BIOORGANIC CHEMISTRY 20, 181–188 (1992)

The Mechanism and Stereochemical Course of Sulfuryl Transfer Catalyzed by the Aryl Sulfotransferase from *Eubacterium* A-44

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Received August 7, 1991

The aryl sulfotransferase from *Eubacterium* A-44 catalyzes the sulfuryl transfer reaction from phenyl [(*R*)-^{16}O,^{17}O,^{18}O]sulfate to *p*-cresol with *retention of configuration at sulfur.* This implies that the sulfuryl transfer reaction involves a sulfoenzyme intermediate which from earlier evidence is probably formed through a tyrosine residue. © 1992 Academic Press, Inc.

The aryl sulfotransferase from *Eubacterium* A-44 (one of the predominant anaerobic bacteria of the human intestine) catalyzes the sulfation of phenolic substrates but does not use 3′-phosphoadenosine 5′-phosphosulfate as the sulfate donor nor is 3′-phosphoadenosine 5′-phosphate a cofactor in the transfer of the sulfuryl group from an aryl sulfate donor (*1*). The enzyme is composed of four identical subunits each of M_r ca. 80,000 and the enzyme activity shows a bell-shaped pH dependence with a pH optimum at about 8.5. Incubation of the enzyme with *p*-nitrophenyl [^{35}S]sulfate in the absence of a phenolic acceptor leads to the covalent attachment of about 2 mol of [^{35}S]sulfate per mole of enzyme, i.e., only two of the four subunits are labeled simultaneously. Hydrolysis of the [^{35}S]sulfoenzyme under basic conditions led to the isolation of [^{35}S]sulfotyrosine as the only ^{35}S-labeled amino acid. The [^{35}S]sulfoenzyme was able to transfer the [^{35}S]sulfuryl group to tyramine *but* only in the presence of added donor, e.g., *p*-nitrophenyl sulfate. Diethyl pyrocarbonate inactivates the enzyme and the enzyme is protected by the donor substrate, *p*-nitrophenyl sulfate, from inactivation by this reagent. On the basis of this evidence a mechanism has been proposed for the aryl sulfotransferase in which a donor substrate first transfers its sulfuryl group to the imidazole group of an active site histidine which is then transferred to an active site tyrosine residue (*2, 3*). Although evidence for a chemically competent sulfoenzyme intermediate has been presented (*2, 3*), no evidence has been provided to demonstrate that the sulfoenzyme intermediate is kinetically competent. The evidence for an active site histidine is based solely on the purported predilection of diethyl pyrocarbonate to react with a histidine residue. Moreover, no evidence was provided to support the involvement of the putative histidine as a site for sulfation by a donor substrate.

In order to explore aryl sulfotransferases we have recently synthesized phenyl

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0045-2068/92 $5.00

[(*R*)-^{16}O,^{17}O,^{18}O]sulfate (*4*) and developed a method for the stereochemical analysis of chiral [^{16}O,^{17}O,^{18}O]sulfate esters using FTIR spectroscopy (*5*). It is expected that each sulfuryl transfer step catalyzed by an enzyme will occur with inversion of configuration at sulfur; it has been found that enzyme-catalyzed phosphoryl transfer steps always occur with inversion of configuration at phosphorus (*6*). If, therefore, a single sulfoenzyme intermediate is involved in the enzymic mechanism, the double inversion expected should lead to overall retention at sulfur. If on the other hand two sulfoenzyme intermediates are involved as proposed (*2, 3*), then overall inversion at sulfur would be expected. We now report an investigation of the stereochemical course of the aryl sulfotransferase from *Eubacterium* A-44 using phenyl [(*R*)-^{16}O,^{17}O,^{18}O]sulfate as the donor substrate and *p*-cresol as the acceptor.

MATERIAL AND METHODS

The infrared spectra were recorded on a Perkin–Elmer 1750 Fourier transform infrared spectrometer with a Perkin–Elmer 7300 professional computer at a resolution of 1 cm^{-1}. All chemicals and reagents were obtained from Aldrich Chemical Co., Ltd, UK.

