Tetrahydrobiopterin regulates monoamine neurotransmitter sulfonation

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Monoamine neurotransmitters are among the hundreds of signaling small molecules whose target interactions are switched “on” and “off” via transfer of the sulfuryl-moiety (−SO3) from PAPS (3′-phosphoadenosine 5′-phosphosulfate) to the hydroxyls and amines of their scaffolds. These transfer reactions are catalyzed by a small family of broad-specificity enzymes—the human cytosolic sulfotransferases (SULTs). The first structure of a SULT allosteric-binding site (that of SULT1A1) has recently come to light. The site is conserved among SULT1 family members and is promiscuous—it binds catechins, a naturally occurring family of flavonoids. Here, the catechin-binding site of SULT1A3, which sulfonates monoamine neurotransmitters, is modeled on that of 1A1 and used to screen in silico for endogenous metabolite 1A3 allosteres. Screening predicted a single high-affinity allosterie, tetrahydrobiopterin (THB), an essential cofactor in monoamine neurotransmitter biosynthesis. THB is shown to bind and inhibit SULT1A3 with high affinity, 23 (±2) nM, and to bind weakly, if at all, to the four other major SULTs found in brain and liver. The structure of the THB-bound binding site is determined and confirms that THB binds the catechin site. A structural comparison of SULT1A3 with SULT1A1 (its immediate evolutionary progenitor) reveals how SULT1A3 acquired high affinity for THB and that the majority of residue changes needed to transform 1A1 into 1A3 are clustered at the allosteric and active sites. Finally, sequence records reveal that the coevolution of these sites played an essential role in the evolution of simian neurotransmitter metabolism.

Significance

Human cytosolic sulfotransferases (SULTs) regulate hundreds of signaling small molecules, yet little is known regarding their small-molecule regulation. Members of the SULT1 family harbor a conserved allosteric site that we hypothesize allows independent regulation of sulfonation in the 11 metabolic areas in which these isoforms operate. This hypothesis is validated using SULT1A3, which sulfonates and inactivates monoamine neurotransmitters. During validation it was discovered that tetrahydrobiopterin (THB), an essential cofactor in monoamine neurotransmitter biosynthesis, allosterically inhibits SULT1A3 with high affinity (Kᵢ, 23 nM) and isoform selectivity. Monoamine neurotransmitter metabolism shapes the behavior and social structures of humans and other primates (25). Although numerous therapeutics target neurotransmitter metabolism (26, 27), none do so by inhibiting sulfonation. The structure of the THB-binding site, presented here, provides an isozyme-specific means of controlling neurotransmitter activity.

Results and Discussion

Discovering the Allosterie. The complexity and biomorphic character of the catechin family and the promiscuity of the SULT1A3

Author contributions: I.C., T.W., and T.S.L. designed research; I.C. and T.W. performed research; I.C., T.W., and T.S.L. analyzed data; and I.C., T.W., and T.S.L. wrote the paper.

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catechin site suggested that it might also bind endogenous metabolite allostere. To explore this possibility, the structure of the SULT1A3 site was modeled on the SULT1A1 site, and both templates were used in an in silico docking study to screen metabolite allostere. The SULT1A1 control template helps gauge the specificity of candidate SULT1A3 allostere. Given the high catalytic efficiency of SULT1A3 toward monoamine neurotransmitters, the screen was limited to the 115 human small-molecule metabolites associated with neurotransmitter metabolism found in the Small Molecule Pathways Database (SMPDB) (28). Screening was performed with the Genetic Optimization for Ligand Docking (GOLD) program (Materials and Methods).

The screens predicted that of the 115 metabolites tested, only THB, an essential cofactor in monoamine neurotransmitter biosynthesis, would bind SULT1A3 with high affinity (44 nM). The studies further predicted that THB would bind SULT1A3 far more tightly (230-fold) than SULT1A1. The compound whose predicted affinity was closest to that of THB is dihydrobipterin (DHB), the oxidized form of THB produced during neurotransmitter biosynthesis. The affinity of DHB is predicted to be ~130-fold weaker than that of THB.

