-nitrophenol, isotopic analysis, and calculation of the isotope effects were the same as previously described,^{53, 78} and are described in the SI.

RESULTS AND DISCUSSION

Determination and interpretation of steady state parameters. Purified PAS WT¹ was used to determine Michaelis-Menten parameters k_{cat} , K_{M} and $k_{\text{cat}}/K_{\text{M}}$ for a series of phenyl sulfate monoesters (compounds **1a-1l**, Scheme 1) with varying leaving group abilities (represented by their $\text{p}K_{\text{a}}$ values, which range from 5.2 to 10.35 (Table S1 and Figures S1 and S2, supporting information, SI). These substrates provide insight into the nature of the transition state (TS) of PAS-catalyzed sulfate monoester hydrolysis by allowing the construction of linear free energy relationships (LFERs), similar to those reported for several phosphoryl transfer enzymes.^{22, 23, 32, 33, 56-59, 72, 81, 105}

The underlying meaning of the steady state parameters used to monitor the bond-making and -breaking processes must be carefully considered. Since the steady-state catalytic rate constant (k_{cat}) was shown to be essentially independent of leaving group ability (Figure 2a, varying between 9-18 s^{-1} , Table S1, SI), it does not reflect the substrate reactivity. Based on the kinetic scheme for enzyme-catalyzed substrate hydrolysis (Figure 1c), the relevant rate constant to probe the nature of the TS should be k_2 . If enzyme-catalyzed cleavage of the sulfate ester bond were fully rate-limiting, the steady state catalytic rate constant k_{cat} would be essentially equal to k_2 (equation S14, SI). If this is not the case, i.e. if $k_2 \geq k_3$, more complex terms arise (see equations S1-3, SI). Assuming that the substrate binding constants ($K_{\text{D}} = k_{-1}/k_1$) are similar for the complete series of sulfate monoesters (Scheme 1), $k_{\text{cat}}/K_{\text{M}}$ reports on all steps from free enzyme and substrate to the first irreversible transition state. In this case the formation of the covalent intermediate between the formylglycine nucleophile and the respective sulfur center of the substrates that leads to leaving group departure is likely to be irreversible, as no significant inhibition by the phenolate product is observed. (See equations S1-S3 in the SI for details on the relation between the Michaelis-Menten parameters and the individual rate constants for the various enzymatic steps).

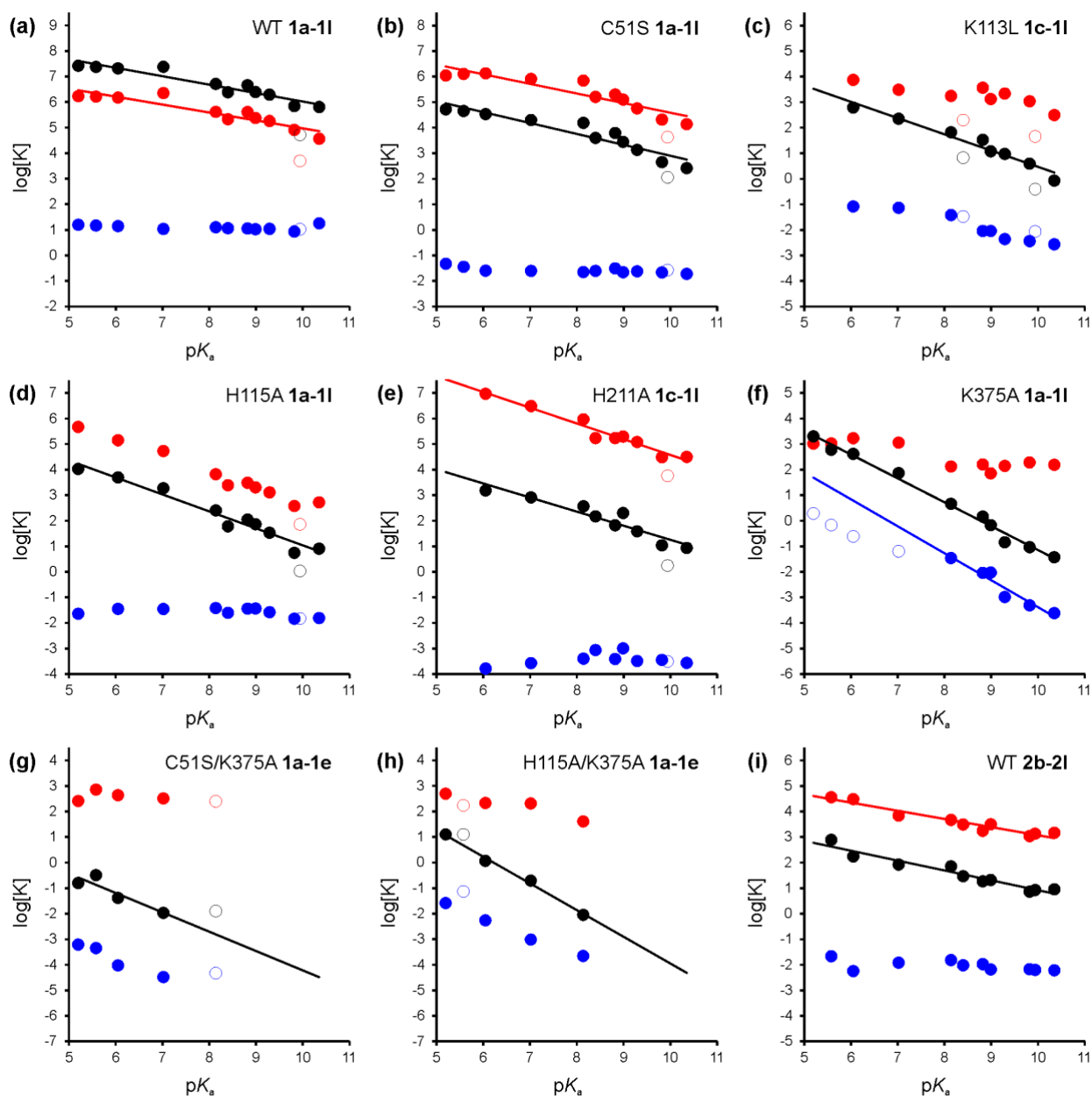


Figure 2. Dependence of catalytic parameters k_{cat} (blue) $1/K_M$ (red) and k_{cat}/K_M (black) (log-values) for PAS-catalyzed hydrolysis of sulfate monoesters on leaving group ability (as represented by the pK_a of the free phenol in solution). (a) PAS WT (see for a superposition of the data measured here with that of Williams *et al.*⁶⁰ in Fig. S6, SI); (b) PAS C51S; (c) PAS K113L; (d) PAS H115A; (e) PAS H211A; (f) PAS K375A; (g) PAS C51S/K375A; (h) PAS

H115A/K375A. (i) PAS WT with phosphate monoesters. All data were obtained in 100 mM Tris-HCl pH 8.0; 500 mM NaCl at 25 °C. The resulting slopes ($=\beta_{\text{leaving group}}^{\text{obs}}$) are listed in Table 1 and S14. The data for the unsubstituted phenol sulfate monoester **1k** deviated significantly from the trend for all enzymes, suggesting a consistent unique difference in binding compared to the other sulfate monoester substrates and were therefore not included in any of the fits. Data points represented by open circles were not included in the fits: e.g. **1f** panel c, in which the fitted value for K_M deviated by an order of magnitude; **1a-1d** in panel f for the fit in blue, where the slope for k_{cat} was only expected to be relevant for the TS if fully representing the rate-limiting step: this is only the case for leaving groups with $\text{p}K_a > 8$, so other points were excluded to reflect the chemical reaction in $\beta_{\text{leaving group}}^{\text{obs}}$ as much as possible, see also Figure S10, SI; **1e** in panel g and **1b** in panel h were excluded, because one parameter deviated strongly, again suggesting idiosyncratic effects in these substrates. All data for the kinetic parameters are listed in the supporting information (Table S1, S3-S10)

LFER for PAS WT-catalyzed sulfate monoester hydrolysis. Plots of k_{cat}/K_M against the leaving group $\text{p}K_a$ (Figure 2) were linear, with a $\beta_{\text{leaving group}}^{\text{obs}}$ of -0.33 (Table 1) that was considerably less negative than that of the rate for the uncatalyzed hydrolysis reaction (k_{uncat}), for which a $\beta_{\text{leaving group}}^{\text{obs}}$ of -1.81 has been measured.³ This work partially reproduces a study of Williams *et al.*⁶⁰, who recently constructed a LFER for PAS, but arrived at a much steeper slope (-0.86). A comparison of both studies (see Figure S6, SI) shows that most points superimpose well with our data, but that the choice of leaving groups with higher $\text{p}K_a$ (specifically the inclusion of the bulky 4-amino-acetyl- and 4-methoxyphenolate), the narrower range of $\text{p}K_a$ -values (three compared to almost five log units covered here) as well as basing the study on overall fewer data

points (7 vs 12 in our work) with worse significance (p-value, 0.026 vs. $<10^{-4}$) and correlation coefficients (R^2 , 0.66 vs. 0.91) seem to have resulted in a distortion of the slope due to idiosyncratic effects of substrates (e.g. due to steric clashes or interactions between active site residues and phenolate substituents). Starting from the quantitatively different values for $\beta_{\text{leaving group}}^{\text{obs}}$ our analysis below naturally differs from that of Williams *et al.*. However, the slope $\beta_{\text{leaving group}}^{\text{obs}}$, is *less* steep compared to that for the solution reaction in both studies, despite the quantitative differences.