Enzyme-Catalyzed Sulfuryl Transfer

An enzyme solution of arylsulfotransferase from *Eubacterium A-44* (400 μl, 15.3 units) was added to a solution containing Tris · HCl buffer (50 mM, pH 8.0, at 37°C, 12 ml), *p*-cresol (55.6 mg) and tetrabutylammonium phenyl [(*R*)-^{16}O,^{17}O,^{18}O] sulfate (29 mg). The mixture was kept at 37°C in a constant temperature bath for 4 h, after which the reaction was terminated by freezing the solution with liquid nitrogen followed by lyophilization. The ratio of phenyl [^{16}O,^{17}O,^{18}O]sulfate: *p*-cresyl [^{16}O,^{17}O,^{18}O]sulfate was 1.7 : 1.0 as determined from reversed phase HPLC (60% 50 mM triethylammonium carbonate, pH 7.5, 40% acetonitrile, Du Pont 21.2 mm × 25 cm, 4 ml/min). *p*-Cresyl [^{16}O,^{17}O,^{18}O]sulfate was isolated by preparative reversed phase HPLC using the same solvent system. The buffer was removed by coevaporation with methanol and 1 eq of 0.154 N tetrabutylammonium hyroxide (167 μl) was added and the solution was lyophilized to give tetrabutylammonium *p*-cresyl [(*R*)-^{16}O,^{17}O,^{18}O]sulfate.

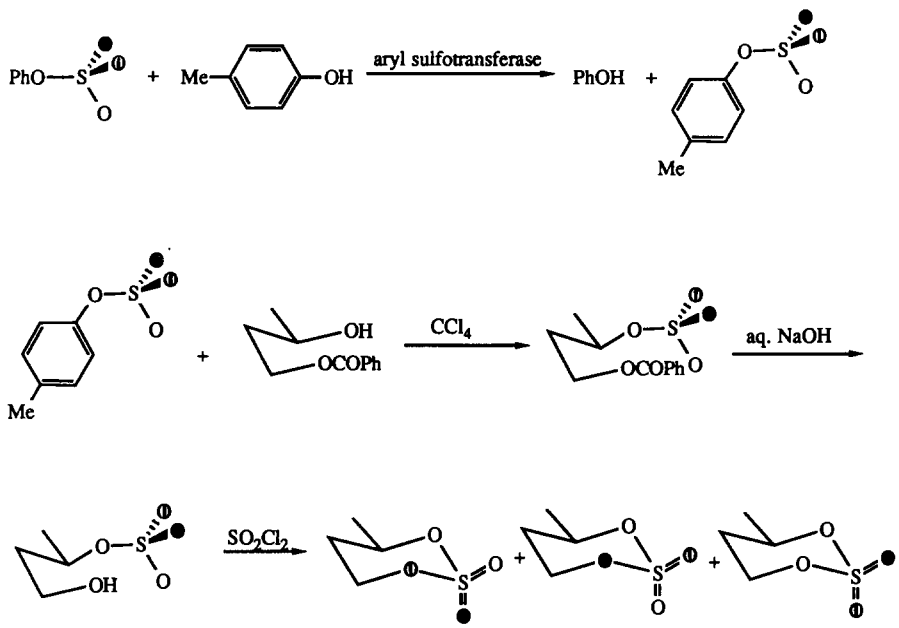
Analysis of the p-Cresyl [^{16}O,^{17}O,^{18}O]sulfate

p-Cresyl [^{16}O,^{17}O,^{18}O]sulfate tetrabutylammonium salt (11.1 mg, 0.026 mmol) was added to a solution of (1*R*)-3-benzoyloxy-1-methylpropan-1-ol (6.7 mg, 0.035 mmol) {prepared from (*R*)-butane-1,3-diol, $[\alpha]^{21}$-31° (*c* = 1, EtOH) by the method of Kim *et al.* (*7*)} in dry carbon tetrachloride (1.0 ml) in a reactivial. The vial was immersed in an oil bath at 100°C for 16 h. The reaction mixture was then evaporated and the residue was dissolved in water (0.5 ml), followed by the addition of 3 eq of 0.1 M sodium hydroxide solution. The solution was stirred for 16 h at room temperature. The reaction mixture was diluted with more water and the solution

