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Mechanistic Studies of the Sulfhydrolases from *Helix pomatia* and *Aerobacter aerogenes*

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The kinetics of reactions of the aryl sulfhydrolases from two nonmammalian sources have been examined with a wide range of substituted aryl and naphthyl sulfates. The hydrolytic routes of the enzymes, one (from *Helix pomatia*) preferring acid and the other (from *Aerobacter aerogenes*) alkaline media, conform to a single general description common to other, bacterial, sulfhydrolases. The data presented here, considered with those from other laboratories, suggest that the rate-determining process is that in which the enzyme–substrate complex releases a phenolic compound as the first product of the hydrolysis.

INTRODUCTION

In a previous report (*1*) we have shown that *m*-nitrophenyl sulfate is a more effective substrate for the aryl sulfhydrolase of the snail *Helix pomatia* than is the *para* isomer. This observation is contrary to the expected order if electronic effects controlled the reaction. It was thus of interest to examine further the mode of action of this sulfatase from a nonmammalian source.

The sulfatase of *H. pomatia* has a pH maximum in the acid region (*2*). As an extension of our work we have also examined the analogous enzyme from *Aerobacter aerogenes*, an enzyme with a preference for alkaline pH (*2*). Mechanistic studies with nonmammalian sulfatases have not been extensive—those with enzymes extracted from two bacterial sources are the only others we have found.

The aryl sulfhydrolase of *Alcaligenes metalcaligenes* has been examined by Dodgson *et al.* (*3*), who reported that K_m decreased with an increase in the electrophilic nature of the substituents on the aryl sulfates, and that simultaneously V increased. The analogous enzyme from *Aspergillus oryzae* has also been examined (*4*) with a variety of substituted substrates; K_m was correlated with electronic effects, but V was unchanged by the nature of the substituents on the aryl sulfates employed.

These data may be interpreted (*3, 4*) on the basis of the mechanism described by:

$$E + S \overset{k_1}{\rightleftarrows} ES' \overset{k_2}{\rightleftarrows} ES'' + P' \overset{k_3}{\rightarrow} E + P''. \qquad [1]$$

For the enzyme of *A. metalcaligenes* the release of P′ would be rate determining, while for that from *A. oryzae* the formation of P″ would be rate controlling.

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However, later results from stopped flow measurements (*5*) suggest that this interpretation for the latter enzyme is not unique, although P″ was shown to be SO_4^{2-}.

We report here an examination of the reactivities of a series of aromatic sulfates on the aryl sulfhydrolases of *H. pomatia* and *A. aerogenes*. Some similarities with the data described above were discerned: the enzyme from *H. pomatia* (like that from *A. oryzae* in preferring acid pH) has V constant, irrespective of the substrate substituents in a series of aryl sulfates; that from *A. aerogenes* (preferring alkaline conditions as does the enzyme of *A. metalcaligenes*) by contrast has a V with dependence on the substituents. However, for both the enzymes examined here K_m values were significantly affected by the steric properties of the substituents, which effects were also important for the values of V for the *A. aerogenes* enzyme. A discussion of these results is presented in this paper with some mechanistic conclusions.

EXPERIMENTAL

Substrates

p-Nitro catechol-, 1- and 2- naphthyl-, and 6-benzoyl-2-naphthyl sulfate were obtained from ICN Laboratories; *p*-acetyl and *p*-nitro phenylsulfate were from Sigma Chemicals. These were used without further purification.

p-Bromo and *p*-methyl phenyl sulfates were obtained by alkaline hydrolysis of the corresponding methyl esters, synthesized from the tetrafluorborate diazonium salts and dimethyl sulfate (*6*, *7*).

The other sulfates used were prepared by the methods of Burkhardt and Lapworth (*8a*) or Jerfy and Roy (*8b*). All sulfates were obtained in good yields and were crystallized from water or 95% ethanol. Partial analyses for them are listed in Table 1.

