para-Nitrophenyl Sulfate Activation of Human Sulfotransferase 1A1 Is Consistent with Intercepting the E-PAP Complex and Reformation of E-PAPS*

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Cytosolic sulfotransferase (SULT)-catalyzed sulfation regulates biological activities of various biosignaling molecules and metabolizes hydroxyl-containing drugs and xenobiotics. The universal sulfuryl group donor for SULT-catalyzed sulfation is adenosine 3'-phosphate 5'-phosphosulfate (PAPS), whereas the reaction products are a sulfated product and adenosine 3',5'-diphosphate (PAP). Although SULT-catalyzed kinetic mechanisms have been studied since the 1980s, they remain unclear. Human SULT1A1 is an important phase II drug-metabolizing enzyme. Previously, isotope exchange at equilibrium indicated steady-state-ordered mechanism with PAPS and PAP binding to the free SULT1A1 (Tyapochkin, E., Cook, P. F., and Chen, G. (2008) Biochemistry 47, 11894–11899). On the basis of activation of SULT1A1 by para-nitrophenyl sulfate (pNPS), an ordered bypass mechanism has been proposed where pNPS sulfates PAP prior to its release from the E-PAP complex regenerating E-PAPS. Data are consistent with uncompetitive substrate inhibition by naphthol as a result of formation of the E-PAPnaphthol dead-end complex; formation of the complex is corroborated by naphthol/PAP double inhibition experiments. pNPS activation data demonstrate an apparent ping-pong mechanism with pNPS adding to E-PAP, and competitive inhibition by naphthol consistent with formation of the E-PAP-naphthol complex. Exchange against forward reaction flux (PAPS plus naphthol) beginning with [35S]PAPS and generating [35S]naphthyl sulfate is also consistent with pNPS intercepting the E-PAP complex. Overall, data are consistent with the proposed ordered bypass mechanism.

Sulfotransferases (SULTs)3 are phase II drug-metabolizing enzymes that catalyze the sulfation (sulfonation) of various hydroxyl-containing compounds: biosignaling molecules such as hydroxysteroid hormones, thyroid hormones, glucocorticoid hormones, bile acids, neurotransmitters, and hydroxyl-containing xenobiotics (1–8). The sulfation proceeds as shown in reaction 1, where the sulfuryl group donor is adenosine 3'-phosphate 5'-phosphosulfate (PAPS), and the reaction products are adenosine 3',5'-diphosphate (PAP) and a sulfated product.

\[
\text{SULTs} \quad R-OH + \text{PAPS} \xrightarrow{\text{E-PAP}} R-\text{OSO}_3\text{H} + \text{PAP}
\]

One of the main biological functions of SULTs is the regulation of various hormones (9). Sulfation of xenobiotics is mainly associated with detoxification, biotransformation of a relatively hydrophobic xenobiotic into a more water-soluble sulfuric ester that is readily excreted. However, in some cases sulfation can also cause bioactivation of procarcinogens and promutagens, leading to possible toxic effects (10, 11).

Studies of the SULTs kinetic mechanisms began to appear in the early 1980s (12). Although many SULT isoforms have been isolated and characterized, their biological functions and catalytic mechanisms are still not well understood. Human phenol sulfotransferase (SULT1A1) is one of the major detoxifying enzymes for phenolic xenobiotics; it also catalyzes the sulfation of endogenous hydroxyl biosignaling molecules. It has very broad substrate specificity and high activity toward most phenolic compounds. SULT1A1 is also widely distributed in the human body. On the basis of isotope exchange at equilibrium, we showed that the kinetic mechanism for human SULT1A1 is steady-state-ordered with PAPS binding to the protein first, and PAP released last (13).

Substrate inhibition by the hydroxyl substrate (sulfate acceptor) is a common feature of most cytosolic SULTs (14, 15). Inhibition of SULT1A1 has been observed by the substrate, naphthol. There are a number of different mechanisms that have been proposed for substrate inhibition, but the mechanism remains unclear. A ternary complex formed between substrate and the enzyme-PAP complex is the most likely possibility in an ordered mechanism, but binding to free enzyme is also possible (12, 16). It is also possible, but unlikely, that substrate could bind to central complexes. In addition, binding of two substrate molecules to the active site has been proposed (4, 14). A SULT1A1 crystal structure was solved that showed two molecules of p-nitrophenol (pNP) in the same active site. However, computer modeling of this structure indicated that the active site could not easily accommodate even one molecule of a larger substrate such as β-estradiol (17). Other SULT crystal struc-