Table 1. Overview of the observed Brønsted constants for leaving group dependence ($\beta_{\text{leaving group}}^{\text{obs}}$) of k_{cat}/K_M for PAS-catalyzed sulfate and phosphate monoester hydrolysis.^a

Reaction	catalyst	$\beta_{\text{leaving group}}^{\text{obs}}$
Sulfate monoesters	Solution (neutral)	-0.27 ^b
	Solution (monoanion)	-1.81 ^b
	WT	-0.33±0.04
	C51S	-0.43±0.05
	K113L	-0.64±0.06
	H115A	-0.67±0.05
	H211A	-0.55±0.07
	K375A	-0.94±0.04
	C51S/K375A	-0.76±0.24
	K113L/K375A	nd ^c
	H115A/K375A	-1.04±0.06
	H211A/K375A	nd ^c
	Phosphate monoesters	Solution (monoanion)

Solution (dianion)	-1.23 ^d
WT	-0.39±0.04

^aAll enzymatic reactions performed in 100 mM Tris-HCl pH 8.0, 500 mM NaCl, 25 °C. ^bEdwards *et al.*,³ H₂O as the nucleophile. ^cNot determined. Activity too low to be detectable. ^dKirby & Vargolis,⁸² H₂O as the nucleophile.

A possible cause for the considerably less negative $\beta_{\text{leaving group}}^{\text{obs}}$ compared to the solution reaction could be that the $k_{\text{cat}}/K_{\text{M}}$ -values do not only represent a chemical step, as shown previously for wild-type *E. coli* alkaline phosphatase (AP).^{35, 53, 83} However, considerations summarized in the SI (section H) rule out diffusion control and suggest that the deviation of $\beta_{\text{leaving group}}^{\text{obs}}$ from the $\beta_{\text{leaving group}}$ of the solution reaction is a genuine effect of substrate binding and turnover by the enzyme.

Previous experimental studies into the nature of the TS of enzyme-catalyzed sulfate transfer using LFERs^{60, 84} typically showed less steep correlations than the corresponding solution reaction,⁸⁵⁻⁸⁸ bringing the Brønsted slopes to values closer to zero. This decrease could be ascribed to interactions with cationic groups in the active site, but no KIEs or mutational data were available to check this hypothesis. In addition a LFER for sulfate transfer has been reported for the promiscuous sulfatase activity of AP WT.³¹ Subsequent KIE studies showed that in this case a dissociative TS is likely.²¹ In AP the less negative $\beta_{\text{leaving group}}^{\text{obs}}$ compared to the solution reaction could arise from interaction of the leaving group with positively charged moieties in the enzyme active site, most likely a divalent metal ion (Zn²⁺). The latter phenomenon has also been reported for AP-catalyzed phosphate monoester hydrolysis.⁸⁹ In several protein tyrosine phosphatases a protonated aspartate was identified as responsible for leaving group stabilization.^{51, 52, 54-56, 59}

Replacement of this aspartate with asparagine restored the leaving group dependence to a value close to that of the solution reaction.⁵⁶ In protein phosphatase 1 (PP1), a histidine was proposed to assume the same role, although practical limitations (low yield and poor activity) prevented experimental verification.⁵⁸

LFER for PAS WT-catalyzed phosphate monoester hydrolysis. The fact that PAS WT is also a proficient phosphatase¹ ($(k_{\text{cat}}/K_{\text{M}})/k_{\text{uncat}} = 2.9 \times 10^{11} \text{ M}^{-1}$ towards phosphate monoester **2d**), opens up the possibility of studying two reactions that proceed through similar TSs in solution^{31, 53, 77, 82, 90, 91} in a single active site, and also facilitates comparisons with the more widely studied phosphatases.

Michaelis-Menten parameters were determined for a series of phosphate monoesters (**2b-2l**, Scheme 1). As for the sulfatase reaction, k_{cat} is practically independent of the leaving group $\text{p}K_{\text{a}}$ (varying between $0.6\text{-}1.2 \times 10^{-2} \text{ s}^{-1}$, Figure 2i and Table S3, SI), suggesting that the rate-limiting step for phosphate as well as sulfate monoesters is not leaving group-dependent. The K_{M} values increase with leaving group $\text{p}K_{\text{a}}$ and range from 0.03-0.92 mM, around ~ 100 -fold higher than for sulfatase activity. The slope of the Brønsted plot $\beta_{\text{leaving group}}^{\text{obs}}$ for $k_{\text{cat}}/K_{\text{M}}$ for phosphate monoester hydrolysis is -0.39 ± 0.04 (Table 1), identical within the error margins to the value observed for the sulfatase reaction (and confirmed by a cross-correlation graph with a slope of unity, see Figure S9, SI). As observed for the sulfatase reaction, enzyme-catalyzed phosphate monoester hydrolysis is considerably less sensitive to leaving group ability than the solution reaction for the phosphate monoester dianion ($\beta_{\text{leaving group}} = -1.23^{82}$). As for sulfate monoester hydrolysis, these considerable deviations can be caused by stabilization/masking of locally developing negative charge, in particular on the leaving group oxygen. This idea is probed in the next paragraph. The less negative value for $\beta_{\text{leaving group}}$ is also consistent with an intrinsically more associative nature of the respective

enzymatic TSs (compared to the solution reaction), possibly reinforced by the presence of cationic groups in the active site. This interpretation assumes the same degree of nucleophilic involvement. If this is not the case, the $\beta_{\text{leaving group}}$ value will only reflect the extent of leaving group cleavage. However, kinetic isotope effect studies (see below for details) discounted these possibilities and suggested that the TS is similar (but not identical) to the solution reaction, as in previously studied phosphatases.^{23, 37, 56, 58, 59}

The effect of mutations on the $\beta_{\text{leaving group}}^{\text{obs}}$ for sulfate monoester hydrolysis. As discussed above, the $\beta_{\text{leaving group}}^{\text{obs}}$ of enzyme-catalyzed phosphate and sulfate transfer reactions can be influenced by compensation by positively charged moieties in the active site of the negative charge build-up that occurs during the TS.^{23, 33, 37, 58, 84} Furthermore the change in nucleophile between the solution (H₂O) and enzyme-catalyzed (formylglycine) reaction can also influence the $\beta_{\text{leaving group}}^{\text{obs}}$. Zalatan *et al.*³⁷ formulated these considerations in order to be able to calculate expected differences in leaving group dependence between enzyme-catalyzed ($\beta_{\text{leaving group}}^{\text{Enz}}$) and solution reactions ($\beta_{\text{leaving group}}^{\text{solution}}$) (equation 1). The expected change in leaving group dependence resulting from a change in nucleophile between the enzyme-catalyzed (fGly) and solution (H₂O) reaction is calculated from the difference in nucleophilicity ($pK_{\text{nuc}}^{\text{Enz}} - pK_{\text{nuc}}^{\text{solution}}$) weighed with the sensitivity of the leaving group dependence of the reaction type to a change in nucleophile (p_{xy}). The second of the contributing factors is leaving group dependent binding of the ground state (GS, $\beta_{\text{bind}}^{\text{GS}}$) and TS ($\beta_{\text{bind}}^{\text{TS}}$). For $k_{\text{cat}}/K_{\text{M}}$ -based $\beta_{\text{leaving group}}$ values the latter two are indistinguishable from each other and are treated as a single variable ($\sum\beta_{\text{bind}} = \beta_{\text{bind}}^{\text{GS}} + \beta_{\text{bind}}^{\text{TS}}$).

$$\beta_{\text{leavinggroup}}^{\text{Enz}} = \beta_{\text{leavinggroup}}^{\text{solution}} + p_{xy} \times (pK_{\text{nuc}}^{\text{Enz}} - pK_{\text{nuc}}^{\text{solution}}) + \beta_{\text{bind}}^{\text{TS}} + \beta_{\text{bind}}^{\text{GS}} \quad (\text{eq. 1})$$

For *E. coli* AP in particular the interaction of the leaving group oxygen with one of the two Zn^{2+} ions during the GS and TS was thought to be mainly responsible for the $\sum\beta_{\text{bind}}$ of +0.33⁸⁹ (3-times larger than the expected contribution of the change in nucleophile from H_2O to the active site serine of +0.11). Selective removal of only the Zn^{2+} -ion responsible for leaving group stabilization is not feasible and therefore experimental assessment of these calculations was not possible. The assignment of the analogous positions of the non-bridging and bridging (leaving group) oxygens in an inorganic sulfate molecule with the active site residues of PAS (as shown in the X-ray structure,⁴⁴ Figure 1), was based on homology with structural data for the enzyme-substrate complex of an inactive variant of human arylsulfatase A.⁷⁵ The majority of the interactions with the non-bridging and leaving group oxygens are expected to be provided by amino acid side-chains, which opens up the possibility of assessing the importance of the correction factors of equation 1 experimentally by determining the leaving group dependence of active site mutants of PAS. To this end the nucleophile (fGly51) and a residue that directly interacts with it (H115) were mutated to assess the contribution of the nucleophile. Furthermore, several positively charged groups expected to provide charge compensation during the GS and TS by interacting with non-bridging (K113 and K375) and leaving group (H211 and K375) oxygens were removed by mutating the respective residues into alanine or leucine.