TABLE 1

PARTIAL ELEMENTARY ANALYSES OF ARYL AND NAPHTHYL SULFATES SYNTHESIZED AS DESCRIBED (7)

Sulfate	Analysis (%)			Theoretical (%)		
	C	H	Halogen	C	H	Halogen
o-Nitro phenyl	28.21	1.60		28.10	1.55	
m-Nitro phenyl	28.18	1.58	14.37			
m-Chlor phenyl	29.10	1.78		29.21	1.63	14.37
p-Chlor phenyl	29.03	1.66	14.44			
p-Fluoro phenyl	31.50	1.77	8.15	31.30	1.75	8.25
p-Phenyl phenyl	50.23	3.29		49.98	3.15	
p-*tert*-Butyl phenyl	44.63	4.87		44.76	4.88	
1-Bromo-2-naphthyl	35.85	2.31		35.23	2.37	
6-Bromo-2-naphthyl	35.09	2.45				
1,6-Dibromo-2-naphthyl	29.27	1.29		28.59	1.21	
3-Hydroxy-2-naphthyl	43.01	2.66		43.16	2.51	

Enzymes

The aryl sulfhydrolase of *H. pomatia* was obtained from Boehringer-Mannheim. Although this preparation contained several protein bands on disc gel electrophoresis (*9*) only a single band of enzymatic activity was observed (*10*), as has been previously reported for this enzyme (*11, 12*). The aryl sulfatase from *A. aerogenes* was from Sigma Chemical Company: a single sulfhydrolase is also obtained from this bacteria (*12*).

Work with the enzyme from *H. pomatia* was done with an acetate buffer, 0.1 *M*, of pH 5.8 unless otherwise noted. Tris–HCl buffer, 0.1 *M*, pH 7.2, was used with the enzyme from *A. aerogenes*. The methanol/buffer mixtures were prepared by adding the alcohol to the acetate buffer to produce 10, 20, and 30% solutions (v/v).

Kinetic Procedures

Determination of first-order rate constants (V/K_m). Reactions proceeded in the cell compartments of Beckman Model 26 or Unicam SP 800 spectrophotometers: the temperature was maintained at 20°C. When reactions had half-lives of less than 5 min product accumulation was recorded at a fixed wavelength (see Table 2): for more prolonged hydrolyses, repetitive scanning was used (*1, 2*). Conventional first-order plots were obtained under the conditions used in these experiments, where the substrates were not at saturating concentrations relative to the enzymes.

Determination of V and of K_m. These parameters were obtained from measurements of the initial (10%) velocities of reactions with substrates at varying concentrations. Double reciprocal plots were prepared, which were estimated to contain a 5% error. Values of K_m and V were obtained from these plots. The ratio of V/K_m obtained by this method was in good agreement (10%) with values obtained by the method described above.

RESULTS AND DISCUSSION

When enzymatic kinetic runs are performed under subsaturating conditions, first-order behavior is obtained. This behavior corresponds to the initial part of a Michaelis–Menten plot of velocity vs substrate concentration (*13*), and it can be shown that the first-order rate constant corresponds to V/K_m. Table 2 contains a list of the arylsulfates studied and the corresponding values of the first-order rate constants V/K_m for the *H. pomatia* aryl sulfatase enzyme. From these results it is apparent that: (i) *meta*-substituted sulfates are more reactive than the *para* isomer (*m*-NPS > *p*-NPS); (ii) bulky groups favor the reaction (*p-t*-BuPS > *p*-MPS); (iii) 2-NS is much more reactive than 1-NS; and (iv) data for *p*-NCS and 3-OH-2-NS require separate consideration because of the presence of the ionizable hydroxylic group next to the sulfate function.