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The abbreviations used are: SULT, sulfotransferase; SULT1A1, simple phenol sulfotransferase; PAPS, adenosine 3'-phosphate 5'-phosphosulfate; PAP, adenosine 3',5'-diphosphate; pNP, para-nitrophenol; pNPS, para-nitrophenyl sulfate.
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SCHEME 1. Proposed ordered bypass mechanism with substrate inhibition by B binding to E-PAP. In the scheme, B, B2, and P2 are naphthol, pNP, and pNPS, respectively, whereas A and Q are PAPS and PAP, respectively. An additional EAP dead-end complex is allowed but not shown.

para-Nitrophenyl sulfate (pNPS) has been used for phenol SULTs enzyme activity assays (24–27). Recently, we have been interested in the mechanisms for pNPS activation of SULT1A1-catalyzed sulfation of other phenol substrates, such as naphthols. On the basis of this activation by pNPS, a mechanism was proposed that requires sulfation of PAP prior to its release, from the E-PAP complex (Scheme 1). i.e. pNPS binds to E-PAP and generates the E-PAPS-pNP complex, which dissociates pNP and generates the E-PAPS complex.

In this work, the proposed mechanism was tested using the double inhibition method of Yonetani and Theorell (28), which provides information on whether binding of two inhibitors is mutually exclusive. Double inhibition experiments have been successful in demonstrating whether the binding of two inhibitors is mutually exclusive, or whether they show interference or synergism in binding (28–32). In addition, substrate inhibition by the hydroxyl substrate and exchange against forward reaction flux were used as probes of the mechanism. Data are discussed in terms of the overall mechanism of SULT1A1.

MATERIALS AND METHODS

Chemicals—1-[^14]C]Naphthol (55 mCi/mmole) was purchased from American Radiolabeled Chemicals, Inc. [35S]Adenosine 3’-phosphate 5’-phosphosulfate (243 Ci/mmole) was purchased from PerkinElmer Life Sciences (Boston, MA). 1-Naphthol, potassium 4-nitrophenyl sulfate (pNPS) and adenosine 3’,5’-diphosphate sodium salt (PAP) were purchased from Sigma-Aldrich, whereas adenosine 3’-phosphate 5’-phosphosulfate, tetralithium salt (PAPS) was purchased from Calbiochem. 2-Naphthol was purchased from Fluka Biochemika. 1-Naphthyl sulfate, potassium salt was purchased from MP Biochemicals, LLC.

Thin layer Partisil KC 18 F, Silica Gel 60 chromatography plates, with a fluorescence indicator (20 cm × 20 cm, 200 μm) from Whatman Schleicher & Schuell, were purchased from Sigma-Aldrich. The plates were channeled using razor blades. A channel width of 1 cm was chosen with a groove width between channels of 2 mm. Deionized water was used in all the experiments. All the other reagents and chemicals were of the highest available grade. Scintillation counting was carried out on a Beckman LS 6500 multipurpose scintillation counter.

Enzyme—SULT1A1 used in this study (7.5 mg/ml, maltose fusion protein) was purified as described previously (25, 26, 33).

pNPS Enzyme Activity Assay—The pNPS assay takes advantage of the fact that phenol sulfotransferases (SULTs) catalyze the transfer of the sulfuryl group of pNPS to PAP, to give PAPS and producing a colored product, p-nitrophenolate (pNP) with maximum absorbance at 401 nm. The amount of pNP is stoichiometrically proportional to the amount of PAP formed in the reaction (1:1). Thus, the extent of the SULT1A1-catalyzed sulfation can be monitored spectrophotometrically by the appearance of pNP (PAP).

