JOURNAL OF PHYSICAL ORGANIC CHEMISTRY
J. Phys. Org. Chem. 2004; **17:** 541–547
Published online in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/poc.775

Investigation of the sulfuryl transfer step from substrate to enzyme by arylsulfatases†,‡

Stuart G. Gibby, Jarod M. Younker and Alvan C. Hengge*

Department of Chemistry and Biochemistry, Utah State University, Logan, Utah 84322, USA

Received 29 July 2003; revised 9 October 2003; accepted 9 October 2003

ABSTRACT: The reactions of the arylsulfatase A (ASA) from *Helix pomatia* and that from *Aerobacter aerogenes* with *p*-nitrophenyl sulfate were examined by determination of the pH dependence of V_{max}/K_m and by measurement of kinetic isotope effects. Both enzymes exhibit bell-shaped pH–rate dependences for V_{max}/K_m. The ASA from *Helix pomatia* exhibits a more acidic pH optimum (pH 4–5) than the ASA from *Aerobacter aerogenes* (pH ~7). The sulfuryl transfer from substrate to enzyme is general acid-assisted in both enzymes, but isotope effects indicate differences in the synchronicity of protonation with S—O bond fission. In the reaction of the *Helix pomatia* enzyme, protonation is synchronous with bond fission and the leaving group is fully neutralized in the transition state. In the reaction catalyzed by the *Aerobacter aerogenes* ASA, protonation of the leaving group lags behind bond fission and the leaving group bears a partial negative charge in the transition state.

KEYWORDS: arylsulfatase; sulfuryl transfer; catalytic mechanism; isotope effect

INTRODUCTION

In recent years there has been a growing realization of the biochemical importance of sulfuryl transfer. In a classic experiment performed more than a century ago, Baumann[1] administered phenol to a patient and showed that it was excreted as phenyl sulfate. This was the first demonstration of biological sulfation as an important detoxification mechanism. The biological roles of sulfation extend much further than detoxification. Sulfate monoesters are found among all classes of natural products, including nucleotides, peptides and proteins, polysaccharides, steroids and lipids. Sulfate monoesters of steroids are key intermediates in the biosynthesis of steroids, where the sulfate moiety enables the intermediates to be kept in circulation as hormone precursors.[2]

Sulfate esters are formed in biological systems by sulfotransferases, enzymes which transfer the sulfuryl group from the phosphoric–sulfuric acid anhydride PAPS (3′-phosphoadenosine-5′-phosphosulfate) to the hydroxyl group of a recipient molecule. This process is counterbalanced by the action of sulfatases, which catalyze the hydrolysis of sulfate esters. Together these two enzyme families regulate the sulfation level of biological molecules in the cell. This regulatory mechanism has strong similarities to that of phosphorylation/dephosphorylation. Despite its biological importance, sulfate ester chemistry has been the subject of comparatively few studies.

Members of the sulfatase enzyme family require post-translational oxidation of the thiol group of a conserved cysteine to an aldehyde, yielding a formylglycine. The amino acid residues surrounding the formylglycine residue in the active sites of members of the arylsulfatase A (ASA) family are highly conserved.[3] A metal ion, thought to be magnesium or calcium, coordinates one of the aldehyde hydrate oxygens, lowering its pK_a. Two lysines, two histidines and an arginine are highly conserved in the active site, in addition to the metal ligands, which are three aspartic acids and an asparagine. The x-ray structure of the human arylsulfatase has been reported.[4]

Experiments with a number of arylsulfatases using ^{18}O-labeled water have shown that sulfuryl transfer proceeds by way of sulfur–oxygen bond cleavage, resulting in ^{18}O incorporation into inorganic sulfate.[5] Kinetic and stereochemical results indicate that the mechanism is stepwise with a covalent sulfoenzyme intermediate.[6] The pH dependence of V_{max} for hydrolysis of *p*-nitrocatechol sulfate by human ASA is bell-shaped, suggestive of acid–base catalysis.[3,7] A proposed mechanism[3] consistent with these observations is shown in Fig. 1. The extent to which the enzymatic aldehyde exists in its hydrate form under

**Correspondence to:* A. C. Hengge, Department of Chemistry and Biochemistry, Utah State University, Logan, Utah 84322, USA.
E-mail: hengge@cc.usu.edu
Contract/grant sponsor: National Institutes of Health; *Contract/grant number:* GM47297.
Contract/grant sponsor: Petroleum Research Fund; *Contract/grant number:* 35690-AC4.
Contract/grant sponsor: Utah State University.
†This paper is dedicated to William P. Jencks, a great scientist and an inspiration to generations of chemists past, present and future.
‡Selected paper part of a special issue entitled 'Biological Applications of Physical Organic Chemistry'.