To test the binding and specificity predictions of the docking studies and whether, like catechins, THB functions as an inhibitor, THB was tested in initial-rate studies as an inhibitor of SULT1A3 and the four other SULTs that, together with SULT1A3, constitute the major SULT isoforms found in human brain (29, 30) and liver (1, 31) (i.e., SULTs 1A1, 1E1, 2A1, and 2B1b). The results (Fig. 1) reveal that THB binds and inhibits SULT1A3 with high affinity, $K_i = 23 \pm 2$ nM, and appears to completely suppress SULT1A3 turnover at saturation. None of the other major brain and liver SULTs are inhibited at concentrations needed to saturate SULT1A3 (Fig. 1), and increasing THB further, to 200 $\mu$M, did not inhibit these SULTs. Thus, THB proved to be a remarkably potent and specific inhibitor of SULT1A3. The allosteric nature of THB inhibition was confirmed in an initial-rate study, which revealed that THB is a noncompetitive inhibitor versus 1-HP (Fig. S1).

THB appears to function both as an essential cofactor in monoamine neurotransmitter biosynthesis and as an allosteric to inhibit the enzyme that inactivates these same transmitters. THB levels found in cells and tissues are consistent with an in vivo role for THB in regulating sulfonation. Examples include the following: (i) THB levels are near its $K_i$ (23 nM) in human cerebrospinal fluid, erythrocytes, and plasma (~15 nM) (32). (ii) THB levels in dopaminergic neurons appear to go from subsaturating in the cell body to well above saturation (~100 nM) in neuronal termini (33)—a scenario that suggests that cytosolic dopamine, which diffuses into the cell body from leaky vesicles located in termini, might only be sulfonated once a suitable point in the THB concentration gradient is reached. (iii) THB levels are potently down-regulated by catechines in a variety of cell types and tissues that express SULT1A3 (34–36). THB levels in normal-patient platelets are ~200 nM (8.7 $K_i$ THB) (36, 37) and expected to decrease significantly upon cytokine stimulation (36). (iv) SULT1A3 is up-regulated in neurons in response to dopamine (30) and prevents toxicity by suppressing dopamine free-radical formation (38). Exogenous THB is known to induce apoptosis via free-radical mechanisms only in dopaminergic cell lines (39) and may do so by preventing dopamine sulfonation.

The THB Binding-Site Structure.

The spin-label triangulation methodology. We have recently developed a spin-label method for determining the structures of SULT ligand-binding sites (12). The technique does not require X-ray crystallography or high-resolution multidimensional NMR and has no protein molecular weight limitations. The method involves identifying six solvent-accessible positions in a SULT scaffold at which cysteine can be inserted and spin-labeled without affecting catalysis. As a set, the individual cyst constructs allow the entire surface of the enzyme to be “coated” in a paramagnetic field of sufficient strength to detect its effects on the 1D solution NMR spectrum of a ligand regardless of where it binds. Paramagnetic effects are detectible when a ligand docks within ~25 Å of a given spin label and exchanges between protein and bulk solvent at rates comparable to or faster than the Larmor frequency difference between free- and bound-ligand NMR peaks. Distances between the spin label and ligand protons are calculated from the magnitudes of the paramagnetic effects. In most cases, effects can be detected from three different spin labels; hence, each ligand proton is separately positioned by triangulation, which allows the ligand to be oriented with respect to enzyme surface. A final, refined structure of the ligand-bound binding site is obtained using NMR distance-constrained molecular-dynamics docking.

The three SULT1A3 cys-insert mutants used in the current study were the same as those used to determine the catechin-site structure of SULT1A1. A model of SULT1A3 with spin labels attached at these positions is shown in Fig. 2. The identical subunits of the enzyme dimer are shown in red and blue. The 25-Å radius of the large, semitransparent spheres (which are centered on nitroxide oxygens) correspond to the approximate maximum distance over which proton/spin-label interactions are detectable. THB was positioned at the catechin site based on predictions from the docking screens described above. As is evident, the putative docking site is coated in a detectable paramagnetic field from each spin label.