Reactions were carried out in 5-ml capacity Pyrex test tubes; the total reaction volume was 250 µl. 2-Naphthol solutions were prepared by dissolving 2-naphthol in 100% ethanol. 5 µl of 2-naphthol solution at the desired concentration was added to each test tube (2% ethanol by volume). The test tubes with different initial concentrations of PAPS, substrate, and pNPS in 50 mM phosphate buffer (pH 6.2) were incubated at 37 °C for 2 min in an ORS 200 Boekel/Grant water bath. Then, 5 µl of 50 mM phosphate buffer (pH 6.2) containing SULT1A1 was added to the reaction mixture to start the reaction. The test tubes were covered with aluminum foil to prevent evaporation. After the reaction was started, the test tubes were incubated for 15 min at 37 °C. Then, the reactions were stopped with 250 µl of Tris base (0.25 M, pH 8.7). The absorbance at 401 nm was measured after the reaction was stopped using a PerkinElmer Lambda Bio UV-visible spectrometer. Plastibrand semi-micro disposable cuvettes (1.5 ml) were used. The measurements were carried out in triplicate, and the average of the absorbance measurements minus the control was used to calculate the enzymatic activity. Two types of controls were carried out. The first one made use of the same protocol as discussed above, but in the absence of SULT1A1, while the second one was conducted in the absence of substrate, 2-naphthol (5 µl of ethanol was added to the second control). No significant difference was observed between the two types of controls. Absorbanes of all controls at 401 nm were low, within the 0.01–0.03 range.

Liquid-Liquid Extraction Protocol—The protocol was used to separate substrate, 14C-labeled 1-naphthol from the reaction product, 14C-labeled 1-naphthyl sulfate. Reactions were carried out in closed microcentrifuge tubes (0.5 ml) to prevent evaporation. The total reaction volume was 50 µl. The tubes with different initial concentrations of PAPS, PAP, and 14C-labeled 1-naphthol in 50 mM phosphate buffer (pH 6.2) were incubated at 37 °C in Fisher Scientific Isotemp 2054FS dry bath incubator. 7.5 µg of SULT1A1 in 50 mM phosphate buffer (pH 6.2) was added to the reaction mixture last to start the reaction. The reaction was stopped with 50 µl of Tris base (0.25 M, pH 8.7). To separate 14C-labeled 1-naphthol from the product, 14C-labeled 1-naphthyl sulfate, 100 µl of chloroform was added to each Eppendorf tube. The mixture was vortexed for 30 s and centrifuged at 9000 × g for 5 min. 1-Naphthol is extracted into the chloroform phase, while 1-naphthyl sulfate remains in the aqueous phase. The chloroform phase was carefully separated from the aqueous phase using a microsyringe and then transferred into a scintillation vial containing 4 ml of scintillation mixture (EcoLite) and counted. The syringe was rinsed with acetone twice between samples. The liquid-liquid extraction
with chloroform was repeated twice. The aqueous phase-containing product was transferred into a scintillation vial containing 4 ml of scintillation mixture (EcoLite) and counted. Two types of controls were carried out. The first one made use of the same protocol as discussed above, but in the absence of SULT1A1, while the second one was conducted in the absence of PAPS and/or PAP. No significant difference was observed between the two types of controls. The counts per minute of the controls were subtracted from those of the samples (aqueous phase only). All the controls and samples were carried out in triplicate; results are given as the mean ± S.D. Substrate and product concentrations were determined from calculated specific radioactivities for each experiment.

\textbf{14C Radioactive Exchange Experiments—}Initial concentrations of pNPS were 0, 5, 10, 20, 30, and 40 μM, whereas the initial concentration of PAPS was 10 μM, and the initial concentration of 1-[14C]naphthol was 20 μM. Substrate and product were separated using the liquid-liquid extraction protocol, and their concentrations were determined from calculated specific radioactivities for each experiment. The initial concentration of PAPS was 10 μM, whereas the initial concentration of PAPS was 10 μM, and the initial concentration of 1-[14C]naphthol was 20 μM. Substrate and product were separated using the liquid-liquid extraction protocol, and their concentrations were determined from calculated specific radioactivities for each experiment as explained above. Controls were carried out as discussed above for the liquid-liquid extraction protocol. The reaction time was 5 min in all the experiments.

\textbf{Double Inhibition Experiments—}The initial concentration of PAPS was 5 μM (~5 times \(K_{\text{PAPS}}\)) (13). The initial concentrations of PAP were 0, 5, 10, 15, and 20 μM. A stock 1-naphthol solution was made in 100% ethanol and doped with 14C-labeled 1-naphthol. This stock solution was then appropriately diluted and used for all the subsequent experiments. The 14C-labeled substrate and product were separated using the liquid-liquid extraction protocol as explained above, and substrate and product concentrations were determined from calculated specific radioactivities for each experiment. Controls were carried out without SULT1A1. The reaction time was 5 min in all of the experiments.