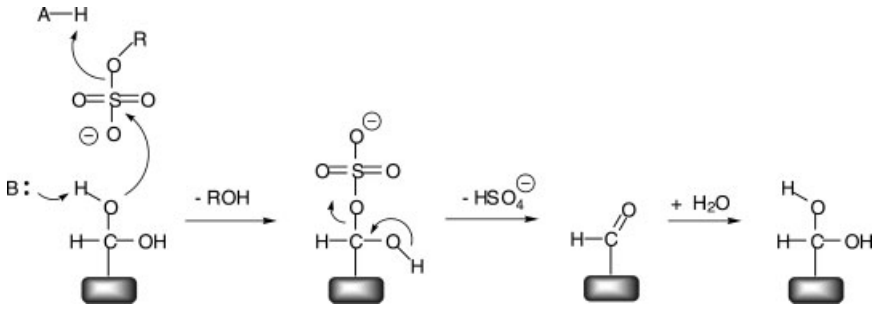

Figure 1. Proposed mechanism of sulfate ester cleavage by sulfatases. In the first step, with general acid–base catalysis, the aldehyde hydrate attacks the sulfate ester forming a sulfoenzyme intermediate. In a second step, sulfate is eliminated with formation of an aldehyde. Subsequently, the aldehyde hydrate is regenerated by the addition of water

physiological conditions is not precisely known. An x-ray structure of the human ASA indicated that it was more likely to correspond to a formylglycine hydrate than to a twofold disordered aldehyde group.[8]

We have measured the pH–rate dependences for V_{max} and for V_{max}/K_m using the substrate *p*-nitrophenyl sulfate (*p*NPS) for the ASAs from *Helix pomatia* and *Aerobacter aerogenes*. The first irreversible step in the overall mechanism is most likely nucleophilic attack by the aldehyde hydrate concerted with loss of the *p*-nitrophenol leaving group. Trans-sulfurylation (transfer of the sulfate group from the sulfoenzyme intermediate to a different phenol) has not been reported for arylsulfatases. No phenyl sulfate was detected when the enzymatic hydrolysis of *p*NPS by the two arylsulfatases in this study were carried out in the presence of phenol (see the Experimental section for details). Mechanistic studies of uncatalyzed sulfuryl transfer indicate that the process is concerted, without a pentacoordinate intermediate.[9] Therefore, the pH dependence of V_{max}/K_m reflects ionizable groups of free enzyme and substrate involved in catalysis of the sulfuryl transfer step from substrate to enzyme.

Kinetic isotope effects (KIEs) on V_{max}/K_m for the ASA-catalyzed hydrolysis of *p*NPS were obtained using

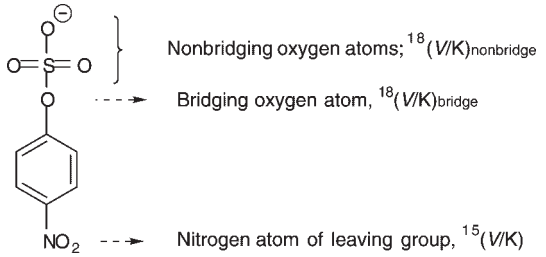

Figure 2. The *p*-nitrophenyl sulfate substrate showing the positions at which kinetic isotope effects were measured and the notation used. The standard notation is used in which a leading superscript designates the isotopic substitution. $^{18}(V/K)_{non\text{-}bridge}$ indicates the isotope effect on *V*/*K* resulting from substitution of the three non-bridging oxygen atoms with ^{18}O. $^{18}(V/K)_{bridge}$ is the KIE at the bridging oxygen position and $^{15}(V/K)$ is the KIE arising from ^{15}N in the nitro group

the competitive method,[10] at the positions indicated in Fig. 2. KIEs in the same positions have been used to analyze transition states for phosphoryl transfer from *p*-nitrophenyl phosphate (*p*NPP),[11] and have been reported for the uncatalyzed hydrolysis of *p*-nitrophenyl sulfate.[12] The primary isotope effect $^{18}(V/K)_{bridge}$ gives a measure of the degree of cleavage of the S—O bond in the transition state. The secondary isotope effect $^{15}(V/K)$ is sensitive to the amount of negative charge borne by the leaving group. The secondary isotope effect $^{18}(V/K)_{non\text{-}bridge}$ distinguishes between a loose transition state in which the transferring sulfuryl group resembles SO_3, versus a tight trigonal bipyramidal transition state in which bond formation to the nucleophile is ahead of bond fission to the leaving group. In the uncatalyzed hydrolysis of the anion, KIE and other data support a concerted reaction with a loose transition state characterized by extensive bond fission to the leaving group and modest bond formation to the nucleophile. Under acidic conditions, the leaving group is protonated in the transition state.[12]