Building and testing cys constructs. Three single-cys insertion mutants were constructed from a SULT1A3 coding region (Materials and Methods). Each construct was covalently labeled to >98% with spin label [TEMPO (2,2,6,6-tetramethylpiperidin-1-oxyl)] or
N-cyclohexylmaleimide—a diamagnetic homolog in which the spin-label Proxyl (2,2,5,5-tetramethyl-1-pyrrolidinoxyl) moiety is replaced by a cyclohexyl-group. The diamagnetic constructs are used as controls in the NMR distance determinations (see Distance Measurements). The initial-rate parameters of the labeled constructs were determined and were similar to those of the wild-type enzyme (Table 1 and Materials and Methods).

**Distance Measurements.** Methods for determining distances between the protons of bound ligands and covalently attached spin labels are well established (40, 41). In favorable cases (when ligand exchange is comparable to or greater than the Larmor frequency difference of the free and bound proton), the effects of the protein environment on ligand–proton relaxation can be detected in the ligand’s solution spectrum. In such cases, observed transverse relaxation rates, \( R_{2,obs} \), can be calculated from solution-phase linewidths, which depend linearly on the fraction of bound ligand, \( F_B \), according to Eq. 1 (42):

\[
R_{2,obs} = (R_{2B} - R_{2F})F_B + R_{2F} + R_{2ex}.
\]

\( R_{2B} \) and \( R_{2F} \) are the transverse relaxation rates for bound and free protons, and \( R_{2ex} \) is the chemical exchange contribution to the relaxation.

The contribution of the electron to the transverse relaxation of the bound proton is required to calculate distances (41). To isolate the electron’s contribution from those of other factors, two \( R_{2,obs} \) versus \( F_B \) plots are created, using the para- and diamagnetic enzyme constructs (43). The electron’s contribution is given by the difference in slopes of the plots (41, 42).

The structure and solution spectrum of THB are given in Fig. 3A, and the effects of the spin-labeled K234C construct on the H3 proton peak of THB are shown in Fig. 3B. A representative set of para- and diamagnetic \( R_{2,obs} \) versus \( F_B \) plots are given in Fig. 3C. Twelve such plot pairs were constructed; the remaining 11 pairs are presented in Fig. S2.

**Refining the structure.** The final structure was obtained by distance-constrained molecular dynamics docking of the THB. Each NMR-determined distance represents a vector between the time-averaged position of the spin-label oxygen (calculated using GROMACS) and a given proton. Three such vectors are associated with each proton, each originates at the oxygen of a different spin label, and all three intersect at the proton. The errors associated with these three measurements constitute an ellipsoid that centers on the proton and whose axis magnitudes are given by the SEs (±1 σ) of the NMR measurements, which are compiled in Table 2. Docking was constrained by applying a 50 kJ-mole^{-1} Å^{-1} restoring force [using distance_restraints, GROMACS (44, 45)] that drives the proton toward the ellipsoid center if any part of its van der Waals surface lies outside the ellipsoid; the restoring force inside the ellipsoid is 0. As is appropriate for NMR spin–spin interaction measurements (42, 46, 47), distance_restraints was parameterized to use time-averaged (1/τ)–weighted restraints. The motions of all five THB protons (H1, H2, H3, H6, and H7) were constrained simultaneously during docking. Docking was repeated 10 times, identical structures were achieved in each case, and the structure did not change once the distance constraints were removed.

**The refined structure.** The MD-refined structure of THB bound to the catechin-binding site of SULT1A3 is shown in Fig. 4A [Protein Data Bank (PDB) coordinates are available at www.modelarchive.org, accession no. 3zswjvneac5ubl7f]. SULT1A3 harbors a conserved ~30-residue active-site cap (shown in light orange) that opens and closes in response to nucleotide binding and a smaller “lower lip” segment (shown in beige) that together with the cap form a molecular pore, or sieve, through which acceptors must pass to enter the active site. The catechin-binding site of SULT1A3, like that of 1A1, is adjacent to the pore and sandwiched between the cap and lower lip. To validate the structure, three residues predicted to be in direct contact with THB (see labeled residues in Fig. 4B) were mutated, and the effects of the mutation on THB binding and inhibition and initial-rate turnover were tested. The results, compiled in Table 3, reveal that although the mutations did not significantly affect the catalytic integrity of the enzyme, neither THB binding nor inhibition could be detected at concentrations as high as 200 μM. When bound, THB directly contacts six residues: P74, Y76, V77, D86, E89, and V243. Two (D86 and E89) are unique to SULT1A3; the remainder are also found in the catechin site of