adjusted to pH 8 with dilute HCl. *p*-Cresol was extracted with ether and the aqueous layer was separated off and evaporated to a small volume under reduced pressure. This was passed through a pyridinium Dowex 50 × 8–100 column, eluting with distilled water. The solution was evaporated under reduced pressure. The solution was coevaporated with dry CH_3CN (4 × 2 ml). The dry residue was dissolved in dry CH_3CN (10 ml) and cooled to −15°C (dry ice/CCl_4) and sulfuryl chloride (21 μl, 10 eq) added. The reaction mixture was removed from the cooling bath and allowed to react for a further 30 min; the solution was evaporated under reduced pressure (water pump). The residue and an approximately equal weight of unlabelled (4*R*)-methyl-2,2-dioxo-1,3,2-dioxathiane were dissolved in a small volume of ether and purified by chromatography on a small column of silica gel (1 g) in a pasteur pipette and eluted with 1 : 1 ether/pentane. After a uv absorbing fraction (probably derived from the reaction of sulfuryl chloride on the benzoic acid byproduct) had been eluted, the solvent was changed to 100% ether. The required cyclic sulfate can be detected by TLC (eluting solvent: ether, blue spot with phosphomolybdate spray at R_f = 0.5). The required fractions were pooled and evaporated under reduced pressure (11 mm Hg). It was necessary to coevaporate the sample several times with CCl_4 to remove the ether. The FTIR spectrum of the isotopomeric mixture of (4*R*)-methyl-2,2-dioxo-1,3,2-dioxathianes in CCl_4 was obtained and the asymmetric and symmetric stretching frequencies were compared with those in the FTIR spectrum obtained from the product derived from direct sulfuryl transfer from phenyl [(*R*)-^{16}O,^{17}O,^{18}O]sulfate to (1*R*)-3-benzoyloxy-1-methylpropan-1-ol (Fig. 2).

RESULTS AND DISCUSSION

We have recently reported the synthesis of phenyl [(*R*)-^{16}O^{17}O^{18}O]sulfate (*4*), and have developed a method for the stereochemical analysis of chiral [^{16}O^{17}O^{18}O] sulfate esters (*5*). Phenyl [(*R*)-^{16}O,^{17}O,^{18}O]sulfate was incubated with the aryl sulfotransferase from *Eubacterium* A-44 in the presence of *p*-cresol at pH 8.0 for 4 h by which time about 37% of the phenyl [(*R*)-^{16}O,^{17}O,^{18}O]sulfate had been converted into *p*-cresyl [^{16}O,^{17}O,^{18}O]sulfate which was isolated by HPLC. The configuration of the *p*-cresyl [^{16}O,^{17}O,^{18}O]sulfate was determined as outlined in Scheme 1. The *p*-cresyl [^{16}O,^{17}O,^{18}O]sulfate was incubated with (1*R*)-3-benzoyloxy-1-methylpropan-1-ol {prepared from (*R*)-butane-1,3-diol, $[\alpha]^{21}$-31° (*c* = 1, EtOH), by the method of Kim *et al.* (*7*)} in carbon tetrachloride and when transfer was complete the benzoyl group was removed by hydrolysis with sodium hydroxide solution and the chiral [^{16}O,^{17}O,^{18}O]sulfate cyclized with sulfuryl chloride, which is known to occur with retention of configuration at sulfur (*5*). The FTIR spectrum of the mixture of isotopomers so generated is shown in Fig. 1. For comparison the FTIR spectrum of the mixture of isotopomers derived directly by the chemical transfer from phenyl [(*R*)-^{16}O,^{17}O,^{18}O]sulfate to (1*R*)-3-benzoyloxy-1-methylpropan-1-ol is shown in Fig. 2. Clearly the phenyl [(*R*)-^{16}O,^{17}O,^{18}O]sulfate and the *p*-cresyl [^{16}O,^{17}O,^{18}O]sulfate have the same configuration and the enzyme-



SCHEME 1. The stereochemical course of the sulfuryl transfer reaction catalyzed by the aryl sulfotransferase from *Eubacterium* A-44. The FTIR spectrum of the mixture of isotopomers of (4*R*)-methyl-2,2-dioxo-1,3,2-dioxathiane is shown in Fig. 1. ⊙ = ^{17}O; ● = ^{18}O.

catalyzed reaction must have proceeded with overall *retention of configuration at sulfur*.