RESULTS

Kinetic isotope effects

The isotope effects measured for the reactions of the two arylsulfatases are shown in Table 1. The $^{18}(V/K)_{non\text{-}bridge}$ values are for substitution of all three non-bridge oxygen atoms. The isotope effects obtained from isotopic ratios of product and those obtained from isotopic ratios of residual substrate agreed within experimental error in all cases and were averaged together to give the results shown. Six or more determinations of each isotope effect were made.

Table 1. KIEs at pH 6.6, 25 °C for the hydrolysis of *p*NPS by ASA from *Helix pomatia* and *Aerobacter aerogenes*

pNPS KIE	*Helix pomatia*	*Aerobacter aerogenes*
$^{18}(V/K)_{non\text{-}bridge}$	1.0024 ± 0.0005	1.0009 ± 0.0005
$^{18}(V/K)_{bridge}$	1.0136 ± 0.0004	1.0191 ± 0.0004
$^{15}(V/K)$	1.0001 ± 0.0002	1.0009 ± 0.0002

pH–rate profiles

The dependences of V_{max}/K_m for the ASAs from *Aerobacter aerogenes* and from *Helix pomatia* are shown in Figs 3 and 4, respectively. For the ASA from *Aerobacter aerogenes* the profile is clearly bell-shaped; a fit of the data to the equation

$$\log\frac{V_{max}}{K_m} = \log\left(\frac{C}{1 + [H]/K_a + K_b/[H]}\right) \qquad (1)$$

which assumes one residue must be protonated and another must be deprotonated for catalysis, yields calculated pK_a values of 4.71 ± 0.09 and 8.78 ± 0.09. The V_{max}/K_m profile for the ASA from *Helix pomatia* is shifted towards lower pH, and a decrease at acidic pH is not well defined (data could not be collected below pH 3.3). The data shown were fitted to both Eqn (1) and Eqn (2):

$$\log\frac{V_{max}}{K_m} = \log\left(\frac{C}{1 + K_b/[H]}\right) \qquad (2)$$

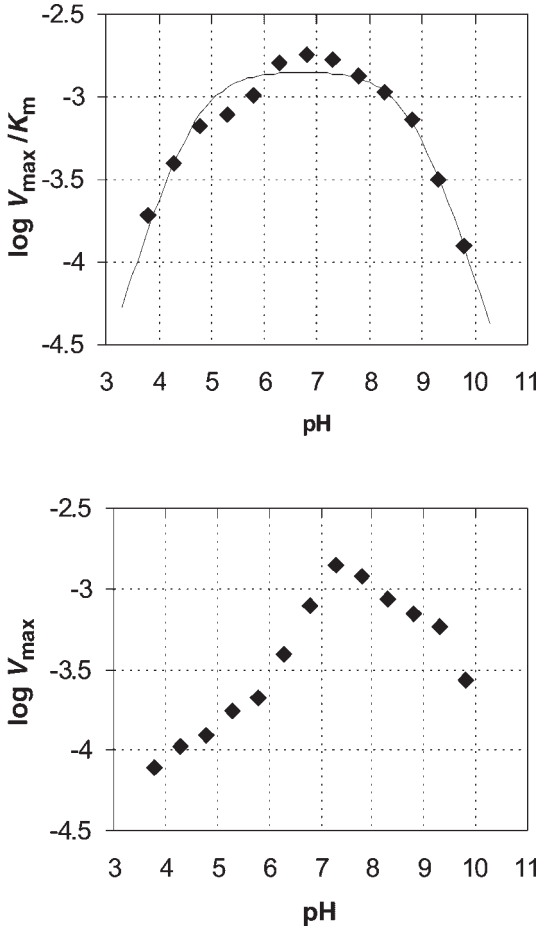

Figure 3. pH–rate profile for reaction of *p*NPS with ASA from *Aerobacter aerogenes*. Top, for V_{max}/K_m; the line drawn through the data represents the fit to Eqn (1). Bottom, pH dependence for V_{max}