All mutants were purified to homogeneity and Michaelis-Menten parameters determined for the same series of sulfate monoesters (**1a-1l**, Scheme 1) as used with the wild-type enzyme. The mutations resulted in 10^3 - 10^8 -fold drops in catalytic efficiencies ($k_{\text{cat}}/K_{\text{M}}$) for the various substrates (Tables S4-S8, SI). For mutants C51S and H211A k_{cat} was still independent of leaving group ability (Figure 2b and 2e respectively), suggesting that, as in the wild-type enzyme, the leaving group-dependent step is not rate limiting. However, the k_{cat} for these mutants was reduced $\sim 10^3$ -fold

(C51S) and $\sim 10^5$ -fold (H211A) compared to PAS WT. The K_M values for PAS C51S and H211A were within an order of magnitude of those for the wild-type enzyme (Tables S4 and S7) and, as observed for wild-type PAS, the $\beta_{\text{leaving group}}^{\text{obs}}$ values for k_{cat}/K_M and $1/K_M$ are nearly identical. For PAS K113L and H115A both k_{cat} and $1/K_M$ decreased with increasing leaving group ability (Figure 2c and d, Table S5 and S6), albeit it only at the higher end of the pK_a spectrum for H115A. For PAS K375A k_{cat} decreases with increasing leaving group pK_a , indicating that the rate-limiting step is largely leaving group dependent. The K_M -values are increased $\sim 10^3$ -fold compared to the wild-type enzyme and are largely constant (varying in a range of 5-10 mM) for the substrates with a leaving group $pK_a > 8$ (Figure 2f, Table S8). The $\beta_{\text{leaving group}}^{\text{obs}}$ for k_{cat}/K_M is nearly identical to that for k_{cat} for $pK_a > 8$. As for PAS WT, the LFERs for k_{cat} and $1/K_M$ for PAS K113L, H115A and K375A could be simulated based on assumed values for the pre-steady state kinetic parameters (Figure S10, SI). In particular for K113L and K375A the break in the LFER for k_{cat} could be explained by a change of the rate-limiting step with increasing $pK_a^{\text{leaving group}}$.

The $\beta_{\text{leaving group}}^{\text{obs}}$ of all active-site mutants was less negative than the $\beta_{\text{leaving group}}^{\text{obs}}$ for the wild type. $\Delta\beta_{\text{leaving group}}$ ($=\beta_{\text{leaving group}}^{\text{obs-WT}} - \beta_{\text{leaving group}}^{\text{obs-mutant}}$) was calculated as the slope of the linear correlation of $\log[(k_{\text{cat}}/K_M)_{\text{WT}}/(k_{\text{cat}}/K_M)_{\text{mutant}}]$ vs. $pK_a^{\text{leaving group}}$ (Table 2, Figure S11a). As described above the difference in leaving group dependencies between enzyme-catalyzed and uncatalyzed sulfate and phosphate monoester hydrolysis is influenced by the nature of the nucleophile and charge compensation effects (equation 1). Based on the pH-rate profile for PAS-catalyzed sulfate monoester hydrolysis the pK_{nuc} of the enzyme is expected to be < 7.2 .¹ The p_{xy} -value for sulfate transfer is not known, but is expected to be similar to the value for phosphate monoesters (0.013).⁹² Assuming that the $pK_{\text{nuc}}^{\text{Enz}} \sim 6$ and $pK_{\text{nuc}}^{\text{solution}} = -1.7$ (nucleophile = H_2O), the effect of the change in nucleophile between the PAS WT-catalyzed and the solution reaction

is expected to be $0.013 \times (6 - (-1.7)) = +0.10$. Assuming the difference in nucleophilicity between fGly (solution $pK_a \sim 13-14^{93}$) and serine ($pK_a \sim 16$, similar to that of ethanol) in solution translates into the same difference in the enzyme active site, a small increase in the contribution of the nucleophile term in equation 1 is expected for mutant C51S. Based on this assumption the $\Delta\beta_{\text{leaving group}}^{\text{WT-C51S}}$ is expected to be small and negative. However, we measure a value of +0.10 (Table 2). Since fGly is interacting directly with the Ca^{2+} -ion, changing it to a serine may cause a change in the charge compensation effects provided by the divalent cation, which could explain the positive value for $\Delta\beta_{\text{leaving group}}^{\text{WT-C51S}}$. Removal of H115 is expected to result in a slight increase in the pK_a of the nucleophile, again predicting a small negative $\Delta\beta_{\text{leaving group}}^{\text{WT-H115A}}$ as a result. However, the measured value of +0.32 suggests that any small effect of the mutation on the nucleophile term is overshadowed by a considerable decrease in $\sum\beta_{\text{bind}}$.

Table 2. Effect of mutations on leaving group dependence for PAS-catalyzed sulfate monoester hydrolysis in PAS WT and K375A

mutation	reaction	effect in	$\Delta\beta_{\text{leaving group}}^{\text{obs}}$
C51S	1a-1l	WT	$+0.10 \pm 0.04^a$
	1a-1d	K375A	$+0.01 \pm 0.33^b$
K113L	1c-1l	WT	$+0.24 \pm 0.05^a$
H115A	1a, 1c-1l	WT	$+0.32 \pm 0.03^a$
	1a-1l	K375A	$+0.15 \pm 0.05^b$
H211A	1c-1l	WT	$+0.16 \pm 0.07^a$
K375A	1a-1e	WT	$+0.61 \pm 0.03^a$

^aDetermined as the slope for $\log[(k_{\text{cat}}/K_M)_{\text{WT}}/(k_{\text{cat}}/K_M)_{\text{mutant}}]$ plotted vs. leaving group pK_a (Figure S10a). ^bDetermined as the slope for $\log[(k_{\text{cat}}/K_M)_{\text{K375A}}/(k_{\text{cat}}/K_M)_{\text{double mutant}}]$ plotted vs. leaving group pK_a (Figure S10b, SI).

The removal of the residues that directly interact with the leaving group oxygen is expected to have a large effect on $\sum\beta_{\text{bind}}$, whereas groups that interact only with the non-bridging oxygens are expected to contribute to $\sum\beta_{\text{bind}}$ at $\sim 10\%$ of the value expected for direct interactions with the leaving group oxygen.³⁷ Mutation of H211 resulted in a $\Delta\beta_{\text{leaving group}}^{\text{WT-H211A}}$ of only +0.16, even though this residue interacts exclusively with the leaving group oxygen. Removal of the nearby K375 results in a $\Delta\beta_{\text{leaving group}}^{\text{WT-K375A}}$ of +0.61. Since the removal of a positive charge is expected to increase the $pK_{\text{nuc}}^{\text{Enz}}$, the actual effects on $\sum\beta_{\text{bind}}$ may be slightly higher than observed. Taken together these data suggest that K375 is largely responsible for leaving group stabilization, either by electrostatic charge offset or by proton transfer. However, unlike in PTPase,⁵⁶ where a protonated aspartate performs the same function, its removal does not result in a near-complete abolition of the difference between $\beta_{\text{leaving group}}^{\text{obs-Enz}}$ and $\beta_{\text{leaving group}}^{\text{obs-solution}}$, since $\Delta\beta_{\text{leaving group}}^{\text{PAS K375A-solution}} = +0.87$. Values of $\sim +0.1-0.2$ would be expected in case of complete removal of charge compensation on the leaving group as a result of the mutation (i.e. $\sum\beta_{\text{bind}} = 0$, only the change in nucleophile results in a small positive $\Delta\beta_{\text{leaving group}}^{\text{obs}}$). This observation could be rationalized by partial compensation of the loss of charge offset (or of partial proton transfer) provided by K375 by nearby H211. The same phenomenon (i.e. that K375 partially assumes the role of H211) could explain the relatively small effect of mutation H211A on $\beta_{\text{leaving group}}^{\text{obs}}$. The combined effects of these two residues find support in the double mutant enzyme PAS H211A/K375A, for which no sulfatase activity was detectable ($k_{\text{cat}}/K_{\text{M}} < 5 \times 10^{-6} \text{ s}^{-1} \text{ M}^{-1}$ for sulfate monoester **1d**, see SI for consideration of the experimental detection limit). This observation strengthens the idea that the effect of each mutation is buffered by the nearby presence of a residue that can take over its function: if the measured reductions in activity for the single mutants were simply additive, the

expected k_{cat}/K_M for sulfate monoester **1d** should be $2.6 \times 10^{-3} \text{ s}^{-1} \text{ M}^{-1}$ (according to equation S21, SI), and still be detectable. By contrast, their cooperativity leads to a larger detrimental effect, when both are removed.

