The Sulfuryl Transfer Mechanism

CRYSTAL STRUCTURE OF A VANADATE COMPLEX OF ESTROGEN SULFOTRANSFERASE AND MUTATIONAL ANALYSIS*

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Estrogen sulfotransferase (EST) catalyzes transfer of the 5'-sulfuryl group of adenosine 3'-phosphate 5'-phosphosulfate (PAPS) to the 3α-phenol group of estrogenic steroids such as estradiol (E2). The recent crystal structure of EST-adenosine 3',5'-diphosphate (PAP)-E2 complex has revealed that residues Lys48, Thr45, Thr51, Thr52, Lys106, His108, and Tyr240 are in position to play a catalytic role in the sulfuryl transfer reaction of EST (Kakuta Y., Pedersen, L. G., Carter, C. W., Negishi, M., and Pedersen, L. C. (1997) Nat. Struct. Biol. 4, 904–908). Mutation of Lys48, Lys106, or His108 nearly abolishes EST activity, indicating that they play a critical role in catalysis. A present 2.2-Å resolution structure of EST-PAP-vanadate complex indicates that the vanadate molecule adopts a trigonal bipyramidal geometry with its equatorial oxygens coordinated to these three residues. The apical positions of the vanadate molecule are occupied by a terminal oxygen of the 5'-phosphate of PAP (2.1 Å) and a possible water molecule (2.3 Å). This water molecule superimposes well to the 3α-phosphate of AMP in the UK-ADP-AMP structure, respectively (13, 14). These structural relationships, as well as an earlier study with phenol sulfotransferase (15), suggest that the catalytic mechanism of sulfuryl and phosphoryl transfers may be similar. The lack of transition state information on the EST structure has made it difficult to visualize the sulfuryl transfer mechanism. The EST-PAP-E2 structure previously identified Lys48, Thr45, Thr51, Thr52, Lys106, His108, and Tyr240 as possible catalytic residues. We have performed site-directed mutagenesis studies on these residues in order to better understand the sulfuryl transfer mechanism of EST, and the roles of these residues in catalysis. Moreover, vanadate has been employed in crystallographic analysis in order to obtain a model of the transition state of EST. Based on these studies, we have identified residues involved in stabilizing the transition state that promotes the sulfuryl transfer reaction.

Sulfuryl transfer reactions are widely observed in various biological processes and are conserved from bacteria to human. As a result, deficiencies due to the lack of metabolic activation of sulfate can be lethal in humans (1). Various biological signaling molecules including neurotransmitters (2), steroid hormones (3, 4), and peptides and proteins (5, 6) can be sulfated to alter biological activity. Growth factors and blood coagulation factors become fully activated after binding to glucosaminoglycan sulfates such as heparan sulfates (7, 8). The sulfation/desulfation balance, thus, regulates cell growth and differentiation as well as neuronal and hormonal homeostasis.

Sulfation generally detoxifies xenobiotics such as pharmaceutical drugs and synthetic and naturally occurring chemicals (9). In some cases, however, xenobiotic sulfation can be used for the in vivo activation of prodrugs or can result in potentiating toxicity and carcinogenicity (10, 11). Sulfation of these diverse substrates is catalyzed by a group of enzymes referred to as sulfotransferases that use adenosine 3'-phosphate 5'-phosphosulfate (PAPS) as the ubiquitous sulfate donor.

The crystal structure of the mouse estrogen sulfotransferase (EST) was the first three-dimensional structure to be solved for the sulfotransferase family (12, 13). The structure revealed the catalytic core of the enzyme to be remarkably similar to those of nucleotide kinases, despite its little to no sequence homology to the kinases. The 5'-phosphate of the inactive cofactor adenosine 3',5'-diphosphate (PAP) and the 3α-phenol group of estradiol (E2) in the EST-PAP-E2 complex superimpose well to the leaving β-phosphate group of ADP and the entering phosphate group of AMP in the UK-ADP-AMP structure, respectively (13, 14). These structural relationships, as well as an earlier study with phenol sulfotransferase (15), suggest that the catalytic mechanism of sulfuryl and phosphoryl transfers may be similar. The lack of transition state information on the EST structure has made it difficult to visualize the sulfuryl transfer mechanism. The EST-PAP-E2 structure previously identified Lys48, Thr45, Thr51, Thr52, Lys106, His108, and Tyr240 as possible catalytic residues. We have performed site-directed mutagenesis studies on these residues in order to better understand the sulfuryl transfer mechanism of EST, and the roles of these residues in catalysis. Moreover, vanadate has been employed in crystallographic analysis in order to obtain a model of the transition state of EST. Based on these studies, we have identified residues involved in stabilizing the transition state that promotes the sulfuryl transfer reaction.