If electronic effects, as defined by Hammett σ parameters, were the predominant factors controlling the reaction, one would expect that *p*-NPS > *m*-NPS; *p*-

TABLE 2

V/K_m VALUES (DETERMINED DIRECTLY AS FIRST-ORDER RATE CONSTANTS) FOR THE SERIES OF ARYLSULFATES IN THEIR REACTION WITH *Helix pomatia* ARYLSULFATASE ENZYME IN 0.1 *M* SODIUM ACETATE, pH 5.8

Arylsulfate	Wavelength at which phenolic product was followed (nm) (not necessarily λ_{max})	V/K_m (10^4 sec^{-1})
p-Methyl phenylsulfate (*p*-MPS)	275	2.6
p-Phenyl phenylsulfate (*p*-PPS)	285	157
p-t-Butyl phenylsulfate (*p*-t-BuPS)	280	47
p-Bromo phenylsulfate (*p*-BrPS)	278	17
p-Chloro phenylsulfate (*p*-ClPS)	276	7
m-Chloro phenylsulfate (*m*-ClPS)	272	100[a]
p-Fluoro phenylsulfate (*p*-FPS)	316	1.5
p-Nitro phenylsulfate (*p*-NPS)	315 (pH 5.80; 400 (pH 7.2)	190
m-Nitro phenylsulfate (*m*-NPS)	340	290
o-Nitro phenylsulfate (*o*-NPS)	276	24
p-Acetyl phenylsulfate (*p*-AcPS)	275	60
p-Nitrocatechol sulfate (*p*-NCS)	450	1000
3-Pyridinium sulfate (3-Pyrs)	315	2.9
1-Naphthylsulfate (1-NS)	300	1
2-Naphthylsulfate (2-NS)	310	123
1-Bromo-2-naphthylsulfate (1-Br-2-NS)	330	94
6-Bromo-2-naphthylsulfate (6-Br-2-NS)	336	360
1,6-diBromo-2-naphthylsulfate (1,6-Br..)	326	263
6-Benzoyl-2-naphthylsulfate (6-Bz-2-NS)	330	113
3-Hydroxy-2-naphthylsulfate (3-OH-2-NS)	280	500[a]

[a] Nonlinear first-order plots.

BrPS = *p*-ClPS; *p*-t-BuPS = *p*-MPS; and 1-NS = 2-NS. Steric effects clearly play an important role in the reaction.

A minimum scheme for the sulfatase hydrolytic reaction would obey Eq. [1]. If the liberation of P′ (phenol) were the rate-limiting step, V would be expected to assume a different value for each substrate. By contrast, if the last step were the limiting one, V would be constant throughout the series of substrates employed, because the complex ES″ would be common in all cases. That is, the bulk of the substrates would not affect V were k_3 rate limiting; however, K_m could be affected. Were k_2 rate controlling, steric factors would be reflected in both K_m and V.

V and K_m have been obtained for the wide range of substrates listed in Table 3. It is observed that V is effectively constant for all the substrates examined except those having a hydroxylic group next to the sulfate center. Figure 1 summarizes these data: log V and log V/K_m are plotted against the Hammett σ substituent parameter. V is constant while V/K_m varies in a pattern not controlled by electronic effects. It thus seems reasonable to conclude, on the basis of Eq. [1], that k_3 is rate limiting.

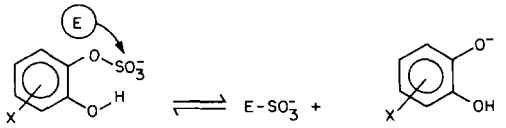
Values of V reported in Table 3 for the hydroxylic substrates *p*-NCS and 3-OH-2-NS are three times higher than those found for other substrates. These results

TABLE 3

V, K_m, AND V/K_m FOR SEVERAL ARYLSULFATES WITH *Helix pomatia* ARYLSULFATASE ENZYME IN 0.1 M SODIUM ACETATE, pH 5.8

Arylsulfate	V (10^6 M sec^{-1})	K_m(10^3 M)	V/K_m(10^4 sec^{-1})
p-NPS	3.2	0.20	175
m-NPS	3.3	0.12	278
p-AcPS	2.7	0.38	71
m-ClPS	2.4	0.18	137
p-ClPS	2.1	1.23	16
p-t-BuPS	2.4	0.48	50
p-MPS	2.5	10.40	2.4
p-BrPS	2.5	1.25	20
6-Br-2-NS	2.7	0.08	350
1-NS	2.0	20	1
2-NS	2.3	0.19	120
p-NCS	7.5	0.08	938
3-OH-2-NS	7.7	0.11	700