\textbf{TLC Separation Protocol—}The protocol was used to separate the co-substrate, \(35\)S-labeled PAPS, and the reaction product, \(35\)S-labeled 1-naphthyl sulfate, which stoichiometrically corresponds to PAP. The method is similar to the liquid-liquid extraction protocol. \(35\)S-PAPS was used as a label instead of 1-[14C]naphthol. Channeled silica gel TLC plates were used to separate \(35\)S-labeled PAPS from \(35\)S-labeled naphthyl sulfate. Termination of the reaction and separation of the labeled reactant and product were different. The reaction was stopped with 50 μl of methanol. The reaction mixture (100 μl) was spotted onto a TLC plate using a micropipette in aliquots of 5 μl. The composition of the mobile phase for the TLC studies was determined experimentally by separating non-radioactive PAPS and the product 1-naphthyl sulfate at 4 and 10 mM, respectively, and viewing the spots with 254 nm UV light. \(35\)S-labeled PAPS and labeled product were best separated using an acetonitrile/methanol/acetic acid/water mixture (25:65:5:5). The TLC plates were developed in a closed glass chamber. After separation of \(35\)S-labeled PAPS and product, the dried TLC plates were subjected to autoradiography (24 h), and the radioactive spots were transferred into scintillation vials containing 4 ml of scintillation mixture (EcoLite) to determine the total amounts of \(35\)S-labeled PAPS remaining and product formed. PAPS and product concentrations were determined from calculated specific radioactivities.

\textbf{\(35\)S Radioactive Exchange Experiments—}Initial concentrations of pNPS were 0, 5, 10, and 20 μM, whereas the initial concentration of PAPS was 10 μM, and the concentration of added [\(35\)S]PAPS was 0.1 μM. The initial concentration of 1-naphthol was 20 μM. The \(35\)S-labeled PAPS and product were separated using the TLC protocol, and PAPS and 1-naphthol concentrations were determined from calculated specific radioactivities for each experiment as explained above. Controls were carried out as discussed above for the liquid-liquid extraction protocol. The reaction time was 5 min in all the experiments.

\textbf{1-, 2-Naphthols—}High specific radioactive labeled 2-naphthol is not commercially available. 14C-Labeled 1-naphthol with high specific radioactivity (55 mCi/mmol) was used in the radioactive exchange experiments. Our experimental results proved that there is no significant difference in the kinetic parameters obtained with 1-naphthol and 2-naphthol. As a result, 1- and 2-naphthols will be called simply naphthol in this report.

\textbf{Data Fitting—}Fitting of plots was done with EnzFitter and Enzyme Kinetics Module 1.2 (SigmaPlot). Data exhibiting uncompetitive substrate inhibition in an ordered mechanism were fitted to Equation 1, while data conforming to competitive inhibition by naphthol in a ping-pong mechanism were fitted to Equation 2. Data for double inhibition by PAP and naphthol were fitted to Equation 3.

\[ v = \frac{V_{\text{AB}}}{K_a K_b + K_c A + AB\left(1 + \frac{B}{K_{ib}}\right)} \]  

\[ v = \frac{V P_2 B}{K_m B\left(1 + \frac{B}{K_{ib}}\right) + K_p P_2 + P_2 B} \]  

\[ v = \frac{V_0}{1 + \frac{B}{K_{ib}} + \frac{Q}{K_{iq}} + \frac{B Q}{\alpha K_{ib} K_{iq}}} \]  

In Equations 1 and 2, \(v\) and \(V\) are initial rate and maximum rate, respectively, \(K_a, K_b, K_p, K_{ib}\) are Michaelis constants for PAPS, naphthol, and pNPS, \(K_{ib}\) is the inhibition constant for PAPS (in an ordered mechanism it is the \(K_d\) for E-PAPS), and \(K_{ib}\) is the substrate inhibition constant for substrate naphthol, and \(A, B, P_2\) are PAPS, naphthol, and pNPS concentrations. In Equation 3, \(v\) and \(V_0\) are observed initial rates at any concentration of inhibitors and zero inhibitors, respectively, \(B\) and \(Q\) are naphthol and PAP inhibitor concentrations, \(K_{ib}\) and \(K_{iq}\) are inhibition constants for \(E\)-naphthol and \(E\)-PAP, and \(\alpha\) is an interaction constant that provides an estimate of the effect of the presence of one inhibitor on the binding of the other.