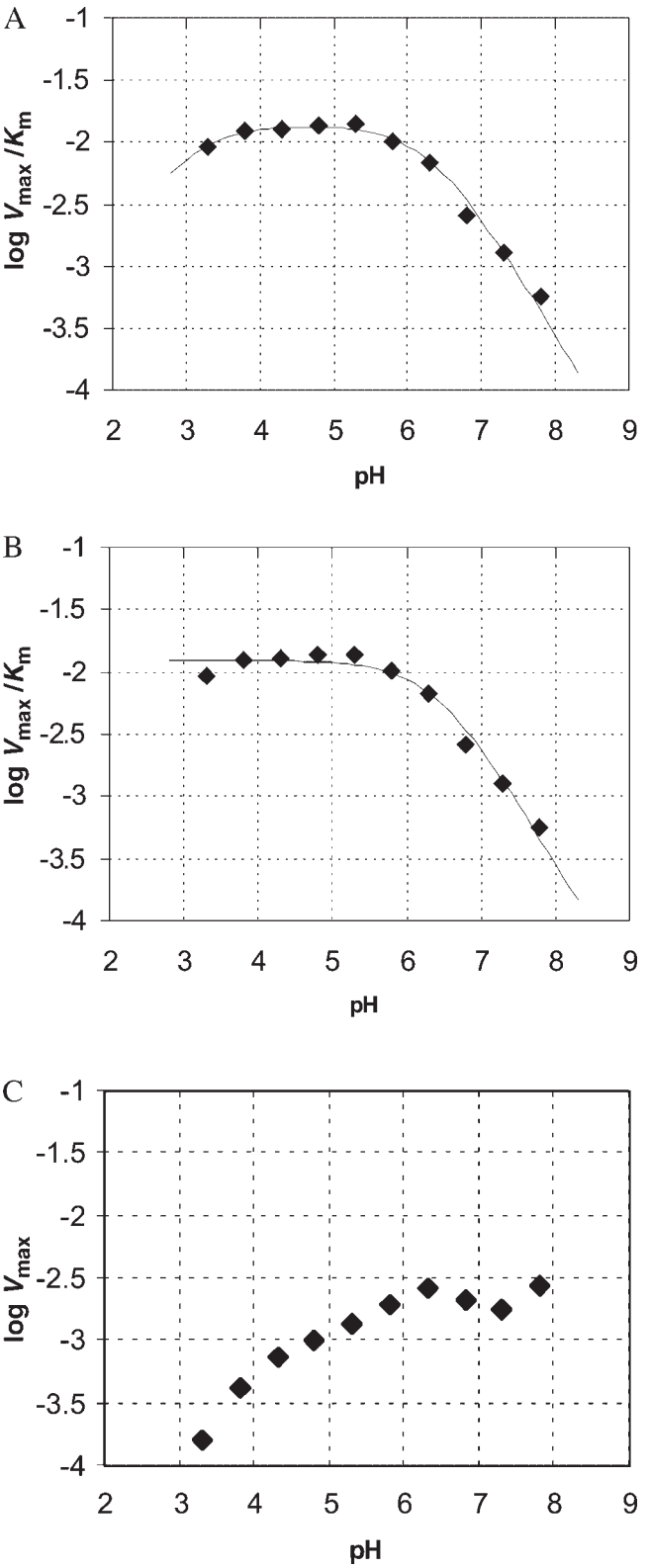

Figure 4. pH–rate profiles for the ASA from *Helix pomatia*. The line drawn through the data in (A) represents the fit of the V_{max}/K_m data to Eqn (1), and that in (B) is the fit to Eqn (2). (C) Dependence of V_{max} on pH

which describes a rate-dependent single residue that must be protonated for catalysis. Equation (1) gives a better fit of the data [$\sigma = 0.15$ versus 0.18 for the fit to Eqn (2)], with pK_a values of 3.0 ± 0.2 and 6.31 ± 0.06. The fit to Eqn (2) gives a pK_a of 6.37 ± 0.06.

The K_m value for *p*NPS increased with increasing pH for both enzymes. The values for K_m at several pH values are given in Table 2.