As discussed above the effect of leaving group dependent ground and transition-state binding ($\sum\beta_{bind}$) is expected to be modest for residues that interact mainly with the non-bridging oxygens: $\sim 10\%$ (for all the non-bridging oxygens combined) of the value expected for leaving group oxygen⁸⁹. This consideration suggests that the maximal combined effect of K113, K375 and Ca^{2+} on $\sum\beta_{bind}$ via interactions with the non-bridging oxygens is expected to be $\sim +0.13$ ($10/110 \times (\beta_{leaving\ group}^{obs-WT} - \beta_{leaving\ group}^{obs-solution}) = 0.09 \times (-0.33 - (-1.81)) = +0.13$). Since removal of a positive charge is expected to increase the pK_{nuc} of the enzymatic reaction, the observed effect on $\sum\beta_{bind}$ of the interaction with non-bridging oxygens, as a result of removing any of these three functional groups is expected to be $< +0.05$. However, mutation K113L results in a $\Delta\beta_{leaving\ group}^{WT-K113L}$ of $+0.24$. This large effect partly explains why removal of H115 has an unexpectedly large $\Delta\beta_{leaving\ group}^{WT-H115A}$ of $+0.32$, since H115 and K113 interact closely in the 3D structure of PAS. The large effect of mutations C51S, K113L and H115A on PAS activity suggests that these mutations influence the interaction of the H211A/K375A pair with the leaving group oxygen. If the effects were electrostatic and isolated from the interactions with the leaving group oxygens, the effect of these mutations would be expected to be identical in K375A and WT, i.e. these three mutations should be additive to mutation K375A. The combined effects of K113L and K375A result in completely inactive enzyme ($k_{cat}/K_M < 5 \times 10^{-6} \text{ s}^{-1} \text{ M}^{-1}$ for sulfate monoester **1d**), which is lower than the expected value ($7.0 \times 10^{-4} \text{ s}^{-1} \text{ M}^{-1}$; based on eq. S21, SI) for an additive effect of both mutations. This can be explained by interaction of both K113 and K375 with the non-bridging oxygens, in which case their simultaneous removal most likely results in complete abolition of

substrate binding. The introduction of mutations C51S and H115A into the K375A variant does result in enzymes with detectable activities. However, the effect on the $\beta_{\text{leaving group}}^{\text{obs}}$ is much lower in the K375A mutant than in the wild-type enzyme, if present at all (C51S in K375A has no significant effect on $\beta_{\text{leaving group}}$, see Table 2 and Figure S10b for details). The non-additive behavior suggests that the unexpectedly large value of $\Delta\beta_{\text{leaving group}}^{\text{WT-mutant}}$ for both these mutations is due to their effect on the interactions between the leaving group oxygen and K375, i.e. this effect cannot be achieved unless K375 is present. Possible explanations for this phenomenon are (i) changes in the overall electrostatic character of the active site as result of the mutations that decrease the strength of the interaction between K375 and the leaving group oxygen or (ii) changes in substrate positioning that cause the optimal configuration of the K375-leaving group oxygen pairing to be distorted, resulting in a lower contribution of this interaction to TS stabilization.

In addition (or as an alternative) to the above considerations of charge compensation, changes in $\beta_{\text{leaving group}}^{\text{obs}}$ can also reflect changes in the nature of the transition state. We discuss these aspects together with kinetic isotope effects below.

Kinetic Isotope Effects (KIEs). Kinetic isotope effects were measured for the bridging (or leaving group) and non-bridging oxygens as well as the nitro group for enzyme-catalyzed hydrolysis of sulfate monoester **1d** and phosphate monoester **2d** (Figure 3; Chart S1, SI), to complement the Brønsted analysis above.^{56, 58} The KIE experiment requires turnover of approximately half of a 100 μmol sample of labeled substrate. With mutants of low activity this can require long reaction times, or unpractically large amounts of enzyme. For that reason, most but not all of the mutants for which kinetic data are presented have accompanying KIE data in Table 3.

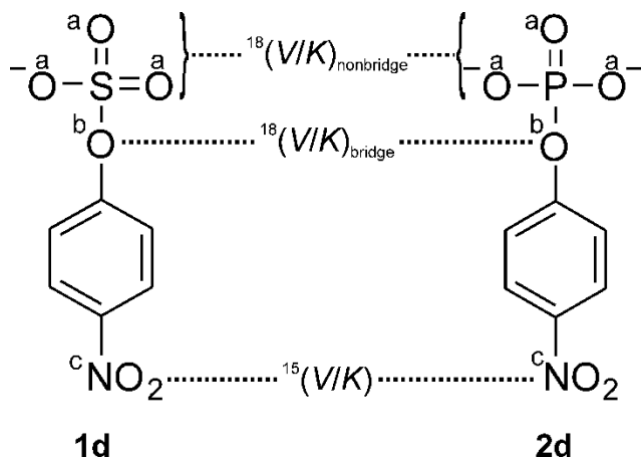


Figure 3. Positions for the kinetic isotope effect measurements in sulfate monoester **1d** and phosphate monoester **2d**. Effects were measured for the non-bridging oxygens (a, $^{18}\text{O}_{\text{nonbridge}}$ KIE), bridging oxygen (b, $^{18}\text{O}_{\text{bridge}}$ KIE), and for the nitro group (c, ^{15}N KIE). See Chart S1 (SI) for the structures of the labeled compounds used.

Because the KIEs were measured by the internal competition method they describe effects on $k_{\text{cat}}/K_{\text{M}}$ (or V/K),⁹⁴ encompassing steps from free enzyme and substrate up to the first irreversible step, regardless of which step in the full mechanism is rate-limiting. In the present case, as described above, this is representative of the rate constant for the first chemical step (k_2 in Figure 1c). Thus, the reported KIEs reflect the TS for the substrate reacting with the formyl glycine nucleophile, even though the overall rate-determining step is breakdown of the intermediate.

The expected ranges of the isotope effects in sulfate monoester **1d** and phosphate monoester **2d** and their interpretation have been discussed in detail previously.^{80, 95} The secondary KIE at the nitrogen atom, $^{15}(V/K)$, reports on negative charge development on the nitrophenolate leaving group in the transition state. The *p*-nitrophenolate anion has contributions from a quinonoid resonance form, with decreased N-O bond order and increased N-C bond order. Because N-O

bonds have tighter vibrational frequencies, the nitrogen atom is more tightly bonded in neutral *p*-nitrophenol (or in substrates **1d** and **2d**) than in the phenolate anion.⁹⁶ Thus, the ¹⁵N KIE for deprotonation of *p*-nitrophenol is normal. When protonation or other interactions maintain the leaving group in a neutral state, there is no isotope effect (KIE = unity). This KIE reaches its maximum value of about 1.003, reflecting a full negative charge, when the leaving group in the TS has a very high degree of bond fission and no interactions neutralize the resulting charge. The KIE at the bridging oxygen atom, ¹⁸(*V/K*)_{bridge}, is a primary isotope effect that arises from S-O or P-O bond fission and is also affected by O-H bond formation, if the leaving group is simultaneously protonated in the TS. Bond fission produces normal isotope effects, primarily due to reduction of the stretching vibration in the TS as the force constant is lowered. Protonation of this atom in the TS gives rise to inverse effects, from the new vibrational modes introduced from the forming bond. A large body of data from phosphate and sulfate ester hydrolysis shows the isotope effect from P-O or S-O bond fission is normally larger in magnitude than the inverse effect from protonation. A normal ¹⁸(*V/K*)_{bridge} effect near its maximum of 1.03 reflects a largely broken S-O or P-O bond in the TS, arising from loss of vibrations involving this bond. In native enzymes utilizing general acid catalysis, or uncatalyzed reactions under acidic conditions, the observed ¹⁸(*V/K*)_{bridge} is reduced by protonation, as shown in Table 3.

The leaving group KIEs ¹⁵(*V/K*) and ¹⁸(*V/K*)_{bridge} report on how leaving group stabilization might be compromised by mutation. Loss of general or Lewis acid catalysis will result in increases in both of these KIEs relative to the native enzyme. The isotope effect on the nonbridging oxygen atoms, ¹⁸(*V/K*)_{nonbridge}, monitors the hybridization state of the transferring sulfuryl or phosphoryl group, which affects the P-O or S-O nonbridging bond orders and hence their vibrational frequencies. A loose transition state gives rise to slightly inverse effects as these bond orders

increase. This isotope effect becomes increasingly normal (i.e. approaching or exceeding a value of 1) as the transition state grows more associative in nature.