\textbf{RESULTS—}Initial Velocity Studies—A double-reciprocal plot of \(v\) versus PAPS at different fixed concentrations of naphthol, at pH 6.2, is shown in Fig. 1. At low concentrations of naphthol, the pattern
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intersects to the left of the ordinate, but high concentrations of naphthol cause the lines to cross. Replots of the slope and intercept are shown in the insets to Fig. 1. The slope is a linear function of 1/naphthol, whereas the intercept replot exhibits substrate inhibition as an increase in the intercept as 1/naphthol approaches zero. Data are indicative of uncompetitive substrate inhibition, consistent with the ordered kinetic mechanism proposed for SULT1A1 previously (13). A fit of the data to Equation 1 gives the kinetic parameters in Table 1. The $K_i$ for substrate inhibition by naphthol is an order of magnitude higher than its $K_m$.

**Double Inhibition by PAP and Naphthol**—Previous data suggested an ordered mechanism for SULT1A1 with PAPS binding to the enzyme first followed by substrate (13). In this mechanism, uncompetitive substrate inhibition by naphthol is common and suggests slow release of last product (PAP), but binding to central complexes is also possible (32). To distinguish between these possibilities, a double inhibition experiment was carried out using naphthol and PAP as inhibitors, with PAPS fixed near its $K_p$ (29, 31, 34). Data were plotted as $1/v$ versus naphthol at different fixed levels of PAP (Fig. 2). The plot gives a series of lines that intersect to the left of the ordinate. Inhibition by naphthol alone is shown as the line in the absence of added PAP, while inhibition by PAP in the absence of added naphthol is given by the ordinate intercept values. The change in slope as PAP is increased is consistent with formation of an $E$-PAP-naphthol complex, in agreement with uncompetitive substrate inhibition in an ordered mechanism. A fit of the data to Equation 3 gives values of $K_{ib}$, $K_{iq}$, and $\alpha$ of $7.0 \pm 0.7$ $\mu M$, $1.0 \pm 0.1$ $\mu M$, and $28 \pm 11$, respectively. The standard error on $\alpha$ parameter is high, because it is not an independent parameter, and it is determined by its covariance with $K_{ib}$ and $K_{iq}$.

When the double inhibition experiment was repeated in the presence of different concentrations of pNPS, inhibition by naphthol was observed at a fixed pNPS concentration. However, upon addition of PAP to the reaction mixture, activation by PAP was observed.

**Activation by pNPS**—With PAPS fixed at 20 $\mu M$ ($\approx 20$ times $K_{PAPS}$) (13) the saturation curve for naphthol exhibits substrate inhibition. As the concentration of PAPS is increased, the rate is increased at all concentrations of naphthol. Because the release of PAP is slow, a bypass kinetic mechanism was proposed for SULT1A1 (Scheme 1). The proposed mechanism suggests direct sulfation of PAP prior to its release from the $E$-PAP product complex, i.e. pNPS intercepts $E$-PAP and converts it to $E$-PAPS, which can then bind naphthol, which is converted to naphthyl sulfate. The result is an apparent ping-pong behavior with pNPS adding to $E$-PAP, followed by release of pNP; naphthol is then added and converted to naphthyl sulfate. A double-reciprocal plot of $v$ versus pNPS is shown in Fig. 3. At low substrate concentrations parallel lines are observed that cross as the concentration of pNPS is increased with the intercepts converging at a single point on the ordinate. Data are characteristic of competitive inhibition by naphthol in a ping-pong mechanism. The same kinetic behavior was observed in the absence of PAPS using a fixed concentration of PAP, and varied concentrations of the hydroxyl substrate and pNPS (data not shown). Data are consistent with the proposed sulfation of $E$-PAP by pNPS. Estimates of kinetic parameters are given in Table 2.
Exchange against Forward Reaction Flux and Activation by pNPS—Activation by pNPS of the SULT1A1-dependent sulfation of naphthol by PAPS was demonstrated above. As a further test of the proposed mechanism of activation, isotopic exchange experiments were carried out. [35S]PAPS was used to assay the sulfation of naphthol in the presence of different concentrations of pNPS. In the absence of pNPS, the sulfuryl group in naphthyl sulfate product is derived solely from PAPS. If the reaction occurs via the proposed bypass mechanism in Scheme 1, the sulfuryl group in naphthyl sulfate will originate from PAPS and pNPS, at low pNPS concentrations, but will originate almost completely from pNPS at high concentrations. As shown in Fig. 4, a decrease in the amount of [35S]-labeled naphthyl sulfate is observed as the concentration of pNPS is increased. When [14C]-labeled naphthol was used in the same assay, activation of the reaction by pNPS was observed (Fig. 5), i.e. the amount of [14C]-labeled naphthyl sulfate increases with increasing pNPS concentration. At concentrations of pNPS > 20 mM the amount of activation begins to decrease, i.e. an apparent substrate inhibition of the reaction is observed. However, at pNPS concentrations up to 20 mM the amount of product obtained via the [14C] and [35S] protocols conducted at virtually the same reaction conditions, compared well (Fig. 6). First order time courses with nearly identical rate constants were observed for the appearance of [35S]- and [14C]-labeled products in the absence and presence of pNPS (data not shown).