DISCUSSION

Kinetic and pH data

One notable difference between the two sulfatases is the difference in their pH optima. The pH optimum for V_{max}/K_m of the *Helix pomatia* ASA, ~4–5, is more acidic by several pH units from that of the *Aerobacter aerogenes* ASA, which is near neutrality. The two enzymes have similar K_m values at their respective pH optima; both exhibit increases in K_m at higher pH. Similar behavior has been reported for the arylsulfatase from *Aspergillus oryzae*.[13] The pH dependence of catalysis was not reported, but the K_m for *p*NPS was found to increase from 0.41 mM at pH 4.02 to 18 mM at pH 7.54.[13] The human ASA has been examined using *p*-nitrocatechol as a substrate, and exhibits a pH optimum of 4.5, where the K_m is 4.2 mM.[3]

The bell-shaped pH dependence of V_{max}/K_m for the *Aerobacter aerogenes* ASA indicates that both acid and base catalysis occur during sulfuryl transfer from substrate to enzyme, to form the sulfoenzyme intermediate. A logical assumption for the roles of such residues is for a general base that deprotonates the aldehyde hydrate nucleophile and a general acid that protonates the leaving group. The V_{max}/K_m pH–rate profiles potentially reflect ionizations on free substrate and free enzyme. However, the pK_a of the substrate is very low; the pK_a of unsubstituted sulfuric acid is of the order of -3[14] and the *p*-nitrophenyl substituent will make this pK_a even more negative. As a result, the anionic form is the only species present in appreciable quantity in the pH ranges measured, and hence is the only viable form of the substrate for catalysis.

The *Aerobacter aerogenes* ASA pH dependence of V_{max}/K_m displays what appears to be a hollow on the acid side of the profile. The appearance of such a hollow can be the result of a situation in which both the substrate and the proton do not dissociate readily from the protonated enzyme–substrate complex, in other words, if both the substrate and proton are 'sticky'.[15] Such a phenomenon can also result from the presence of a second residue whose protonation has a minor but measurable effect of reducing *V*/*K*; in such a case the slope of the asymptote will still go to unity. The $^{18}(V/K)_{bridge}$ is the largest KIE and thus the most sensitive one to the suppression that would occur in the event of a sticky substrate. Its magnitude is very high for a situation in which the leaving group is protonated by general acid catalysis in the transition state, as discussed in detail below. For this reason, we doubt that substrate stickiness, which would suppress the intrinsic KIE, is the likely cause of the hollow in the pH–rate profile.

The pH dependence of V_{max}/K_m for the *Helix pomatia* ASA does not clearly drop off at low pH [Fig. 4(A) and (B)]. Attempts to gather kinetic data below pH 3.3 were hampered by gradual enzyme inactivation, as evidenced by curved absorbance versus time plots (data not shown). In the pH range that could be examined, it was noted that while V_{max} decreases as pH is lowered [Fig. 4(C)], K_m does also, resulting in only a small apparent trend toward a decrease in V_{max}/K_m. Nevertheless, a small drop in V_{max}/K_m at the lowest pH at which data could be obtained can be observed in Fig. 4, and a fit of the data to the equation assuming a bell-shaped pH dependence [Fig. 4(A)] results in a somewhat better fit of the data. Although suggestive, the results are far from conclusive. Thus, although general acid catalysis is clearly indicated for both enzymes, whether catalysis by the *Helix pomatia* ASA also depends on a basic residue that must be deprotonated is uncertain.

Background to interpretation of the isotope effects

The previously reported[12] isotope effects for the uncatalyzed hydrolysis of *p*NPS are shown in Table 3. The large difference in reactivity under alkaline and acidic conditions precluded measurement of these KIEs at the same temperature. The data at pH 9 represent those for a reaction in which the leaving group departs as the phenolate anion. Based on reactions with the phosphate

Table 2. Variation of K_m for *p*NPS with pH for the ASA enzymes in this study

pH	*Helix pomatia* K_m (μM)	*Aerobacter aerogenes* K_m (μM)
4.3	60	263
5.3	102	223
6.3	387	244
7.3	1432	824
7.8	4970	883
9.8		2163

Table 3. Kinetic isotope effects for the aqueous hydrolysis of *p*NPS:[12] values for $^{18}k_{non\text{-}bridge}$ are the KIEs for all three non-bridge atoms labeled; standard errors in the last decimal place(s) are shown in parentheses