Table 3. Kinetic isotope effects for PAS-catalyzed hydrolysis of sulfate monoester **1d** and phosphate monoester **2d**.

substrate	catalyst	$^{15}(V/K)$	$^{18}(V/K)_{\text{bridge}}$	$^{18}(V/K)_{\text{nonbridge}}$
1d	solution (neutral) ^a	1.0004 (1)	1.0101 (2)	1.0098 (3)
	solution (monoanion) ^b	1.0026 (1)	1.0210 (10)	0.9951 (3)
	WT	1.0006 (4)	1.0088 (9)	1.0064 (15)
	C51S	1.0012 (6)	1.0097 (19)	1.0039 (4)
	H115A	1.0013 (2)	1.0216 (17)	1.0004 (6)
	H211A ^c	-	-	-
	K375A	1.0023 (5)	1.0244 (23)	0.9958 (11)
2d	solution (monoanion) ^d	1.0004 (2)	1.0087 (3)	1.0184 (5)
	solution (dianion) ^d	1.0028 (2)	1.0189 (5)	0.9994 (5)
	WT	1.0007 (4)	1.0102 (9)	0.9912 (8)

^aRecorded in 10 N HCl, 15 °C.⁷⁸ ^bRecorded at pH 9.0, 85 °C.⁷⁸ ^cActivity too low to be determined.
^dRecorded at 95 °C.⁵³

The small $^{15}(V/K)$ of 1.0006 (Table 3), for PAS WT-catalyzed hydrolysis of sulfate monoester **1d** suggests nearly complete neutralization of the negative charge developing on the leaving group from S-O bond fission. Similar neutralization occurs by intramolecular pre-equilibrium proton transfer during the uncatalyzed hydrolysis of neutral sulfate monoesters under acidic conditions⁷⁸ ($^{15}(V/K) = 1.0004$). The $^{18}(V/K)_{\text{bridge}}$ KIE for the PAS-catalyzed reaction is similar to that of the uncatalyzed hydrolysis of the neutral monoester⁷⁸ and the AP-catalyzed sulfate monoester

hydrolysis²¹ (Table 3, Figure 4a). In both cases significant masking of leaving group charge development occurs due to intramolecular proton transfer, or interaction with a Lewis acid, respectively. The magnitude of $^{18}(V/K)_{\text{bridge}}$ is consistent with significant S-O_{bridge} bond fission concomitant with leaving group stabilization, either by interaction with a positively charged group (Lewis acid) or protonation. A similar observation has been made for phosphate monoester hydrolysis catalyzed by AP,²¹ protein phosphatase 1⁵⁸ (PP-1) and several protein-tyrosine phosphatases (PTPs)^{51, 52, 54, 55, 97} (Figure 4a, Table S15).

In the PAS WT-catalyzed hydrolysis of phosphate monoester **2d**, the $^{15}(V/K)$ and $^{18}(V/K)_{\text{bridge}}$ KIEs also indicate near complete neutralization of the charge on the leaving group, and significant P-O_{bridge} bond fission (Table 3, Figure 4a). The $^{18}(V/K)_{\text{nonbridge}}$ KIE for PAS WT-catalyzed hydrolysis of phosphate monoester **2d** was more inverse than for the uncatalyzed solution reaction of the dianion (0.9912 vs 0.9994; Table 3, Figure 4b), and is similar to the value for its hydrolysis by the superfamily member AP R166S (0.9925).³⁶ In the case of AP the inverse shift in this KIE was attributed to interactions of the phosphoryl group with the metal ions and hydrogen bonding residues at the active site. In the PAS reaction similar interactions are possible (Figure 1). For the PAS WT-catalyzed hydrolysis of sulfate monoester **1d** the $^{18}(V/K)_{\text{nonbridge}}$ is normal (1.0064, or +0.64%, Table 3 and Figure 4b), in contrast to the inverse $^{18}(V/K)_{\text{nonbridge}}$ for the uncatalyzed hydrolysis of the **1d** monoanion⁷⁸ (-0.49%). This value is closer to the $^{18}(V/K)_{\text{nonbridge}}$ for the hydrolysis of the neutral sulfate monoester⁷⁸ (+0.98%). However, in this case, the normal KIE arises from deprotonation of the sulfuryl group (i.e. proton transfer from S-O-H to the leaving group). In previous investigations of the TS for enzymatic sulfate and phosphate transfer the drop in the normal $^{18}(V/K)_{\text{bridge}}$ was accompanied by a more inverse²¹ or mostly unchanged $^{18}(V/K)_{\text{nonbridge}}$ KIE^{51, 52, 54-56, 58, 97} (compared to the $^{18}(V/K)_{\text{nonbridge}}$ for uncatalyzed phosphate

dianion hydrolysis, i.e. ≤ 0.9994), and suggests that the TS in these enzymatic reactions is still largely dissociative despite the less negative $\beta_{\text{leaving group}}^{\text{obs}}$ and drop in normal $^{18}(V/K)_{\text{bridge}}$ KIE compared to the uncatalyzed reaction, which arise from neutralization of the charge developing on the leaving group.

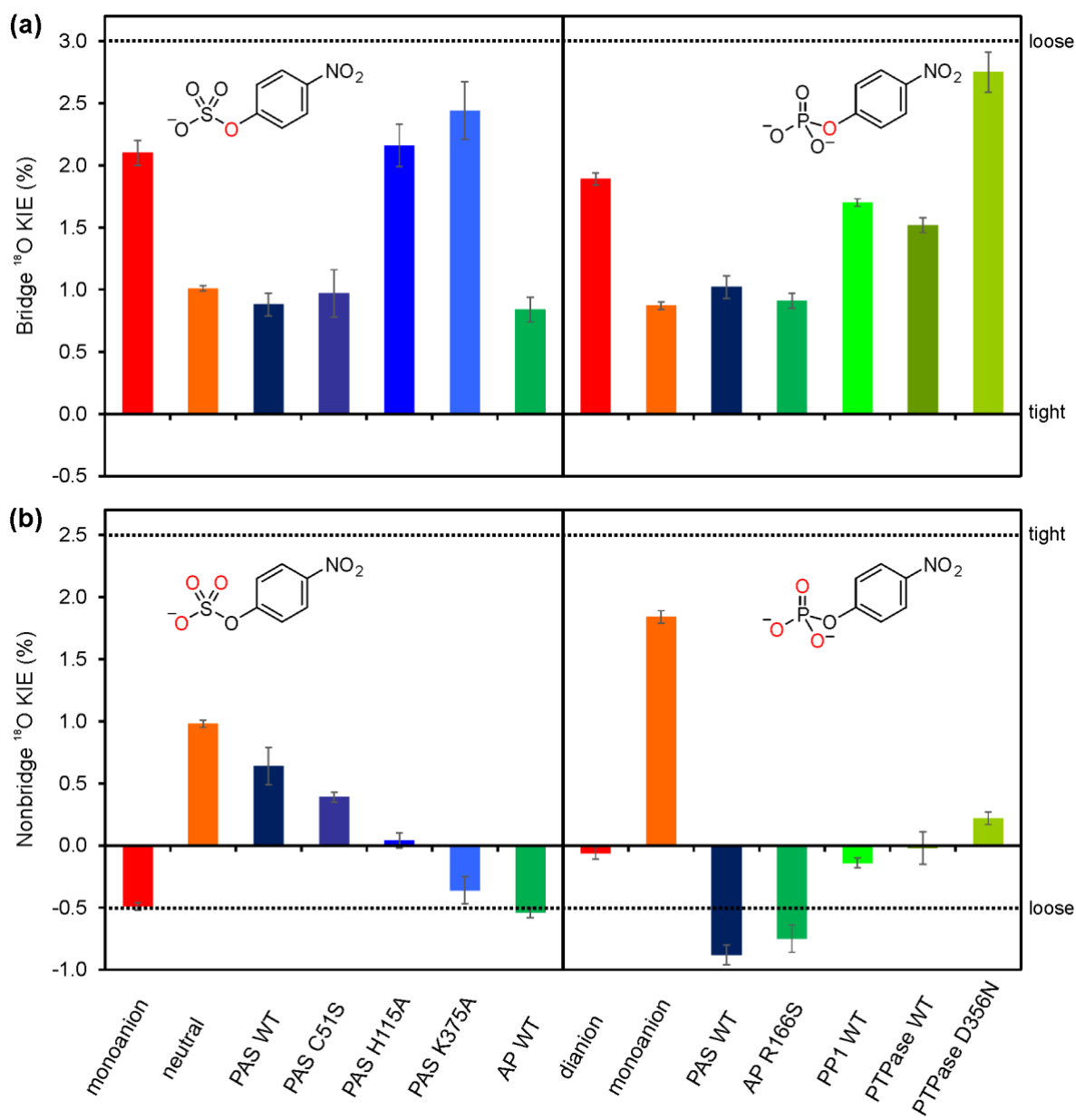


Figure 4. Kinetic Isotope Effects (KIEs) for PAS-catalyzed hydrolysis of sulfate monoester **1d** and phosphate monoester **2d**. The values are listed in Table 3 (PAS and solution data) and Table S15 (all other enzymatic data, SI). (a) The ^{18}O -KIEs for the bridging (or leaving group) oxygen. (b) The ^{18}O -KIEs for the nonbridging oxygens. The dotted lines indicate extremes of ^{18}O KIEs for tight (^{18}O bridge = 0%, ^{18}O non-bridge = +2.5%) and loose (^{18}O bridge = +3.0%, ^{18}O non-bridge = -0.5%) transition states.⁸⁰ AP: *E. coli* Alkaline phosphatase,²¹ PP1: Protein Phosphatase 1,⁵⁸ PTPase: *Yersinia* Protein Tyrosine Phosphatase.⁵⁴

The normal $^{18}(V/K)_{\text{nonbridge}}$ is unlikely to arise from the same origin as in the uncatalyzed hydrolysis of the neutral sulfate monoester, since protonation of the sulfuryl group oxygens will not occur, except under extremely acidic conditions. A more plausible explanation is that the PAS WT-catalyzed TS is more associative than the solution reaction of the monoanion. The latter scenario could also partly explain the unexpectedly large effect of the removal of residues that only interact indirectly with the non-bridging leaving group oxygen on $\beta_{\text{leaving group}}^{\text{obs}}$.