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>( k_{cat} ), min^{-1}</th>
<th>( K_m ), mM</th>
<th>( K_I ), THB, nM</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>120 (17)</td>
<td>22 (3.1)</td>
<td>23 (1.5)</td>
</tr>
<tr>
<td>26*</td>
<td>110 (15)</td>
<td>25 (3.5)</td>
<td>25 (2.7)</td>
</tr>
<tr>
<td>151</td>
<td>115 (16)</td>
<td>26 (2.2)</td>
<td>20 (2.2)</td>
</tr>
<tr>
<td>234</td>
<td>109 (15)</td>
<td>24 (3.2)</td>
<td>24 (1.6)</td>
</tr>
</tbody>
</table>

Values in parentheses indicate SD.

* Cys residue at which spin label is attached.
The NMR measurements. The enzyme is saturated (μM). The solution pH 7.4, 25°C. The enzyme is saturated with THB (100 μM), KPO4 (50 mM), DTT (>50 mM), D2O (>98%), pH 7.4, 25 ± 1°C. (b) Spin-label effects on the peak of the H3 protons of THB. The solution 1H-NMR spectrum (600 MHz) of the H3 peak of THB is shown as a function of the percent of THB bound to spin-labeled C234-SULT1A3. Conditions: THB (100 μM (brown), 200 μM (blue and black), 400 μM (green), and 800 μM (red)), spin-labeled C234-SULT1A3 (20 μM monomer or 0 μM (black)), PAP (500 μM, 17 mM KCl), KPO4 (50 mM), D2O (>98%), pH 7.4, 25 ± 1°C. The enzyme is saturated (≥4,400 Kd) at all THB concentrations (Kd THB = 23 nM). Peak amplitudes are normalized to THB concentration. (c) Line width versus fraction THB-bound plots. The effects of paramagnetic and diamagnetic C234-SULT1A3 constructs on the line width on the H3 proton peak of THB are plotted as a function of fraction of enzyme-bound THB. Conditions are described in B.

SULT1A1. The SULT1A3-specific residues are negatively charged and contact THB at the three hydrogen atoms that are linearly aligned along the N2–N1–N8 edge of its 2-amino-pterin ring (Fig. 4C). The charge distribution of THB, predicted by Automated Topology Builder (48, 49) using the B3LYP/631G* basis set, reveals that these three hydrogen atoms carry significant positive charge. Thus, their interactions with D86 and E89 are likely to contribute substantially to the site’s THB selectivity. The conversion of THB to DHB during catecholamine biosynthesis results in a subtle structural change and extends the resonance of the pterin ring, which delocalizes and thus diminishes charge at the N1–N2–N8 protons (Fig. 4C). This decrease in charge density is expected to weaken interactions with D86 and E89. The affinity of DHB was determined, by fluorescence titration, and found to be 130-fold weaker than that of THB (Fig. S3)—a value that agrees remarkably well with the ~130-fold change predicted by GROMACS docking studies. The D86 and E89 ionic interactions engender both THB specificity and the ability to respond selectively to amino acid hydroxylase substrates, which report the cellular demand for neurotransmitter synthesis.

The metabolic imperative of the THB-binding site. If THB allosterism is essential to the proper functioning of neurotransmitter metabolism, one might expect that the allosteric and active sites, whose interdependent functions are coupled at different points in the same metabolic pathway, evolve as a single unit of metabolic circuitry until a more evolutionarily advantageous solution could be found. To evaluate the conservation and coevolution of these two sites, their evolutionary lineage was traced through the 14 SULT1A3 sequences available in the NCBI database. SULT1A3 made its evolutionary debut at some point in the ~10 My interval between the separation of monkeys (whose genomes encode the allosteric and active sites of SULT1A3; Fig. 4D) and lemurs (which encode neither) and the separation of old-world monkeys and apes. The interval begins 56–66 My (50)—a time that immediately follows the last mass extinction and is associated with a burst in mammalian speciation caused by a lack of competition in the many niches vacated by the extinction (51).