*p*NPS KIE	pH 9.0, 85 °C	10 M HCl, 15 °C
$^{18}k_{non\text{-}bridge}$	0.9951 (3)	1.0098 (3)
$^{18}k_{bridge}$	1.0210 (10)	1.0101 (2)
^{15}k	1.0026 (1)	1.0004 (1)

ester *p*NPP, when the leaving group is *p*-nitrophenol the magnitude of $^{18}k_{bridge}$ reaches a maximum value of about 1.03 at room temperature, when bond fission is extensive and the leaving group is not protonated.[11] Because protonation of *p*-nitrophenoxide is associated with an inverse ^{18}O isotope effect of 0.985, protonation in the transition state reduces the magnitude of the bridge-^{18}O KIE. If both bond fission and protonation are far advanced, a net KIE of about 1.015 is observed. This net normal effect results from loss of bending and torsional modes associated with the phosphoryl group that are absent in the phenol. (For similar reasons, the ^{18}O fractionation factors between water and alcohols are 2–3% and are normal, in the direction from alcohol to water).[16] The gas-phase ^{18}O fractionation factor between *p*-nitrophenol and *p*NPP has been calculated to be $\sim$1.011 and that between *p*-nitrophenol and *p*-nitrophenyl acetate 1.014. The latter compares well with an experimental value of 1.012. Since the fractionation factors between *p*-nitrophenol and the acetate and phosphate esters are similar, that between *p*-nitrophenol and *p*NPS is expected to be of comparable magnitude. Thus a similar value for $^{18}(V/K)_{bridge}$ would be expected in a transition state in which S—O fission and proton transfer are both far advanced.

The presence of negative charge on the leaving group reveals itself in the presence of a normal $^{15}(V/K)$. The *p*-nitrophenolate anion has contributions from a quinonoid resonance form with less N—O bond order and more N—C bond order. Because N—O bonds are stiffer than N—C bonds in terms of vibrational frequencies, the nitrogen atom is more tightly bonded in the neutral species than in the phenolate anion. Hence the ^{15}N equilibrium isotope effect (EIE) for deprotonation of *p*-nitrophenol is normal, 1.0023 ± 0.0002.[17] In reactions where protonation is synchronous with bond fission, no negative charge develops and the ^{15}N KIE is unity. Thus, in the acid hydrolysis of *p*NPS, in which a proton is thought to be transferred to the leaving group in the transition state, this KIE is much smaller than in the reaction of the *p*NPS anion (the very small ^{15}k that is observed may represent the EIE between *p*NPS and nitrophenol, or may indicate that protonation lags slightly behind S—O bond fission).

The secondary non-bridge-^{18}O KIE in sulfuryl transfer reactions may also be considered in part by analogy with the body of data for phosphoryl transfer, since the changes in hybridization and in bonding to the non-bridge oxygen atoms in sulfuryl and phosphoryl transfer reactions are analogous. In phosphoryl transfer reactions, the non-bridge-^{18}O KIE reveals whether the phosphoryl group resembles metaphosphate in a loose transition state, in which case this KIE is small and inverse, or if it has a phosphorane-like structure in a tighter transition state, in which case this KIE is normal.[11] In phosphate triester reactions, which have tight phosphorane-like transition states, $^{18}k_{non\text{-}bridge}$ reaches values of 3% (1.03).[18] Calculations at the B3LYP6–31++G** level of the expected equilibrium isotope effect between *p*NPS and sulfur trioxide (P. Czyryca and A. C. Hengge, unpublished results) yield a value of 0.9910. The experimental value of 0.9951 for the hydrolysis of the *p*NPS anion is consistent with expectations from the calculations and from KIEs from analogous phosphoryl transfer reactions, for a transition state in which the sulfuryl group resembles SO_3. In the acid hydrolysis reaction, the normal $^{18}k_{non\text{-}bridge}$ KIE reflects deprotonation of the sulfuryl group as this proton is transferred to the leaving group during reaction, and is also affected by the fractionation factor for protonation of the labeled and unlabeled sulfuryl groups. Since such protonation is highly unlikely in the enzymatic reactions that are the subject of this paper, these are not relevant.

Mechanism and transition state of sulfuryl transfer from pNPS to enzyme

The KIE data for the ASA from *Helix pomatia* are unity for $^{15}(V/K)$ and 1.0136 for $^{18}(V/K)_{bridge}$, consistent with a transition state in which S—O bond fission and protonation of the leaving group are both far advanced. The small normal value for $^{18}(V/K)_{non\text{-}bridge}$ of 1.0024 contrasts with the inverse value of 0.9951 for the uncatalyzed hydrolysis of the *p*NPS anion. By analogy with phosphoryl transfer reactions, this indicates an increase in nucleophilic participation.