The observed retention of configuration at sulfur in the aryl sulfotransferase-catalyzed reaction is consistent with the involvement of a single sulfoenzyme intermediate, inversion occurring in the transfer of the sulfuryl group from the donor substrate to the enzyme and in the transfer from the sulfoenzyme intermediate to the phenolic acceptor. Thus the stereochemical evidence suggests that the enzyme follows a ping pong kinetic mechanism. Since this work was completed, Kim and Kobashi (*8*) have reported kinetic data which suggest that the enzyme adopts a ping pong bi bi mechanism. Although enzyme kinetics is an unreliable method of establishing a ping pong type mechanism, since the statistical error on lines drawn through experimental points means that the lines can never be *proven* to be parallel, gratifyingly the kinetic mechanism (*8*) and the stereochemical evidence reported here are in accord. Evidence has been presented that a sulfoenzyme intermediate is formed through a tyrosine residue (*2, 3*). The authors actually suggested that two sulfoenzyme intermediates were involved and that the first to be formed was a transient species in which the sulfuryl group was attached to the imidazole group of a histidine residue (*2, 3*); this statement has recently been reiterated (*8*). However, the evidence for this suggestion is tenuous, being based on the observation that the enzyme was inhibited by diethyl pyrocarbonate. Even if this reagent did specifically modify an active site histidine this does not imply