EXPERIMENTAL PROCEDURES

Crystallographic and Structure Determination—Bacterially expressed mouse EST was co-crystallized with PAP for the EST-PAP complex and E2 was soaked into the crystals for the EST-PAP-E2 complex as described in our previous studies (12, 13). Diffraction data were collected on the synchrotron at Brookhaven National Laboratories beamline X9b. To obtain the EST-PAP-vanadate complex, EST-PAP crystals were soaked with 1 mM Na3VO4 for 10 min prior to data collection using an R-axis IV area detector. For all data sets, data were processed using Denzo and Scalepack (16). All refinements of the data were carried out using positional, torsion_angle, and b-factor refinement in X-plor 3.851 (17). Noncrystallographic symmetry restraints were applied to the two molecules in the asymmetric unit for all three refinements as well as bulk solvent corrections. Residues for which electron density existed in the EST-PAP structure were 7–67 and 73–294 for molecule A and 7–64 for molecule B. In the EST-PAP-E2 structure, residues 7–65

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† The atomic coordinates and structure factors (for EST + PAP, 1bo2; for EST + PAP + E2, 1bo3; for EST + PAP + VO4, 1bo5) have been deposited in the Protein Data Bank, Brookhaven National Laboratory, Upton, NY.

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1 The abbreviations used are: PAPS, adenosine 3'-phosphate 5'-phosphosulfate; PAP, adenosine 3',5'-diphosphate; E2, estradiol; EST, estrogen sulfotransferase.
and 73–294 in molecule A and 7–64 and 72–293 in molecule B were visible. Residues reported for the vanadate structure are 6–65 and 73–294 in molecule A and 7–64 and 71–294 in molecule B.

Site-directed Mutagenesis—Site-directed mutagenesis experiments were performed using a QuickChange kit (Stratagene) with the EST sequence in pGEX-4T-3 plasmid as the template. Pairs of proper oligonucleotide primers were used for the polymerase chain reaction to introduce mutations. *Escherichia coli* XL blue cells were transformed with a polymerization mixture. Several single colonies were selected to produce 3–4-ml overnight cultures in 100 µl ampicillin-supplemented LB medium. Subsequently, plasmid DNA was isolated and subjected to sequencing with pGEX-5’ or pGEX-3’ primers to verify the mutations.

Bacterial Expression of the Mutated Enzymes—Overnight bacterial cultures (0.1–1.0 ml) were used to inoculate 0.2–1 liter of 2xYT medium supplemented with 100–150 µg/ml ampicillin. Cells were grown at 37 °C in an incubator shaker for 6–8 h (or up to *A*~0.0~ 0.4–0.8 absorbance unit), followed by addition of isopropl1-thiol-p-galactopyranoside (10–50 µM). The cells were then allowed to grow on shakers overnight at room temperature. Using previously described methods (18), the expressed enzyme was extracted by sonication of the bacterial suspension and purified using glutathione-Sepharose (Amersham Pharmacia Biotech) and thrombin cleavage (Sigma). Typically 2–10 mg of pure EST were obtained from a 1-liter culture.