may also be explained on the basis of Eq. [1]. It is assumed that P′ (catechol product) from these substrates associates, by hydrogen bonding, with the enzyme in a position proximate to the active center. The result of this secondary binding to the substrate in the complex would be similar to that produced in conventional acid catalysis (*14*) in which a preequilibrium step involving protonation of the phenolic oxygen occurs: reaction is facilitated. The low values of K_m for the hydroxylic substrates suggest that there is a strong enzyme/substrate interaction with these compounds, supporting the idea of a secondary (hydrogen bounded) binding. At high pH values the phenolic groups of these compounds are dissociated (pK values 6.4 and 8.7 for *p*-NCS and 3-OH-2-NS, respectively): Consequently as the H-bonding system described can no longer exist, the acceleration caused by it would be lost, as observed previously (*2*). Dissociation of the phenolic group in *p*-NCS may itself produce loss of binding ability by the sulfohydrolases, as the free R-O^- may be incapable of an essential bond formation to produce ES, or the negative charge may preclude the normal interaction of functional groups of the enzyme with the substrate. A plausible alternate mechanism[1] for the substrates with hydroxyl group in position 2 is intramolecular acid catalysis associated with enzyme catalysis, as shown:



[2]

[1] Suggested by one of the referees of this contribution.

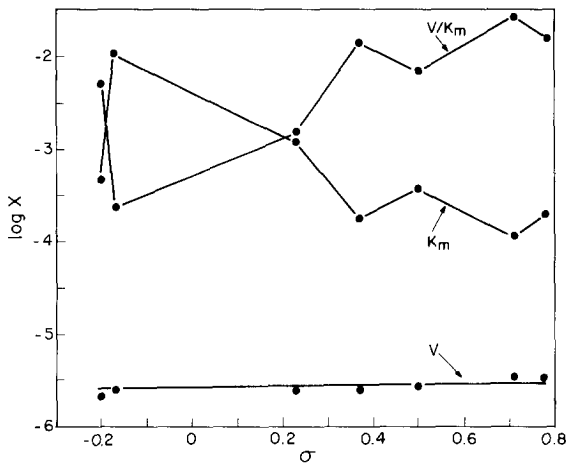


FIG. 1. Variation of log V, log K_m, and log V/K_m for *Helix pomatia* aryl sulfatase with electronic substituent effects (Hammett σ parameter).

The effect of the ionization of the hydroxylic substituent groups was studied with measurements in aqueous-methanolic solvents. The use of such a solvent system is not expected to introduce heterogeneity to a degree which prevents the acquisition of kinetic information; also, effects on the enzyme were minimized by comparing ratios of V/K_m for *p*-NPS and *p*-NCS (only the latter substrate having an ionizable phenolic group). Table 4 contains the V/K_m values for these substrates at solvent compositions of 0, 10, 20, and 30% (v/v) methanol at pH 6.0. The addition of methanol diminishes the dielectric constant of the reaction medium, so decreasing the dissociation of the phenolic group of *p*-NCS. This, from the argument above, is the form preferred by sulfhydrolases, so an increased concentration of methanol should have less effect on enzymic hydrolyses of *p*-NCS than of *p*-NPS, as is reported in Table 4.

The results with the sulfohydrolase of *A. aerogenes* are contained in Table 5. Presented there are V, K_m, and V/K_m values obtained from initial velocity experiments, together with V/K_m constants obtained directly using first-order kinetics.

TABLE 4

EFFECT OF INCREASING METHANOL CONTENT IN THE SOLVENT SYSTEM ON V/K_m (DETERMINED DIRECTLY AS FIRST-ORDER RATE CONSTANT) AT pH 6.0, FOR *p*-NPS AND *p*-NCS ($[AcO^-] = 0.1\ M$)

	V/K_m (10^4 sec^{-1})		
MeOH(%, v/v)	*p*-NPS	*p*-NCS	V/K_m(*p*-NCS)/(*p*-NPS)
0	14.37	28.08	1.95
10	8.84	21.20	2.40
20	1.94	17.14	8.84
30	1.23	16.70	13.58