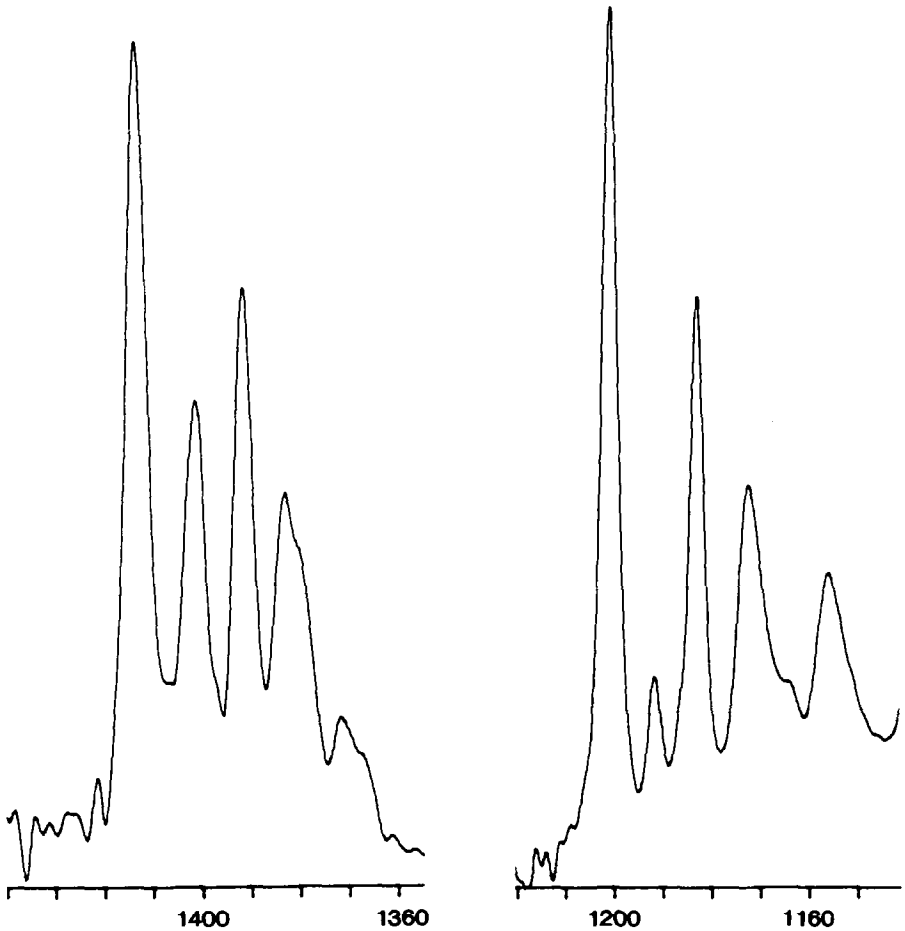


FIG. 1. The FTIR spectrum showing the antisymmetric (1414–1378 cm^{-1}) and symmetric (1201–1157 cm^{-1}) $\rangle SO_2$ stretching frequencies of the mixture of isotopomers derived by cyclizing (1*R*)-3-hydroxy-1-methylpropyl [^{16}O,^{17}O,^{18}O]sulfate (obtained from *p*-cresyl-[^{16}O,^{17}O,^{18}O]sulfate, Scheme 1) with sulfuryl chloride. The scale is in cm^{-1} and the resolution was enhanced by Fourier deconvolution; for the antisymmetric stretching region a linewidth of 20 cm^{-1} and an enhancement factor of 2.0 were used, whereas for the symmetric stretching region a linewidth of 10 cm^{-1} and an enhancement factor of 1.5 was used. Isotopes in a bridging position do not influence the $\rangle SO_2$ stretching frequencies (*5*).

that its role would be to function as a sulfuryl acceptor, rather the group could simply function as a general acid–general base catalyst. Thus in the attack by the enzyme active site tyrosine hydroxyl group on the donor substrate, a histidine residue could facilitate the process by acting as a general base–general acid catalyst. Although it is possible that a single histidine (or some other amino acid residue) could function as a general base–general acid catalyst, it seems more likely that two amino acids (one or both of which may be histidine residues) could function *in concert* as general base–general acid catalysts as depicted in Scheme 2.

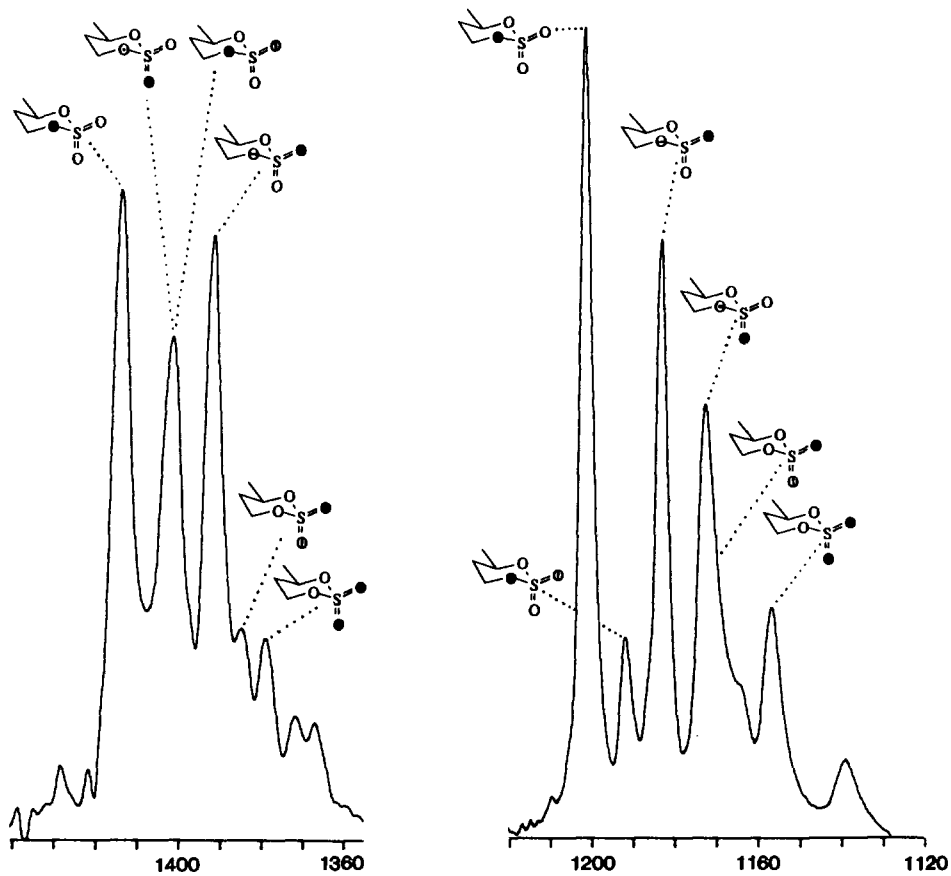
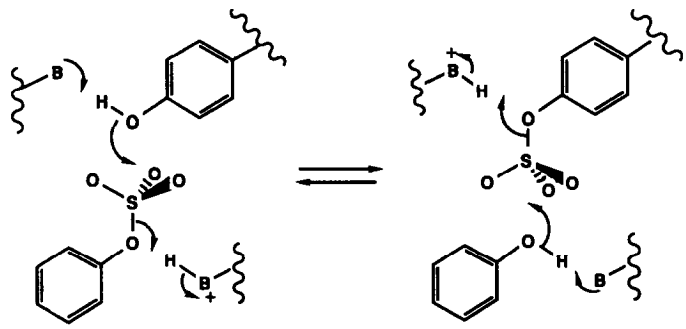