DISCUSSION

Kinetic Mechanism of SULT1A1—Previous studies, using isotope exchange at equilibrium, suggested the kinetic mechanism of human SULT1A1 is a steady state ordered with PAPS binding to the enzyme first (13). Uncompetitive substrate inhibition by naphthol is consistent with a bi-bi ordered sequential mechanism (34, 35), with naphthol binding to the E/PAP product complex, slowing the release of PAP. Although binding of substrate to central complexes has been observed, it is rare. Northrop and Cleland (32) showed that, in the direction of isocitrate formation, -ketoglutarate exhibits uncompetitive inhibition versus NADPH as a result of binding to the central complexes, but they suggested this was a result of Schiff base formation between the ketone of -ketoglutarate and a lysine near the active site of pig heart isocitrate dehydrogenase. Thus, although technically naphthol binding to the central complexes, E-PAPS-naphthol and/or E-PAP-naphthyl sulfate is possible, it is highly unlikely and most likely is not the mechanism of inhibition. The most common mode of substrate inhibition
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in an ordered mechanism is the former, binding of the second substrate, B (naphthol), to the EQ (E-PAP) complex. This is especially true if A and Q are similar in structure, as are PAPS and PAP (34). It is not surprising that naphthol has affinity for the E-PAPS and E-PAP complex.

With PAPS maintained near its $K_{mut}$ double inhibition by naphthol and PAP indicate formation of an E-PAP-naphthol dead-end complex, consistent with substrate inhibition by binding of naphthol to E-PAP. In Fig. 2, inhibition by high concentrations of naphthol (in the substrate inhibition range) was observed in the absence of added PAP as a result of the E-PAP that builds up in the steady state, i.e. release of PAP from E-PAP is slow and contributes to limitation of the overall reaction. Inhibition by added P is observed as an increase in the value of the ordinate intercept of the plot; the naphthol concentration is high, but not in the substrate inhibition range. The concentration of free enzyme under these conditions is still significant, because the concentration of PAPS is low. As PAP increases the concentration of E-PAP increases as PAP ties up more and more of the free enzyme available. The inhibition by naphthol is enhanced as PAP is added, and this is shown by the increase in slope of the $1/v$ versus naphthol plot. Data are indicative of synergism in binding of PAP and naphthol, and the estimated value of $\alpha$ is an indicator of the amount of synergism.

Data are consistent with Reaction 2, which shows ordered addition of PAPS and naphthol, followed by release of naphthyl sulfate and PAP, and inhibition by naphthol binding to E-PAP. Reaction 2 is contained in Scheme 1. In Reaction 2, A, B, P, and Q are PAPS, naphthol, naphthyl sulfate, and PAP, respectively, $k_1$ and $k_3$ are on-rate constants for binding of A and B, respectively, while $k_5$ and $k_9$ are off-rate constants for A from EA and B from EAB, respectively. Interconversion of central complexes is represented by $k_4$ and $k_6$, while $k_2$ and $k_8$ represent the off-rates for P and Q from the EPQ and EQ complexes, respectively. The substrate inhibition constant for B is given by $K_{ib}$ (this is not a thermodynamic parameter and depends on the amount of EQ in the steady state).

\[
\begin{align*}
  E &\rightleftharpoons EA \\
  &\xrightleftharpoons[k_2]{k_1} B \xrightleftharpoons[k_4]{k_3} EAB \\
  &\xrightleftharpoons[k_6]{k_5} EPQ \xrightleftharpoons[k_7]{k_9} EQ \xrightleftharpoons[k_8]{k_6} E
\end{align*}
\]

REACTION 2

The release of PAP from the E-PAP complex contributes to rate limitation at saturating concentration, and thus $V$ will have a significant contribution from $k_9$. Using the net rate constant method (36), Equations 4 and 5 were derived. Expressions for $V$ and $V/K_i$ are given in Equations 4 and 5; $V/K_{PAP}$ is equal to $k_1$.