The PAS K375A mutation results in a reaction for which the $^{15}(V/K)$ and $^{18}(V/K)_{\text{bridge}}$ KIEs are largely identical to those of the uncatalyzed hydrolysis (Table 3, Figure 4b), suggesting this residue is largely responsible for leaving group neutralization in the TS. This is consistent with the effect of the mutation on the $\beta_{\text{leaving group}}^{\text{obs}}$, and similar to the value observed for the removal of an aspartic acid performing a similar role in PTPs.^{52, 54, 55} The data suggest that this residue either protonates the leaving group directly, or, that its mutation results in a dislocation of H211 (Figure 1), interfering with its function in this role, implying synergy between these two residues, as discussed earlier. The $^{18}(V/K)_{\text{nonbridge}}$ for PAS K375A is identical to the uncatalyzed hydrolysis, which could be explained by the loss of coordination of K375 to the sulfuryl group.

The relatively modest effect of the C51S mutation on $^{15}(V/K)$ and $^{18}(V/K)_{\text{bridge}}$ compared to the wild type reaction is consistent with the modest effect of this mutation on $\beta_{\text{leaving group}}^{\text{obs}}$. As stated above, the TS of the PAS WT-catalyzed sulfate monoester hydrolysis has a more associative character than in the uncatalyzed reaction, i.e. the nature of the nucleophile is thought to be more important, based on the change to a normal $^{18}(V/K)_{\text{nonbridge}}$. However, the change in nucleophile from fGly to serine has a much smaller effect on $^{18}(V/K)_{\text{nonbridge}}$, than the removal of K375, which is thought to interact directly with the nonbridging oxygens (Figure 1).

The mutation of H115 to alanine has a large effect on the KIEs compared to WT, despite the absence of direct TS interaction between H115 and the substrate (Table 3, Figure 4). The $^{18}(V/K)_{\text{bridge}}$ is nearly as large as that observed for PAS K375A. The $^{15}(V/K)$ shows partial charge neutralization on the leaving group, intermediate between the WT reaction and that of K375A. A possible explanation is suboptimal orientation of the substrate relative to the residues mainly responsible for leaving group stabilization, K375 and H211. The reaction of the H115A mutant shows a $^{18}(V/K)_{\text{nonbridge}}$ that is also intermediate between the WT and K375A, also consistent with less than optimal coordination of the non-bridging oxygens to K375. The fact that mutation H115A has a larger effect on $^{18}(V/K)_{\text{nonbridge}}$ than C51S confirms that the large change in $^{18}(V/K)_{\text{nonbridge}}$ for the PAS WT-catalyzed reaction compared to the sulfate monoester monoanion uncatalyzed hydrolysis is most likely not dependent on the nucleophile, but the result of interactions between the nonbridging oxygens and positively charged functional groups (K113, K375 and Ca^{2+}). The nature of the contribution of the latter interactions to TS stabilization appears to be unique for PAS WT-catalyzed sulfate monoester hydrolysis, since the effect of the enzyme on the $^{18}(V/K)_{\text{nonbridge}}$ for phosphate monoester hydrolysis is completely different. However, both reactions show a similar degree of leaving group stabilization.

Leaving group dependence in D₂O. The considerably less negative $\beta_{\text{leaving group}}^{\text{obs}}$ and a ¹⁵N KIE near unity for the PAS WT-catalyzed sulfate monoester hydrolysis could be caused by protonation of the leaving group oxygen, as in PTPs.^{52, 54-56, 59} Lewis acid charge neutralization by metal ions can have the same effect.²¹ The available data for PAS all point to K375 as the main residue responsible for stabilization of charge that develops on the leaving group oxygen in the TS. Direct proton transfer involving lysine as the donor in enzyme active sites is rare, and charge compensation by Lewis acid interaction with the cationic charge of the protein shared between H211 and K375 is a potential alternative. The PAS WT-catalyzed sulfate monoester hydrolysis in D₂O is more sensitive to the leaving group ($\beta_{\text{leaving group}}$ is more negative) compared to the same reaction in H₂O (Figure S12). The observed difference in leaving group dependence ($\Delta\beta_{\text{leaving group}}^{\text{H-D}}$) is $+0.06\pm 0.03$, which corresponds to a $k_{\text{H}}/k_{\text{D}}$ ratio ranging from ~ 1.7 at $\text{p}K_{\text{a}}^{\text{leaving group}} = 5.5$ to ~ 3.3 at $\text{p}K_{\text{a}}^{\text{leaving group}} = 10$. These data suggest that with increasing demand for leaving group stabilization, a proton transfer event becomes more rate-limiting. This would suggest that the degree of S-O_{bridge} bond fission during the TS is reduced with increasing leaving group ability (i.e. S-O_{bridge} bond fission is almost complete prior to proton transfer for the low $\text{p}K_{\text{a}}$ leaving groups that require charge offset assistance less). However, the ¹⁵N KIE for PAS WT-catalyzed hydrolysis of sulfate monoester **1d** ($\text{p}K_{\text{a}} = 7.02$) suggests almost complete charge compensation on the leaving group oxygen, suggesting K375 is mainly responsible for charge compensation without transferring its proton during catalysis. For PAS K375A, there is no difference in $\beta_{\text{leaving group}}^{\text{obs}}$ when recorded in H₂O or D₂O (Figure S13). However, a $\text{p}K_{\text{a}}$ -independent $k_{\text{H}}/k_{\text{D}}$ of ~ 2 for all reactions was observed, suggesting that a leaving group independent proton transfer event is rate-limiting for this mutant. The solvent isotope effect is consistent with the suggested catalytic role

for K375 as the main residue responsible for leaving group stabilization during bond-breaking in the TS, since any leaving group dependent proton transfer event will most likely be fast compared to the severely slowed down S-O_{bridge} bond fission. Indeed, the heavy-atom isotope effects suggest that these two events occur in the same step.

IMPLICATIONS AND CONCLUSIONS

Assignment of catalytic roles to active site residues. A mechanistic pathway for PAS WT-catalyzed sulfate monoester hydrolysis (Figure 1b) had been suggested by structural analysis, but can now be firmly established on the basis of the results presented in this study, including a reassessment of the contributions of the active site residues. The presence of 9 highly interconnected polar residues and a divalent metal cation, all of which could contribute to catalysis, makes assignment to specific function necessarily difficult, even when a high resolution structure is available and patterns of amino acid conservation can be discerned.⁹⁸ The importance of each of these residues was further established in mutational scanning experiments (by changing them to either asparagine or aspartate),⁴⁹ but the observed v_{\max} decreases and K_M increases implied that each of them was critical, leaving an ambiguity about their respective roles. An abundance of potentially functional residues is frequently observed. This abundance has been convincingly explained by Kraut *et al.*⁹⁹ to be a result of the cooperativity of active sites that prevents a simple quantitative assignment of mechanistic contributions to individual residues. This present work exemplifies how LFERs can be employed to probe the role of catalytic residues comparatively in mutants, providing insights that amino acid scanning experiments evaluated by measurement of Michaelis-Menten parameters for one substrate do not provide. For example, leaving group stabilization by the proposed general acid H211 is much less important than that of K375 (based

on the much smaller effect on $\beta_{\text{leaving group}}^{\text{obs}}$ upon alanine scanning). The LFERs serve to measure the effective charge ‘seen’ at the leaving group oxygen on moving from ground to transition state¹⁰⁵ and quantify the charge compensation achieved by the enzyme *via* its side chains: in the absence of a charge compensating residue a larger charge is ‘seen’ at this oxygen. The combined effect of removing both these residues was larger than the sum of its effects in wild-type enzyme, suggesting a high degree of interdependence between them with regard to leaving group stabilization, possibly by sharing a proton (Figure 5). The LFERs for k_{cat} and $1/K_M$ for PAS WT-catalyzed sulfate monoester hydrolysis and the pre-steady-state measurements with sulfate monoester **1d** suggest that the leaving group dependent sulfate ester bond fission (S-O_{bridge} fission; step 1 in Figure 1b) is much faster than the cleavage of the hemiacetal intermediate (step 2, Figure 1b).