FIG. 2. The FTIR spectrum showing the antisymmetric (1414–1378 cm^{-1}) and symmetric (1201–1157 cm^{-1}) $\rangle SO_2$ stretching frequencies of the mixture of isotopomers derived by cyclizing (1*R*)-3-hydroxy-1-methylpropyl [^{16}O,^{17}O,^{18}O]sulfate (obtained from phenyl [(*R*)-^{16}O,^{17}O,^{18}O]sulfate) with sulfuryl chloride. The scale is in cm^{-1} and the resolution was enhanced by Fourier deconvolution; for the antisymmetric stretching region a linewidth of 20 cm^{-1} and an enhancement factor of 2.0 was used, whereas for the symmetric stretching region a linewidth of 10 cm^{-1} and an enhancement factor of 1.5 was used. Isotopes in a bridging position do not influence the $\rangle SO_2$ stretching frequencies (5). ⓓ = ^{17}O; ● = ^{18}O; ⊖ = ^{17}O and ^{18}O; ⊙ = ^{16}O, ^{17}O and ^{18}O.

The observation that *p*-nitrophenyl [^{35}S]sulfate labels only two of the four subunits and that transfer of the sulfuryl group from the sulfoenzyme intermediate to the acceptor phenol requires a donor substrate to be present (*2, 3*) is typical of half-the-sites reactivity (*9*). In this case it implies that the donation of the sulfuryl group to the enzyme in two of the subunits is coupled to the transfer of the sulfuryl group from the sulfoenzyme intermediate to the acceptor phenol in the other two subunits. This suggests that the intrinsic binding energy of the donor substrate induces a conformational change in the sulfated subunits which facilitate sulfuryl transfer to the acceptor phenol. Thus while donation of the sulfuryl group to the



Enzyme-Donor substrate complex **Sulfo-enzyme Acceptor substrate complex**

SCHEME 2. A proposed ping pong mechanism for the aryl sulfotransferase-catalyzed reaction by way of a sulfoenzyme intermediate involving a tyrosine residue. One or both of the general acid–general base catalysts may be histidine residues. It is envisaged that two subunits are present as enzyme–donor substrate complexes and two subunits as sulfoenzyme acceptor complexes at any time.

enzyme occurs at two of the subunits, transfer from the sulfoenzyme intermediate to the acceptor substrate occurs at the other two subunits (Scheme 2).

It is not surprising that an aryl sulfotransferase adopts a ping pong type mechanism to transfer sulfuryl groups between phenols since the donor substrate and the product have similar structural features and can be expected to be accommodated by the same binding site. Thus by providing a refuge on the enzyme for the sulfuryl group (and the phenolic hydroxyl of a tyrosine seems to be an ideal choice thereby not changing the sulfuryl transfer potential of the substrate) it is only necessary for the enzyme to evolve a single binding site for donor and acceptor substrate. This would be an evolutionary advantage and provides a rationale for the ping pong type mechanism adopted (*10*).

ACKNOWLEDGMENTS

The authors gratefully acknowledge support for this work from the SERC and the award of a Violette and Samuel Glasstone Research Fellowship (to C.L.L.C.). We are also grateful to Professor K. Kobashi, Toyama Medical & Pharmaceutical University, Japan for a gift of the aryl sulfotransferase from *Eubacterium* A-44.

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