Note that $K_{ib}$ in Equation 4 is for substrate inhibition by B, while $K_{ib}$ in Equation 5 is the dissociation constant for EAB.

\[
\frac{V}{K_b} = \frac{k_9 k_5/k_4}{1 + \frac{k_6}{k_5} + \frac{k_9}{k_7} + \frac{B}{K_{ib}}} = \frac{k_9}{1 + \frac{B}{K_{ib}}}
\]  
(Eq. 5)

In the limit where $k_9$ is the slowest step along the reaction pathway with reactant at saturating concentrations, Equation 4 reduces to the expression on the right. This situation is approximated by the SULT1A1 reaction.

Mechanism of Activation by pNPS—In the presence of pNPS, SULT1A1 exhibits ping-pong kinetics as predicted by the Scheme 1 with PAPS fixed at a high concentration and the concentrations of naphthol and pNPS varied. Under these conditions, the mechanism in Scheme 1 reduces to that shown in Reaction 3. Release of PAP from E-PAP is slow (see above), and pNPS and naphthol compete for the E-PAP complex. As long as the naphthol concentration is not much greater than its $K_f$ for substrate inhibition, pNPS can combine with E-PAP and transfer its sulfuryl group to PAP. p-Nitrophenol is then released, naphthol binds to E-PAPS, naphthyl sulfate is produced and released to regenerate E-PAP. As long as the concentration of pNPS is high enough, PAP may not be released from the E-PAP complex. However, substrate inhibition by naphthol will still be observed, because pNPS and naphthol compete for E-PAP, and in this case competitive substrate inhibition is observed by naphthol versus pNPS, consistent with competition for E-PAP.

In agreement with the proposed mechanism, qualitatively identical kinetic behavior is observed in the absence of PAPS, using PAP at a fixed concentration, with pNPS, and naphthol as substrates.

If regeneration of E-PAPS is much faster than release of PAP from E-PAP, activation of SULT1A1 will occur. The maximum activation of SULT1A1 in the presence of pNPS is about an order of magnitude. However, $K_{pNPS}$ is high (1 mM) compared with that of naphthol (35 $\mu$M), and this may be the reason activation by pNPS was not reported previously.

According to Scheme 1, pNPS could bind to E-PAPS, competing with naphthol, and forming a dead-end E-PAP-pNPS complex or to E-PAP, resulting in regeneration of E-PAPS through the bypass mechanism. In agreement, Fig. 3 shows that increased pNPS to high concentrations decreased substrate inhibition by naphthol as a result of competition for E-PAP. The double inhibition experiment in the presence of pNPS is also consistent with the proposed mechanism. As the concentration of PAP increased in the reaction mixture, so did the concentration of the E-PAP complex, which pNPS binds leading to regeneration of E-PAPS and activation of SULT1A1.

Exchange against forward reaction flux using $[35S]$PAPS and unlabeled pNPS is also consistent with the proposed mechanism. As the concentration of pNPS increased the amount of $[35S]$naphthyl sulfate decreased consistent with pNPS intercepting the E-PAP complex and transferring an unlabeled sulfuryl group to PAP to form unlabeled PAPS, which led to unlabeled naphthyl sulfate (Fig. 4). Results are corroborated using $[14C]$naphthol and PAPS in the presence of different concentrations of pNPS (Fig. 5). As the concentration of pNPS increased, the amount of $[14C]$naphthyl sulfate produced increased, and correlated with the decrease in the amount of $[35S]$naphthyl sulfate (Fig. 6). In addition, the same first order
rate constant, within error, was obtained for the decrease in \([^{35}\text{S}]\)naphthyl sulfate and increase in \([^{14}\text{C}]\)naphthyl sulfate at a varied concentration of pNPS, consistent with both these courses reflecting the same process.