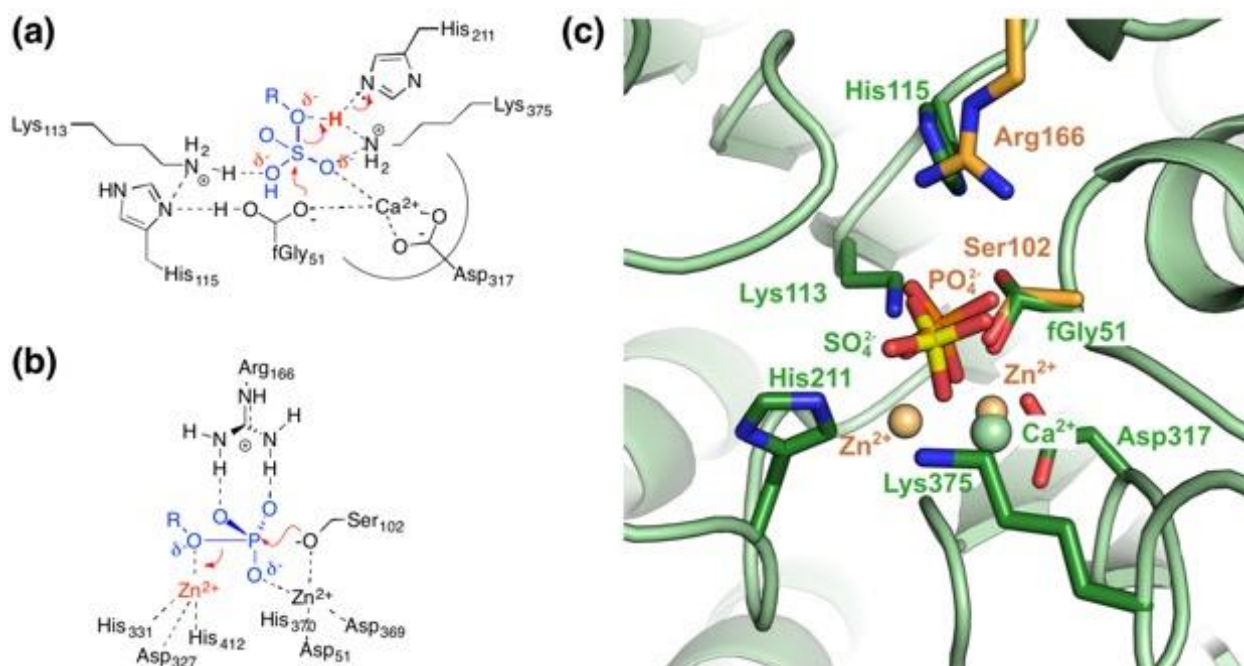


Figure 5. Comparison of mechanisms of leaving group stabilization in the transition states of sulfate monoester hydrolysis by PAS and phosphate monoester hydrolysis by alkaline

phosphatase. **(a)** During fission of the sulfate ester bond negative charge develops on the leaving group and is offset by a proton, held by the H211-K375 pair. **(b)** In *E. coli* alkaline phosphatase (AP)^{26, 29} the charge developing on the leaving group as a serine nucleophile attacks is offset by a metal ion. **(c)** Structural superposition of PAS⁴⁴ (pdb entry 1HDH) and AP¹⁰⁰ (3TG0), including bound product in the active site (inorganic sulfate and phosphate, respectively). The functionally homologous residues align well, showing that the second divalent metal ion (Zn²⁺) of AP is providing charge offset in a similar position as the proton held by H211 and K375 in PAS.

The strongest effect on leaving group dependent catalysis was seen for the K375A mutants ($\Delta\beta_{\text{leaving group}}^{\text{obs}} = +0.61$, Figure 2f, Table 2), further underlining that K375 is the most important residue for stabilization of the negative charge that develops on the leaving group oxygen during catalysis. This conclusion was also supported by the following observations: *(i)* The ¹⁵N and ¹⁸O_{bridge} KIEs for mutant K375A are essentially the same as for the solution reaction. The wild-type showed almost complete charge compensation on the leaving group (¹⁵N KIE essentially unity) and a lowered normal ¹⁸O_{bridge} KIE (Table 3, Figure 4). *(ii)* For PAS K375A the leaving group dependent step becomes increasingly more rate-limiting with decreasing leaving group ability (Figures 2f, S10b), pointing to its involvement in leaving group stabilization. *(iii)* The unexpectedly large effects on $\beta_{\text{leaving group}}^{\text{obs}}$ for active site mutants C51S and H115A are dependent on the presence of K375 (Table 2), suggesting that the main function of the other active site residues is to position the substrate for optimal interaction with K375. *(iv)* Structural alignment of PAS⁴⁴ with AP¹⁰⁰ shows that K375 occupies a similar position as the divalent metal ion thought to provide leaving group stabilization in AP (Figure 5).

Compensation of charge development on the leaving group oxygen by an amino acid side chain may be expected to involve direct protonation of the leaving group oxygen. The cleavage of this S-O_{bridge} ester is dependent on, and occurs in concert with, a near-complete neutralization of the charge on the leaving group for sulfate monoester **1d** ($pK_a^{\text{leaving group}} = 7.03$), as evidenced by a ¹⁵N KIE near unity (Table 3). This would suggest near-complete proton transfer during the TS, but transfer of the proton would only occur once the S-O_{bridge} bond cleavage is well advanced, if at all (Figures 5a and b). Charge compensation at the leaving group oxygen is nearly complete for PAS WT-catalyzed hydrolysis of sulfate monoester **1d**. As the proton is shared between H211 and K375 (Figures 5a and b) it may be that actual proton donation from lysine does not take place and instead transfer to H211 results in the observed compensation. Comparison of the leaving group dependences in H₂O and D₂O showed an increasing k_H/k_D ratio for k_{cat}/K_M with decreasing leaving group ability (Figure S12, SI), suggesting that proton transfer is becoming increasingly more important for S-O_{bridge} ester bond fission. The absence of the leaving group dependent change in k_H/k_D for PAS K375A (Figure S13) further supports the importance of K375 for S-O_{bridge} bond fission, since it is in agreement with S-O_{bridge} ester bond fission being fully rate-limiting for leaving group departure in this mutant. (This solvent isotope effect is relevant for the first chemical step, while the isotope effect on k_{cat} reflects the overall rate-determining step, i.e. breakdown of the intermediate).

An alternative mechanistic scenario in which additional steps prior to the first irreversible S-O_{bridge} ester bond fission are rate limiting for wild-type, but the chemical step becomes limiting in a mutant may be considered. Such an explanation has been advanced for FLAP endonuclease, where pH-rate profiles suggested rate-limiting physical steps after substrate binding resulted in a commitment to catalysis that suppressed the magnitude of $\beta_{\text{leaving group}}^{\text{obs}}$ and was reduced or

eliminated by a mutation that slowed the chemistry step.¹⁰¹ However, this scenario is inconsistent with the observed KIEs on PAS catalysis. A commitment factor would reduce all the observed KIEs in equal proportion.^{94, 102} Thus, if making the chemical step more rate-limiting (reducing a commitment factor) were the cause of the larger Brønsted slope, the KIEs would increase in the same proportion. Instead, the K375A mutant reaction gives a ¹⁵N KIE that is 4-fold higher than wild-type PAS; the bridge ¹⁸O effect is 2.8-fold greater; and the non-bridge ¹⁸O effect goes from normal to inverse. The substrates in FLAP and PAS also show significant differences: DNA substrates of FLAP may require unpairing or helical arch ordering, whereas for the small molecule substrates of PAS no analogous steps are conceivable. In addition, the high K_M -values are consistent with reversibility and a rapid equilibrium between bound and unbound states (contrasting with the nM binding in FLAP). The LFERs and KIEs for PAS use k_{cat}/K_M comparisons that reflect the first irreversible step of the reaction, which thus is unlikely to be associated with binding.

Although the exact residues and functional groups involved differ between several previously characterized phosphatases^{21, 23, 24, 33, 36, 51, 52, 54-56, 58, 59, 97} and PAS, all these enzymes rely on leaving group stabilization by charge compensation, resulting in less negative $\beta_{\text{leaving group}}^{\text{obs}}$ values and lowered, but still normal ¹⁸O_{bridge} KIEs. However, the KIEs for PAS are consistent with a more associative mechanism than previously reported phosphatases, because of the observed change of ¹⁸O_{nonbridge} KIE from inverse (-0.49%) to normal (+0.62%) compared to the solution reaction (Table 3, Figure 4b). In phosphatases the small inverse ¹⁸O_{nonbridge} KIE was either more inverse²¹ or virtually unchanged^{51, 52, 54, 55, 58, 97} (Figure 4b, Table S15). The possible change from a dissociative to a more associative TS does not apply to all PAS-catalyzed conversions, since the promiscuous enzyme-catalyzed hydrolysis of phosphate monoester **2d** showed a more inverse

$^{18}\text{O}_{\text{nonbridge}}$. Enzymatic specificity towards sulfate over phosphate monoester cannot be primarily based on overall charge, demand for leaving group stabilization or nucleophile strength, since these either provide no discrimination or will favor the more highly charged phosphate monoesters. The difference in effect on the $^{18}\text{O}_{\text{nonbridge}}$ KIE suggest that the subtle differences in geometry between the S- $\text{O}_{\text{nonbridge}}$ and P- $\text{O}_{\text{nonbridge}}$ bonds are responsible for making PAS specific toward sulfate monoesters.

Relationship to computational studies. The existence of computational studies attempting to model aspects of PAS catalysis *in silico*^{38, 42, 43} provides an opportunity for a comparison of their conclusions with our experimental results. One report has taken a shortening of the S- O_{lg} bond distances (i.e. the $^{18}\text{O}_{\text{bridge}}$ in our study) as an indication for a shift to a more associative transition state.³⁸ However these distances are not equivalent to shifts in TS. We find that the $^{18}\text{O}_{\text{bridge}}$ KIE is similar in both sulfate monoesters and phosphate monoesters, yet the non-bridging oxygen data (for which no computational results are available) differ widely. Importantly, only limited information on the individual contributions of active site residues has been provided by computational studies. The residue that we show is central to both leaving group stabilization and shifting the nature of the TS for PAS-catalyzed sulfate monoester hydrolysis, K375, has not been discussed in the context of QM/MM studies to date. Instead, Marino *et al.*⁴² highlight the effect of H211, but assign its role as the sole general acid catalyst, while no such effect is ascribed to K375. Here, and in the work of Luo *et al.*,³⁸ residues that – in our analysis - are assigned only indirect roles affecting the nature of the TS and leaving group stabilization (K113, H115, fGly51) feature more prominently. A third study⁴³ uses molecular dynamics simulations (based on active site group ground state $\text{p}K_a$ values validated by PROPKA) to define a model that features K375, but not His211.