The immediate evolutionary progenitor of SULT1A3 is SULT1A1 (52)—their primary sequences are 295 residues in length, 92.9% identical, and 98.6% conserved. Eleven of the 21 nonidentical residues are clustered in two regions (Fig. 4D): the lower lip region of the THB allosteric site (seven residues), and the catechol-binding region of the active site (four residues), which is largely responsible for changing the substrate specificity of 1A1 to that of 1A3 (53). The remaining 10 residues are scattered throughout the scaffold and have not been assigned function. The active-site cluster is perfectly conserved in all 14 SULT1A3 sequences; however, the amino acids that line the SULT1A3 catechin-binding site drift slightly. Two of the six residues that directly contact THB (V77 and E89 in the human isoform) have drifted either to I77/D89 or M77/E89. To assess whether these conserved substitutions alter THB binding, they were inserted, using PCR mutagenesis, into the human SULT1A3, and the THB affinities of the hybrids were determined. The hybrids’ affinities were within a factor of 1.7 from that of the native construct (see Table 4). Hence, the allosteric site has drifted only so far as to maintain its THB-binding properties. It appears that species that have lost either function have not survived the challenges of Darwinian selection to the current day—only those that retain the ability to allosterically down-regulate SULT1A3 turnover in response to THB remain.

Conclusions
The central hypothesis of this manuscript—that the so-called catechin allosteric site of SULT1A3 can be used by endogenous metabolites—has been proven. THB, an essential metabolite in the synthesis of monoamine neurotransmitters,
binds the SULT1A3 catechin site with high and physiologically relevant affinity (23 nM) and has no detectible affinity for four other major SULT isoforms. The binding site is highly selective for THB over DHB, its conjugate product, and has thus evolved to respond to the substrate side of the rate-limiting step in the biosynthesis of such transmitters. These findings suggest a classical feedback mechanism in which increased cellular demand for transmitters, which increase THB levels (54), results in allosteric inhibition of the enzyme that would otherwise have inactivated the transmitter and shorted the supply/demand circuitry.

The structure of the THB-bound binding site of SULT1A3 was determined and confirms that THB binds the catechin site of SULT1A3. THB is sandwiched between the active-site cap (gold) and lower lip (sand) of the SULT1A3. The transparent surface of the binding pocket was calculated using Hollow. The donor (PAPS) and acceptor (dopamine) are labeled. (B) Residues used to verify the structure. Mutations at these positions prevent THB binding and inhibition but do not alter the initial-rate parameters of the enzyme (Table 3). (C) THB and DHB charge distributions. Distributions were calculated with Automated Topology Builder (49), which uses the B3LYP/6-31G* basis set. Charges are color-coded in electron units according the scale shown on the right. The protons that interact with D78 and E81 are labeled with their charges, and the nitrogen atoms bound to the protons are numbered (i.e., 1, 2, and 8). (D) Residues responsible for the allosteric and active-site specificity of SULT1A3. The sequences of SULT1A3 and SULT1A1 (the evolutionary progenitor of SULT1A3) differ at 21 positions, 11 of which, shown in red, cluster in two regions. Changes in the lower lip result in high affinity and specificity for THB; changes in the specificity region shift the substrate specificity from that of SULT1A1 to SULT1A3.

Fig. 4. The THB-bound catechin-binding site of SULT1A3. (A) THB bound to the SULT1A3 allosteric-binding site. THB is sandwiched between the active-site cap (gold) and lower lip (sand) of the SULT1A3. The transparent surface of the binding pocket was calculated using Hollow. The donor (PAPS) and acceptor (dopamine) are labeled. (B) Residues used to verify the structure. Mutations at these positions prevent THB binding and inhibition but do not alter the initial-rate parameters of the enzyme (Table 3). (C) THB and DHB charge distributions. Distributions were calculated with Automated Topology Builder (49), which uses the B3LYP/6-31G* basis set. Charges are color-coded in electron units according the scale shown on the right. The protons that interact with D78 and E81 are labeled with their charges, and the nitrogen atoms bound to the protons are numbered (i.e., 1, 2, and 8). (D) Residues responsible for the allosteric and active-site specificity of SULT1A3. The sequences of SULT1A3 and SULT1A1 (the evolutionary progenitor of SULT1A3) differ at 21 positions, 11 of which, shown in red, cluster in two regions. Changes in the lower lip result in high affinity and specificity for THB; changes in the specificity region shift the substrate specificity from that of SULT1A1 to SULT1A3.
neuronal synapses, and modulating these levels is a major goal of the pharmaceutical industry (57). Classical therapeutic strategies aim to increase neurotransmitter levels either by preventing neurotransmitter reuptake from synapses (57) or by inhibiting enzymes that inactivate neurotransmitters, particularly monoamine oxidases (MAOs) (58) and catechol-O-methyltransferases (COMTs) (59). MAOs attach to mitochondrial outer membranes with their active sites facing the cytosol, and COMTs are embedded in the outer membranes of pre- and postsynaptic neurons with their catalytic domains oriented extracellularly (60). Thus, these catalysts operate in spatially separate tissue compartments—cytosols and extracellular fluids.