**Sulfotransferase Assay**—Sulfotransferase activity was assayed as described previously (18). For the determination of K~m,E2~ reaction mixtures (200 µl of 100 mM Tris-HCl buffer pH 8.0) contained 100 µM PAPS and various E~2~ concentrations of 3.45, 6.9, 10.8, 14.7, 22.5, 38.2, 69.4, 131.9, 256.9, and 506.9 nM. For the K~m,PAPS~, the E~2~ concentration was fixed at 1 µM and the PAPS concentrations varied from 19.5, 39, 78, 156, 312, 625, 1250, 2500, 5000, and 10000 nM. For the K~m,cat~ determination corresponding to Lys48 in EST (6, 19, 20), mouse tryosylprotein sulfotransferase have an Arg at the position corresponding to Lys48 in EST (6, 19, 20).

**RESULTS AND DISCUSSION**

**Mutagenesis of Residues in the Catalytic Center**—Residues Lys~48~, Thr~45~, Thr~51~, Thr~52~, Lys~106~, and Tyr~240~ are located in the catalytic center of EST (Fig. 1). These residues were subjected to mutational analysis in order to investigate their possible roles in the sulfuryl transfer reaction. Various mutants of EST were bacterially expressed and their steady-state kinetics characterized. The wild-type EST displayed K~m,E2~, K~m,PAPS~, and K~cat~ 17 nm, 0.3 µM, and 0.023 s~−1~, respectively (Table I).

**TABLE I**

<table>
<thead>
<tr>
<th>Enzymes</th>
<th>K<del>m,E2</del> [nM]</th>
<th>K<del>m,PAPS</del> [µM]</th>
<th>k<del>cat</del> [s~−1~]</th>
<th>k<del>cat</del>K<del>m,E2</del> [10<del>3</del>/nM]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
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<td>0.30</td>
<td>23.5</td>
<td>1.3</td>
</tr>
<tr>
<td>K48R</td>
<td>18.0</td>
<td>0.35</td>
<td>5.6</td>
<td>0.31</td>
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<tr>
<td>K48M</td>
<td>ND</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>T45V</td>
<td>21.4</td>
<td>0.35</td>
<td>4.2</td>
<td>0.2</td>
</tr>
<tr>
<td>T51V</td>
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<td>2.3</td>
<td>4.5</td>
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<td>T52V</td>
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<td>K106R</td>
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<td>0.2</td>
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<td>K106S</td>
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<td>K106A</td>
<td>119.2</td>
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<td>0.3</td>
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<tr>
<td>K106K</td>
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<td></td>
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<td>ND</td>
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<tr>
<td>H108K'</td>
<td>54.3</td>
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<td></td>
</tr>
<tr>
<td>Y240F</td>
<td>23.3</td>
<td>0.95</td>
<td>21.8</td>
<td>0.94</td>
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</table>

* a The K~m~ and k~cat~ values were calculated using the activities with E~2~ concentrations of 6.4, 10.8, 14.7, and 22.5 nM.
* ND, activity not detectable.
* b The freshly prepared glutathione S-transferase fusion enzyme was used for the assays.

* c Lower than the wild-type enzyme. This turnover number is probably underestimated since the activity of this mutant was inhibited by high E~2~ concentrations (i.e., >40 nM). Thus, residue 48 may require a positively charged side chain to confer the high sulfotransferase activity. In fact, amino acid sequence alignment has shown that bacterial sulfotransferase NodH and mouse tryosylprotein sulfotransferase have an Arg at the position corresponding to Lys48 in EST (6, 19, 20).

**FIG. 1.** Schematic representation of the key residues and their coordinations in the active site. All possible backbone and side chain coordinations of Lys~48~, Thr~45~, Thr~51~, Thr~52~, Lys~106~, and His~108~ from the EST-PAP-E2 complex are shown. Hydrogen bonds are indicated by dotted lines. The hydroxy group of Tyr~240~ is located 6.7 Å from the 5’-phosphate of PAP and 5.2 Å from the 3α-hydroxyl group of E2.