In computational studies with AP different authors obtained diverging predictions for enzymatic TSs that were either more associative⁴⁰ or more dissociative.²⁷ It is possible that the calculated relatively flat calculated energy surfaces (e.g. as shown in ^{39, 103}) make precise assignment of a TS position in More O’Ferrall/Jencks-type diagrams difficult *in silico*, as many energetically near-equivalent paths of bond-making and breaking seem to exist, yet these ambiguities may also be artefacts of the computational methods. Experimentally the notion of flat calculated energy surfaces is not supported: even in promiscuous enzymes the nature of the TS is close to that in solution,^{23, 32, 36, 37} suggesting it is hard to change. Computational results from modeling physical processes are not direct experimental observations and should not be used as substitutes of experimental reality. A more confident assignment of the nature of the TS requires resolution by experimental evidence of the sort presented here, i.e. a kinetic analysis involving juxtapositions of LFERs and KIEs.

In comparing experimental and computational results it is important to bear in mind that ground state binding effects (contributing to K_M) are excluded and that elementary rate constants are calculated that may be implicit in Michaelis-Menten parameters, but hard to disentangle. Multiple potentially rate-limiting steps may make these parameters complex functions of rate constants, preventing straightforward reconciliation of data. In some cases the computed energy levels do not reproduce the actually measured rates, indicating possible shortcomings in capturing the solution reaction,¹⁰³ but in the case of enzymatic reactions - also leading to postulation of different rate limiting steps. For example, the rate-limiting step of the PAS reaction had been predicted to be k_2 ,⁴² which our analysis suggests to be incorrect. Our stopped flow data suggest that k_2 (i.e. the relevant step in the LFERs) is at least >100-fold faster than k_{cat} . All these factors necessitate the

judicious evaluation of both experimental and computational data with respect to functional conclusions, with many aspects necessitating experimental confirmation or resolution.

A quantitative measure for the efficiency of general acid or charge compensation catalysis.

The correlation of reaction rates and the measured values for $\beta_{\text{leaving group}}^{\text{obs}}$ (Figure 6) establishes a direct link between catalytic effects by charge compensation (and/or proton transfer) and catalytic efficiency: the more the catalytic effect of the protonation of an active site group is removed (as indicated by larger effective charge changes at the leaving group oxygen), the more the overall rate suffers. This effect is more pronounced for unreactive substrates that require interaction with the proton held by K375 and H211 to a greater extent (Figure S15), such as the presumed natural substrates, sulfated sugars.

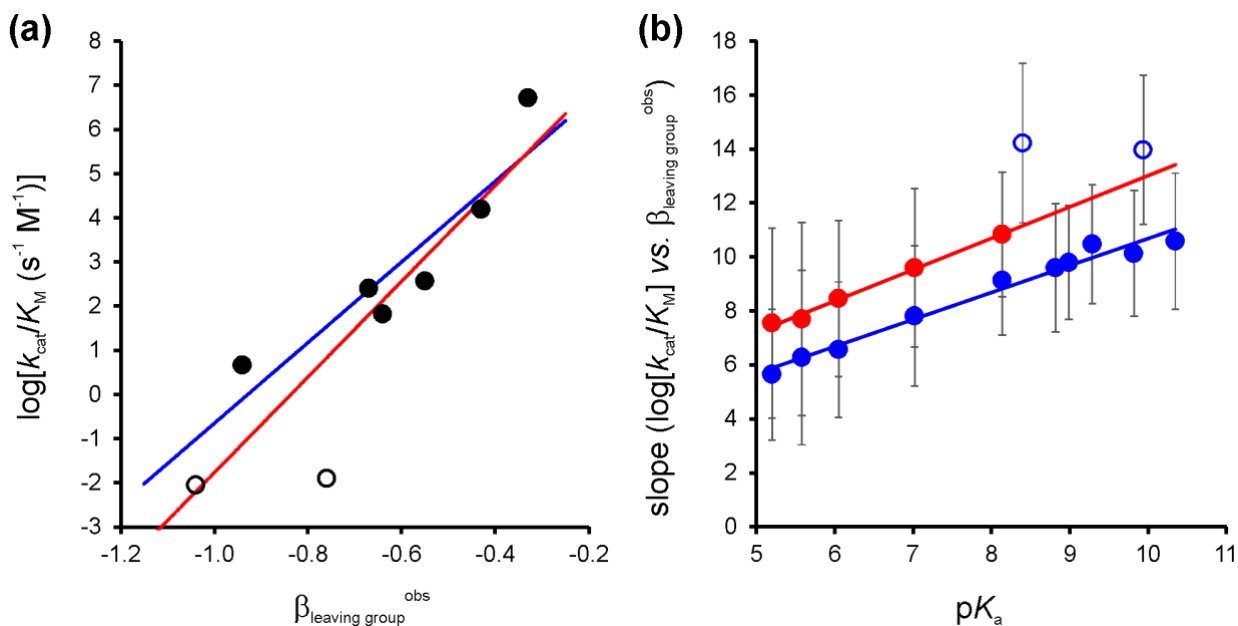


Figure 6. PAS variants with lower catalytic efficiencies are more sensitive to the leaving group ability. (a) Correlation between catalytic efficiency (represented by $\log[k_{\text{cat}}/K_{\text{M}}]$) and leaving group dependency ($\beta_{\text{leaving group}}^{\text{obs}}$) of the various PAS variants (closed symbols: PAS WT and its single mutants, open symbols: double mutants) for 3-nitrophenyl sulfate **1e**. Linear correlations

including *all* variants (red line, $R^2 = 0.79$; $p = 0.0033$) and only based on the single mutants and wild type (i.e. excluding the open symbols; blue line, $R^2 = 0.83$; $p = 0.011$) show slopes of 10.8 ± 2.3 and 9.1 ± 2.0 respectively. (b) Correlation between the response slope as shown in panel (a) and pK_a values of the substrate's leaving group (see Figure S14 for the corresponding red and blue correlation lines from which the slopes were obtained). The slopes for sulfate monoesters **1f** and **1k** (open blue symbols) clearly deviate from the observed trend (suggesting idiosyncratic active site interactions) and were therefore not included in the fit (see Figure S15, SI, for a fit that includes all data points). Red fit: slope: 1.16 ± 0.05 ; R^2 : 0.99; p : 2.2×10^{-4} . Blue fit: slope: 1.0 ± 0.06 ; R^2 : 0.97; p : $< 10^{-4}$.

The correlation of catalyst and substrate reactivity provides a further measure of sensitivity of catalysis to substrate reactivity changes and should be used to quantitatively assess the efficiency of general acid catalysis. In the future it will be interesting to contrast slopes in such double reactivity plots (as in Figure 6b) for several enzyme systems and use its slopes to quantify the sensitivity of catalytic effects in different active site arrangements.

More generally, this work shows that physical-organic approaches can continue to contribute to the understanding of enzyme catalysis¹⁰⁴ by providing a quantitative picture of elusive transition state interactions, through the measurement of effective charge changes *via* Brønsted plots.¹⁰⁵ These, combined with isotope effects studies, are applied here systematically for the first time to studies of mutational effects to quantitatively assess the individual involvement of particular residues in PAS catalysis.

ASSOCIATED CONTENT

Supporting information available. Synthetic procedures, mathematical descriptions for enzyme kinetics and expected detection limits, determination of kinetic isotope effects and data analysis. Michaelis-Menten plots for sulfate substrates hydrolyzed by PAS WT, tables of experimentally measured steady-state kinetic parameters, additional LFERs, stopped flow data and primers used for mutant construction.

AUTHOR INFORMATION

Corresponding Author

*E-mail: fh111@cam.ac.uk

*E-mail: alvan.hengge@usu.edu

ORCID

Bert van Loo: 0000-0003-4253-2163

Florian Hollfelder: 0000-0002-1367-6312

Alvan Hengge: 0000-0002-5696-2087

Present Addresses

[§]BvL: Institute for Evolution and Biodiversity, University of Münster, Germany

[‡]UB: Faculty of Tropical Medicine, Mahidol University, Thailand

[⊥]MG: Institute of Biochemistry, Faculty of Medicine, University of Ljubljana, Slovenia

Funding Sources

This research was funded by the Biological and Biotechnological Research Council (BBSRC, BB/I004327/1) and the Engineering and Physical Sciences Research Council (EPSRC, EP/E019390/1) and NIH grant GM 47292 to ACH. FH is an ERC Investigator (695669), UB received a studentship from the Royal Thai Government and MG was supported by a postdoctoral Marie-Curie fellowship from the EU.

ACKNOWLEDGMENT

We thank Marko Hyvonen for his help in the preparation of structural figures.

ABBREVIATIONS

AP, alkaline phosphatase; LFER, linear free energy relationship; KIE, kinetic isotope effect; PAS, *Pseudomonas aeruginosa* arylsulfatase.

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