TABLE 5

V, K_m, AND V/K_m VALUES DETERMINED FROM RECIPROCAL PLOTS FOR *Aerobacter aerogenes* WITH SEVERAL ARYLSULFATES, AND DIRECTLY DETERMINED V/K_m VALUES[a]

Arylsulfate	V (10^5 M sec^{-1})	K_m (mM)	V/K_m (10^4 sec^{-1})	Direct determination first-order rate constant
p-NPS	0.120	1.4	9	9
m-NPS	1.200	4.0	30	30
3-Pyr-S(3-pyridine sulfate)	0.033	0.5	6.5	6
p-ClPS	0.020	0.5	4	3.7
p-BrPS	0.330	3.3	10	10
p-MPS	0.009	0.4	2.3	1.7
p-t-Bu-PS	0.025	0.8	3.1	3.4

[a] 0.1 M Tris–Cl, pH 7.2.

It is apparent that both V and K_m are affected by substrate substituents. This finding suggests that in Eq. [1] k_2 is the limiting step in the process. It is clear that the variation of V does not follow electronic effects. Instead, steric factors are most important, as is observed from a comparison of the last two pairs in Table 5: larger groups induce higher V values. The variations of K_m also show a relationship to steric effects, since substrates with smaller substituents have lower K_m values. When k_2 is considered as the slow step in Eq. [1], $V = k_2E_0$ and $K_m = (k_2 + k_{-1})/k_1$. Since both V and K_m seem to follow related patterns regarding steric effects, and since k_2 is the only rate constant influencing V, it is likely that only k_2 is controlled by steric effects in the K_m expression. Thus substrate size appears to have opposing effects on K_m and V: smaller substrates may better fit the active site, but they react more slowly than larger ones. It is for this reason that V/K_m reflects the steric differences between substrates to a lesser extent than do V or K_m individually.

It has been previously reported that *A. aerogenes* sulfatase could not hydrolyze 1-NS (*15*). However, our results consistently show enzyme activity toward this substrate, although at a much lower rate than with 2-NS. This difference is interpretable within a hypothesized general steric selectivity of these enzymes, as follows: a substrate would bind the enzyme by means of a multipoint attachment process which would define the steric relationship between the sulfate group, the substituents, and the functional groups at the active site of the enzyme. This relationship seems to be optimal when position 3 with respect to the sulfate group is substituted. In this scheme *meta*-substituted phenyl sulfates and 2-naphthyl-sulfates belong to the same category and respond to the same steric selectivity of the enzyme.

Figure 2 shows pH–rate profiles for 3-OH-2-NS and *p*-NPS measured with the sulfohydrolases of *H. pomatia* (Fig. 2A) and *A. aerogenes* (Fig. 2B). It is seen that pH_{max} of 3-OH-2-NS with *A. aerogenes* is 7.1, contrasting with the value of 6.4 found for *p*-NCS (*2*). It was previously reported that *p*-NCS is reactive only as its undissociated form; and since its pK_a is 6.4, at 7.1 it would be mostly dissociated

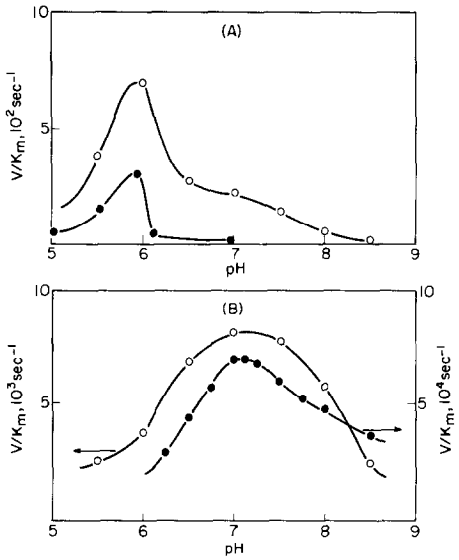


FIG. 2. pH–rate profiles for *p*-NPS (●) and 3-OH-2-NS (○) with (A) *Helix pomatia* aryl sulfatase and (B) *Aerobacter aerogenes* aryl sulfatase. 0.1 *M* sodium acetate buffers were used for pH values under 7, while 0.1 *M* Tris–HCl buffers were used for pH values equal to and larger than 7.