**Lys~106~—**We mutated Lys~106~ to arginine, serine, or alanine. Depending on the type of residue at position 106, the mutant enzymes decreased K~m,E2~ only up to 7-fold compared with the wild-type EST, while their K~m,PAPS~ remained unchanged (Table I). Despite the fact that these mutant enzymes displayed lower than the wild-type enzyme. This turnover number is probably underestimated since the activity of this mutant was inhibited by high E~2~ concentrations (i.e., >40 nM). Thus, residue 48 may require a positively charged side chain to confer the high sulfotransferase activity. In fact, amino acid sequence alignment has shown that bacterial sulfotransferase NodH and mouse tryosylprotein sulfotransferase have an Arg at the position corresponding to Lys48 in EST (6, 19, 20).
small or no changes in their $K_m$ values, they exhibited dramatically decreased $k_{cat}$ (i.e. 200–3000-fold). The residual activities of these mutant enzymes were generally not associated with a unique chemical property of any of the mutated amino acid residues.

$\text{His}^{108}$—This histidine is located within hydrogen bond distance to the $3\alpha$-phenol group of $E_2$ (Fig. 1). Thus, $\text{His}^{108}$ is suggested as the catalytic base that may deprotonate the $3\alpha$-phenol group, resulting in a nucelophile phenoxide (13). In addition, this histidine residue is found to be totally conserved in all of the known cytosolic sulfotransferases (see the sequence alignments in Weinshilboun et al. (21)). $\text{His}^{108}$ was mutated to serine, leucine, lysine, or arginine to examine the role of this histidine residue in catalysis. No detectable activity was observed with any of the mutant enzymes, suggesting that $\text{His}^{108}$ plays a critical role in catalysis (Table I). Given the possibility that the $\text{His}$ mutants are structurally unstable, we measured the activity using a freshly prepared glutathione $S$-transferase-H108K fusion enzyme. The fusion enzyme displayed sulfotransferase activity with a 100-fold lower $k_{cat}$ than the wild-type enzyme (Table I). This $k_{cat}$ value, however, might have been underestimated because of the instability of this mutant enzyme.

$\text{Try}^{240}$—Since this residue is in close proximity to the probable position of the sulfate moiety of PAP (Fig. 1), $\text{Try}^{240}$ was suggested to possibly play a role in catalysis (13). The sulfate group, for example, could be transferred to this residue forming a sulfotyrosine intermediate. Such a sulfotyrosine has previously been suggested for an aryl sulfotransferase from $\text{Eubacterium}$ $\text{A44}$ (22, 23). Substitution of phenylalanine for tyrosine at position 240 of mouse EST did not alter the $K_m$ or $k_{cat}$ values, indicating that EST does not form the sulfotyrosine intermediate during this reaction. $\text{Try}^{240}$, therefore, does not appear to play a role in the sulfate transfer reaction of mouse EST.

Further Refinement of the EST-PAP and EST-PAP-E$_2$ Structures—New data sets were collected on the synchrotron and the structures refined on the EST-PAP and EST-PAP-E$_2$ complexes at 1.75 and 1.6 Å, respectively (Table II). The overall $R$ factor for the EST-PAP model is 21.8% ($R_{free} = 24.8\%$). For the EST-PAP-E$_2$ structure the $R$ factor is 21.8% ($R_{free} = 25.1\%$). The improved resolution revealed a sequencing error at position 113. Resequencing of the vector confirmed that the residue at position 113 is indeed a leucine rather than valine. The orientation of Arg$^{257}$ that forms hydrogen bonds with the $3\'\text{-phosphate}$ of PAP was optimized to better match the density. In this newly refined structure, the side chain nitrogens $\text{Ne}$ and $\text{Np2}$ of Arg$^{257}$ are in positions to form hydrogen bonds with the $3\'\text{-phosphate}$ oxygens (3.1 and 3.3 Å, respectively).

Crystal Structure of the EST-PAP-Vanadate Complex—Some metal oxoanions such as aluminum fluoride and orthovanadate are inhibitors of enzymes such as kinases, phosphatases, and sulfatase. They are believed to be able to visualize the transition state of the reaction by forming a trigonal bipyramidal geometry in the active site of these enzymes (24–28). To employ this visualization to obtain the transition state information of EST, $\text{Na}_3\text{VO}_4$ was tested to see whether vanadate inhibits EST activity. Indeed, vanadate was found to be a competitive inhibitor of EST with a $k_i$ value of 1 mM. Subsequently, the structure of a vanadate-soaked crystal of the EST-PAP complex has been solved at 2.2 Å resolution (Table II).