The leaving group KIE data for the ASA from *Aerobacter aerogenes* indicate that a fractional negative charge resides on the leaving group in the transition state (as evidenced by the 1.0009 value for $^{15}(V/K)$, about one-third of the value of the ^{15}N EIE for deprotonation of *p*-nitrophenol). The $^{18}(V/K)_{bridge}$ KIE is larger than that for the *Helix pomatia* ASA by about one-third of the value for the ^{18}O EIE for deprotonation of *p*-nitrophenol [recall that protonation reduces the magnitude of $^{18}(V/K)_{bridge}$]. Thus, a transition state is indicated in which protonation of the leaving group is concerted, but not completely synchronous, with S—O bond fission. The small normal $^{18}(V/K)_{non\text{-}bridge}$ again suggests more nucleophilic participation than in the uncatalyzed reaction of the anion, but less than in the reaction catalyzed by the *Helix pomatia* ASA.

CONCLUSIONS

The pH–rate dependences of V/K for both enzymes indicate general acid–general base catalysis of the sulfuryl transfer from *p*NPS to enzyme particularly for the *Aerobacter aerogenes* ASA. The *Aerobacter aerogenes* ASA shows a higher pH optimum than the ASA from *Helix pomatia*. The two enzymes also differ in the synchronization of leaving group protonation and S—O bond fission. The two processes are fully synchronous in

the *Helix pomatia* ASA, with the result that the leaving group is neutral in the transition state. In the *Aerobacter aerogenes* ASA reaction, about one-third of a charge resides on the leaving group, indicating that proton transfer lags behind S—O bond fission.

EXPERIMENTAL

Arylsulfatase A from *Helix pomatia* (type H-5) and from *Aerobacter aerogenes* (type VI) were obtained from Sigma-Aldrich and were used as received.

Syntheses of unlabeled *p*-nitrophenyl sulfate and of the isotopic forms used for KIE determinations were carried out as described previously.[12]

Kinetics experiments

The potassium salt of *p*NPS was used in all kinetic experiments. All kinetic runs were performed at 25 °C and the concentrations of *p*NPS used ranged from 0.01 to 4.0 mM. These reactions were examined in buffer solutions consisting of 0.05 M Tris, 0.05 M Bis-Tris and 0.2 M acetate, ranging in pH from 3.3 to 9.8. This buffer system maintains a constant ionic strength of 0.1 M throughout the pH range. The stock solution of the *Aerobacter aerogenes* ASA was prepared by adding 100 µl of the commercial enzyme prep (in 50% glycerol and 0.01 M Tris, pH 7.5) to 1 ml of pH 6.3 Bis-Tris–acetate buffer. For *Helix pomatia* ASA, a stock solution was prepared by dissolving 4.0 mg of the lyophilized enzyme in 1 ml of pH 6.3 buffer. Reactions were initiated by addition of 50 µl of this enzyme solution to 3 ml of buffer containing *p*NPS. Periodically, 400 µl aliquots were removed and added to 800 µl of 1 M NaOH, measuring the evolution of *p*-nitrophenolate spectrophotometrically at 400 nm.

Test for trans-sulfurylation activity

Each of the arylsulfatases was incubated with *p*NPS substrate in the presence of an equimolar quantity of phenol and allowed to react for approximately two half-lives, as judged by assaying release of *p*-nitrophenol. At this time the reaction was stopped, titrated to pH 2 and extracted with diethyl ether to remove free phenols. The aqueous fraction was concentrated and proton NMR was used to look for the presence of phenyl sulfate. NMR showed remaining unreacted *p*NPS but no detectable phenyl sulfate. For *Helix pomatia* the reaction conditions were 1 mM potassium *p*-nitrophenyl sulfate, 1 mM phenol, pH 6.3 and for *Aerobacter aerogenes* 0.4 mM potassium *p*-nitrophenyl sulfate, 0.4 mM phenol, pH 7.3.

Isotope effect determinations

Kinetic isotope effects were measured using the methodology reported previously for the uncatalyzed hydrolysis of *p*NPS.[12]

p-Nitrophenyl sulfate (26 mg) was added to 5 ml of 0.1 M Bis-Tris–0.2 M acetate buffer at room temperature in three large test-tubes. Enzyme stock solution (180 µl) was then added (with *A. aerogenes* the commercial enzyme solution was used as received; with *H. pomatia*, the enzyme prep was prepared as 4.0 mg enzyme in 1 ml of pH 5.8 Bis-Tris–acetate buffer). The reaction progress was monitored by recording periodic measurement of the absorbance of 5 µl of the above solution in 3 ml of 1 M NaOH at 400 nm. When the individual samples had reached fractions of reaction ranging from 30 to 60%, each was diluted with 5 ml of H_2O. A 50 µl aliquot of this solution was then added to two separate tubes, one containing 0.5 ml of 1 M HCl and the other 0.5 ml of 1 M NaOH. *p*-Nitrophenol was assayed by measuring the absorbance at 400 nm, which was immediately measured on the basic sample. The acidic sample was kept at 40 °C overnight to ensure complete hydrolysis of the remaining substrate before the absorbance was read. For both samples, absorbance was recorded of a 50 µl aliquot in 3 ml of 1 M NaOH.