(*2*). Spectrophotometric measurements indicate that 3-OH-2-NS has a pK_a of 8.7, which allows the substrate to be in its undissociated form at the pH of maximum activity of the enzyme of *A. aerogenes*. The above results give further support to the fact that hydroxyl groups vicinal to the sulfate grouping enhance the reactivity of substrates when in their undissociated form.

The pH dependence of *H. pomatia* sulfatase activity suggests that the enzyme has two dissociable groups involved in the catalysis: one with a pK_a at about 5.2 and the other with a pK_a of about 6.2, as indicated by the pH–rate profiles using *p*-NPS, *p*-NCS (*1, 2*), and 3-OH-2-NS. For these three substrates the pH_{max} is 5.8 (Fig. 2). Similar examination of the sulfhydrolase from *A. aerogenes* is complicated by the ionization of *p*-NCS, as previously discussed (*2*). However, if substrates with no dissociable groups are considered, there are indications of two ionizable groups, at pH values of 6.6 and 7.5, being involved in the enzymic activity. The range of pK_a values thus found for the two enzymes discussed is small, from 5.2 to 7.5. Such overlapping of different possibilities occurs (*13*) so as to make valueless specific assignments of functional groups in the enzymes on the basis of the pK_a values defined above.

Our results indicate that there is no inhibition of either enzyme by sulfate or the phenolic products, in agreement with previous reports (*15*). However, at much higher initial concentrations of substrates than those used in the work reported here there are indications of substrate inhibition. This phenomenon is being further investigated.

Comparison of results presented here with studies using other nonmammalian aryl sulfatase enzymes may be useful. For example, the enzyme from *A.*

metalcaligenes shows linear Hammett plots for both K_m and V (3), so that the maximum rate would be controlled by electronic effects. The aryl sulfatase from *A. aerogenes* is similar in that both show substituent effects in V and in K_m. However, while for V these effects are steric, for K_m they are electronic in nature.

The aryl sulfatase of *A. oryzae* (*4*) apparently resembles that of *H. pomatia* in that V seems to be constant, and the differences in reactivities are determined by different K_m values. However, the existence (*5*) of a linear correlation between log V/K_m and log k_H+ (the catalytic constant for the nonenzymatic acid-catalyzed reaction) suggests that electronic effects are much more important with the former enzyme.

It is interesting to note that the two enzymes showing alkaline pH_{max} have k_2 as the rate-limiting step. It may be that this is because the greater nucleophilicity of the medium facilitates the last step of Eq. [1] for sulfohydrolases preferring alkaline pH to those preferring acid pH. Sulfohydrolases displaying maximum activity at acid pH may, as the discussion above suggests, have the final step (k_3) of Eq. [1] rate determining. However, Sampson *et al.* (*5*) were unable to detect ES″ when working with the enzyme from *A. oryzae,* using several experimental procedures. They suggested that here, too, k_2 is rate determining with the enzyme effectively "leveling" substituent effects. This explanation may also apply to the sulfatase of *H. pomatia,* if it is modified to incorporate the postulated secondary binding of hydroxylated substrates discussed above.

Recent results with model studies of the hydrolysis of sulfate esters in the presence of macrocycles are of interest in considering the role of steric factors in the work reported here. Those studies (*16*) suggest that tight incorporation of a substrate into the catalytic cavity results in greater strain and the development of hydrophobicity in the guest molecule, these effects facilitating its hydrolysis. Comparable behavior in the aryl sulfhydrolases of *H. pomatia* and *A. aerogenes* could account for some of the results reported above including those with 3-pyridine sulfate, which is hydrolyzed very slowly by the aryl sulhydrolases studied (Tables 2,5). Model building also suggests that the preference shown for *meta*-substituted substrates as well as for the 2-NS series may be a result of these compounds being in preferred conformations.

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