The simulated annealing omit map of the PAP, vanadate, and proposed water molecules in the active site of the EST-PAP-vanadate structure is shown in Fig. 2. The vanadate molecule clearly adopts a trigonal bipyramidal geometry in the active site of EST. The three equatorial positions of the bipyramid are occupied by a terminal oxygen of the vanadate molecule clearly adopts a trigonal bipyramidal geometry in the active site of EST. The three equatorial positions of the bipyramid are occupied by a terminal oxygen of the vanadate molecule (Fig. 3). The observed structure of the vanadate molecule displays the characteristics predicted for the transition state.

Fig. 3 shows the possible coordinations of the vanadate molecule to residues at the active site. The backbone nitrogens of $\text{Lys}^{18}$ is coordinated with a water molecule in the equatorial oxygen as well as with the terminal $5\'\text{-phos-}
that runs through the center of the vanadium atom (mimicking the transferring sulfate group) perpendicular to the equatorial oxygens. This positioning is consistent with a structure for the transition state of an in-line transfer reaction.

**General Discussion**—The transition state structure has strongly suggest that Lys\(^{48}\) and His\(^{108}\) are involved in catalysis of the sulfuryl transfer reaction in EST. Mutagenesis of these residues elicits profound alterations in EST activity. Consistent with this suggestion, Marsolais and Varin (29) have previously mutated Lys\(^{59}\) (Lys\(^{48}\) in EST) of flavonol 3-sulfotransferase to arginine (K59R) or alanine (K59A) and found that the K59R mutant retained approximately 6.5% of the wild-type activity, whereas the K59A mutant was basically inactive. Thus, Lys\(^{59}\) was suggested to play an important role in stabilizing a reaction intermediate of this sulfotransferase (29). Our transition state structure has directly implicated Lys\(^{48}\) as one of the catalytic residues in EST, since this residue is directly coordinated to both the bridge oxygen of the leaving group and an equatorial oxygen of the transferring group. Supporting the finding that this residue should be positively charged, all sulfotransferases have either lysine or arginine at the corresponding site (20).

The transition state coordination of Lys\(^{48}\) to both the phosphate-sulfate bridge oxygen and the equatorial oxygen of the vanadate molecule are similar to those of Arg\(^{789}\) in GTPase-activating protein of the Ras-GTPase-activating protein complex structure (30). In that structure, an Arg\(^{789}\) guanidinium is also found within hydrogen bond distance to the \(\beta-\gamma\) bridge oxygen of GTP and a fluoride atom of AlF\(_3\) mimicking the transferring phosphoryl group at the transition state with shorter distance to the fluoride than to the bridge oxygen. This structural information has led the authors to suggest that the major role of this residue is to stabilize the transition state rather than to donate a hydrogen bond to the bridge oxygen to help dissociating the leaving group (30). In contrast, in our EST structure Lys\(^{48}\) is almost equidistant from these atoms in the leaving and transferring groups. Taking into account the mutational and structural studies as well as the sequence conservation in all known sulfotransferases, Lys\(^{48}\) plays an essential role in catalysis, probably through the stabilization of the transition state. Additionally, it is possible that the hydrogen bond interaction of Lys\(^{48}\) with the bridge oxygen acts as a catalytic acid to enhance the dissociative nature of the sulfuryl transfer reaction. Such dissociative mechanisms have been intensively discussed for the phosphor transfer reaction of kinases and phosphatases (31, 32).