Next, each solution was titrated with 1 M HCl to pH <3. The *p*-nitrophenol was then extracted with diethyl ether (3×25 ml). The organic layers were dried over anhydrous magnesium sulfate and the ether was removed by rotary evaporation. The aqueous layer containing the residual substrate solution was then acidified by adding 1.2 ml of 10 M HCl and kept at 40 °C for 24 h for complete hydrolysis of residual *p*NPS. Subsequently, the *p*-nitrophenol liberated from residual substrate was extracted with diethylether as described for the product samples.

All *p*-nitrophenol samples were then sublimed at 100 °C and subsequently subjected to isotope ratio analysis, using a Europa 20–20 isotope ratio mass spectrometer. Data analysis was carried out as reported previously for the uncatalyzed hydrolysis reactions of the same substrate.[12]

Acknowledgments

Funding for this research came from the National Institutes of Health (GM47297) and the Petroleum Research Fund of the American Chemical Society (35690-AC4) to A.C.H. and an Undergraduate Research and Creative Opportunities Grant from Utah State University to J.M.Y.

REFERENCES

1. Baumann E. *Ber. Dtsch. Chem. Ges.* 1876; **9**: 54–58.
2. Bernstein S, Solomon S. *The Chemistry and Biochemistry of Steroid Conjugates*. Springer: Berlin, 1970.
3. Waldow A, Schmidt B, Dierks T, von Bulow R, von Figura K. *J. Biol. Chem.* 1999; **274**: 12284–12288.
4. Lukatela G, Krauss N, Theis K, Selmer T, Gieselmann V, von Figura K, Saenger W. *Biochemistry* 1998; **37**: 3654–3664.

5. Spencer B. *Biochem. J.* 1958; **69**: 155–159; Sampson EJ, Vergara EV, Fedor JM, Funk MO, Benkovic SJ. *Arch. Biochem. Biophys.* 1975; **169**: 372–383.
6. Burns GRJ, Galanopoulou E, Wynn CH. *Biochem. J.* 1977; **167**: 223–227; Chai CLL, Loughlin WA, Lowe G. *Biochem. J.* 1992; **287**: 805–812.
7. Knaust A, Schmidt B, Dierks T, von Bulow R, von Figura K. *Biochemistry* 1998; **37**: 13941–13946.
8. von Bulow R, Schmidt B, Dierks T, von Figura K, Uson I. *J. Mol. Biol.* 2001; **305**: 269–277.
9. Hopkins A, Day RA, Williams A. *J. Am. Chem. Soc.* 1983; **105**: 6062–6070; Bourne N, Hopkins A, Williams A. *J. Am. Chem. Soc*. 1985; **107**: 4327–4331.
10. O'Leary MH, Marlier JF. *J. Am. Chem. Soc.* 1979; **101**: 3300–3306.
11. Hengge AC. *Acc. Chem. Res.* 2002; **35**: 105–112.
12. Hoff RH, Larsen P, Hengge AC. *J. Am. Chem. Soc*. 2001; **123**: 9338–9344.
13. Benkovic SJ, Vergara EV, Hevey RC. *J. Biol. Chem.* 1971; **246**: 4926–4933.
14. Shriver DF, Atkins P, Langford CH. *Inorganic Chemistry* (2nd edn). Freeman: New York, 1994; Luder WF, Zuffanti S. *The Electronic Theory of Acids and Bases* (2nd edn). Dover: New York, 1961.
15. Cleland WW. In *Investigations of Rates and Mechanisms of Reactions*, vol. 6, Bernasconi CF (ed). Wiley: New York, 1986; 791–870; Cleland WW. In *Advances in Enzymology*, vol. 45. Wiley: New York, 1977; 273–387.
16. Rishavy MA, Cleland WW. *Can. J. Chem.* 1999; **77**: 967–977.
17. Hengge AC, Cleland WW. *J. Am. Chem. Soc.* 1990; **112**: 7421–7422.
18. Anderson MA, Shim H, Raushel FM, Cleland WW. *J. Am. Chem. Soc.* 2001; **123**: 9246–9253.