Data are consistent with Reaction 3, which showed addition of pNPS to E-PAP, conversion of E-PAP to E-PAPS, release of pNPS, followed by addition of naphthol and eventual production of PAP. In Reaction 3, B, B2, P2, and Q are naphthol, pNPS, PAPS, and PAP, respectively. For P2, k1 and k12 are on- and off-rate constants for P2, k13 and k14 represent interconversion of E-PAP·pNPS and E-PAPS·pNPS central complexes, and k15 is the off-rate constant for pNP. Rate constants k1 to k5 are as defined in Reaction 2.

k1 and k3 are on-rate constants for binding of A and B, respectively, while k4 and k5 are off-rate constants for A from EA and B from EAB, respectively. Interconversion of central complexes is represented by k5, while k3 and k4 represent the off-rates for P and Q from the E and EQ complexes, respectively. The substrate inhibition constant for B is given by KnB (this is not a thermodynamic parameter and depends on the amount of EQ in the steady state), which is included in Scheme 1.

\[
\begin{align*}
\text{EQ} & \xrightleftharpoons[k_{12}]{k_{11}} \text{EOP}_2 \xrightarrow[k_{14}]{k_{13}} \text{EAB}_2 \xrightarrow[k_6]{k_5} \text{EQ} \\
\text{E} & \xrightarrow[k_9]{k_8} \text{A} \\
\text{B} & \xrightarrow[k_2]{k_1} \text{B} \\
\text{EA} & \xrightarrow[k_4]{k_3} \text{B} \\
\text{EAB} & \xrightarrow[k_{14}]{k_{15}} \text{EAB}_2 \\
\text{EPQ} & \xrightarrow[k_7]{k_6} \text{EQ}
\end{align*}
\]

\text{REACTION 3}

Overall, data from substrate and double inhibition and exchange against forward reaction flux are in agreement with Scheme 1. At low concentrations of pNPS (P2), the reaction is limited by the pathway in which A adds to E, followed by addition of B and the eventual release of P from EPQ. Under these conditions, there is no observable effect of pNPS on the rate of the reaction as shown by the parallel lines in Fig. 3 for naphthol concentrations of 2 to 8 μM; the rate is determined by \( (V/K_n)B \). However, the intercepts of the lines at these concentrations do exhibit activation by pNPS, and this reflects the second half of Reaction 3 i.e. the steps represented by rate constants k11 to k15. Under these conditions, it is unlikely that release of PAP (Q) from E-PAP contributes to the overall reaction. At saturating P2, the rate is limited by the net rate constant for conversion of EQP2 to EA and B2. At high concentration of B, a E-PAP·B dead-end complex forms, giving competitive substrate inhibition by B versus P2. This is shown in Fig. 3 by the lines for 150–600 μM naphthol concentration that cross those at the lower concentrations of 2–8 μM, and intersect on the y axis.

The expression for V in Reaction 3 is given in Equation 6.

\[
V = \frac{k_{13}k_{15}}{k_{13} + k_{14} + k_{15}}
\]  
(Eq. 6)

The activation by pNPS thus results from bypassing the release of PAP from E-PAP by direct sulfonyl transfer from pNPS to PAP on enzyme. Therefore, \( k_{13}k_{15}/(k_{13} + k_{14} + k_{15}) > k_9 \). Understanding the kinetic mechanism of SULT1A1 is important to an understanding of drug metabolism and xenobiotic detoxification by SULT1A1. The novel bypass mechanism proposed by us for SULT1A1-catalyzed sulfation could also apply to some other phase II SULTs and may have special biological significance. For instance human SULT1A3 and rat SULT1A1 (aryl sulfotransferase IV) show very similar kinetic properties to those of human SULT1A1. Most phase II transferases have high affinity for the co-substrate (endogenous donor). Through the bypass mechanism, PAP may not need to be released once a sulfonyl group has been transferred from the protein-co-substrate complex, thus increasing the efficiency of the enzyme. This mechanism also utilizes low affinity (more hydrophilic and easier to be excreted) products to conjugate more hydrophobic substrates for detoxification when the co-substrate (PAPS) supply is limited. It is known that in vivo PAPS concentrations are low, and it can be a limit factor for drugs detoxification in certain cases (37). The low affinity products could also be used to conjugate endogenous substrates, which could be beneficial or cause problems. When low affinity products reach high concentrations under special conditions (therapeutic medicine, drug abuse, diet, etc.), these products will change the catalytic activities of the enzymes, which in turn may disrupt the normal biological functions of these transferases.

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