The transition state structure has clearly demonstrated that His\(^{108}\) is in position to both stabilize the transition state and/or to function as the catalytic base. Our mutational analysis of
His<sup>108</sup> has also provided functional evidence to indicate that His<sup>108</sup> is critical in catalysis. Marsolais and Varin (33) have previously reported that an Arg mutant of the equivalent histidine of flavonol 3-sulfotransferase (His<sup>118</sup>) retained approximately 10% activity of the wild-type enzyme. The H108K mutant of EST displayed only 1% activity of the wild-type EST. The role of His<sup>108</sup> as the base to deprotonate the nucleophile could be important to achieve the high sulfotransferase activity. Transition state coordinations of His<sup>108</sup> to the transferring sulfuryl group may provide EST with sufficient catalytic efficiency to confer the low activity. Including our His<sup>108</sup> mutants, various mutations of His<sup>108</sup> (in EST) and of its corresponding His<sup>118</sup> (in flavonol 3-sulfotransferase) and His<sup>90</sup> (in hydroxysteroid sulfotransferase ST-40) (34) appear to be structurally unstable. Thus, the site-directed mutagenesis is not likely to provide definitive evidence for the role of these His residues as a catalytic base. Due to the high pK<sub>a</sub> value (64 9.0) of the entering phenol group of substrates such as E<sub>2</sub> and flavonols, a base to deprotonate the phenol group to a phenoxide may be necessary. The resulting nucleophile could then attack the sulfur atom of the transferring sulfuryl group in order to achieve the high catalytic efficiency. His<sup>108</sup> is located within hydrogen bonding distance to the 3a-phenol group in the EST structure, consistent with a role as catalytic base. Lys<sup>106</sup> is the only other residue that is close enough to form a hydrogen bond to the 3a-phenol group, but it is an unlikely candidate for a base, and it is not conserved in all cytosolic sulfotransferases. The main role of Lys<sup>106</sup> appears to be stabilization of the transition state. We propose that His<sup>108</sup> may not be essential, but it is necessary as a base in order to confer high sulfotransferase activity to EST and may also be involved in the stabilization of the transient state.

Consistent with the mutational analysis of the threonine residues at positions 45, 51, and 52, these threonine residues are not found to be in position to coordinate either the bridge oxygen of the leaving group, the vanadate molecule, or the entering phenol group directly. The possible side chain interaction of Thr<sup>45</sup> to both Thr<sup>51</sup> and His<sup>108</sup> (N<sub>a</sub>) had once prompted us to suggest that it may play an important role in distributing a proton from the 3α-phenol group to the leaving phosphate group (13). The present mutational analysis indicates that this side chain interaction is not essential for sulfotransferase activity, although these side chain interactions may attenuate the catalytic efficiency of the enzyme. Thr<sup>51</sup> and Thr<sup>52</sup> are coordinated via their side chain oxygens and backbone amides to the oxygens (not the phosphate-sulfate bridge oxygen) of the 5′-phosphate of PAP (Fig. 3). Moreover, these coordinations are identical in the three different EST structures, EST-PAP, EST-PAP-E<sub>2</sub>, and EST-PAP-vanadate. The coordination of these threonine residues do not appear to play a critical role in catalysis, but Thr<sup>51</sup> may be important in catalysis by assisting in the binding of PAPS to EST.

Taking the EST-PAP-vanadate structure and our mutation study into consideration, sulfuryl transfer appears to proceed through an in-line transfer reaction mechanism (Fig. 4). The distances of the vanadium atom to the apical ligands are practically identical, 2.1 and 2.3 Å to the bringing oxygen of the leaving group and the entering 3c-phenol group, respectively. These distances are too long for a covalent bond but too short for allowable van der Waals contacts, implying that partial bond cleavage between the transferring sulfuryl and the leaving phosphate groups may be occurring at the same time as bond formation between the 3c-phenol group of the substrate and the sulfuryl group. These reaction steps of the sulfuryl transfer reaction require residues Lys<sup>48</sup>, Lys<sup>106</sup>, and His<sup>108</sup> to confer high sulfotransferase catalytic efficiency to EST. Lys<sup>48</sup> may protonate the sulfate-sulfate bridge oxygen of PAPS to enhance the dissociation of PAP, while His<sup>108</sup> may act as a base to facilitate the deprotonation of the 3c-phenol group. Stabilization by Lys<sup>48</sup>, His<sup>106</sup>, and Lys<sup>108</sup> of the transition state may also be essential for the high catalytic efficiency.

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REFERENCES
Catalytic Mechanism of Sulfotransferase

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