SULT1A3 is, like MAO, located in neuronal cytosols (29) and inactives catecholamines. Recent work reveals that sulf-conjugates predominate among serotonin metabolites in fluids extracted from brain cortices of living humans and are major metabolites in brain-ventricle and spinal fluids (61). Studies using subsections of rat brain reveal that MAO inhibition routes dopamine metabolism substantially through the suffonation pathway and demonstrate the interactivity of the MAO and SULT systems (62, 63). These findings strongly recommend SULT1A3 as a candidate for controlling neurotransmitter activity, particularly when used in conjunction with MAO inhibitors. It is notable that nearly half of patients with neurocognitive disorders are not fully responsive to classical therapy but can be treated successfully with combination therapy (56, 64). To our knowledge, SULTs inhibitors have not been tested as therapeutics. The THB-binding site offers a structurally well-defined, isozyme-specific, allosteric target for developing SULT1A3 inhibitors that can be used to control neurotransmitter activity.

We are just beginning to appreciate the importance of small-molecule SULT allostery. With the exception of the current work, there appear to have been no studies aimed specifically at understanding SULT metabolism but also as molecular templates for the design of isoform-specific SULT allosteres—molecular strategies that inactivate neurotransmitters, particularly monoamine oxidases (MAOs) (58) and catechol-O-methyltransferases (COMTs) (59). MAOs attach to mitochondrial outer membranes with their active sites facing the cytosol, and COMTs are embedded in the outer membranes of pre- and postsynaptic neurons with their catalytic domains oriented extracellularly (60). Thus, these catalysts operate in spatially separate tissue compartments—cytosols and extracellular fluids.

Table 3. Initial-rate and THB binding and inhibition parameters for WT and mutant SULT1A3

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>$k_{cat}$ min$^{-1}$</th>
<th>$K_m$ 1-HP mM*</th>
<th>$K_i$ THB mM*</th>
<th>$K_d$ THB mM*</th>
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<tr>
<td>WT</td>
<td>120 (17)$^3$</td>
<td>24 (3.8)</td>
<td>25 (2.7)</td>
<td>23 (2.3)</td>
</tr>
<tr>
<td>D68A</td>
<td>97 (18)</td>
<td>27 (3.6)</td>
<td>ND$^3$</td>
<td>ND$^3$</td>
</tr>
<tr>
<td>E89I</td>
<td>79.5 (4)</td>
<td>22 (2.1)</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Y76A</td>
<td>107 (19)</td>
<td>25 (3.0)</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

Values in parentheses indicate SD.

*Initial-rate protocols are described in Materials and Methods.

Materials and Methods

The materials and sources used in this study are as follows: DTT, deuterated DTT, 5,5'-dithiobis-2-nitrobenzoic acid (DTNB), ethylenediamine-tetraacetic acid (EDTA), glutathione (reduced), 1-hydroxypropane-1(HP), imidazole, isopropyl-thio-o-p-galactopyranoside (IPTG), LB media, lysozyme, β-mercaptoethanol, 3-maleimido-PROXYL, N-cyclohexylmaleimide, peptatin A, potassium phosphate at the highest grade available from Sigma. Ampicillin, Hepes, KOH, KC, MgCl$_2$, and phenylmethylsulfonyl fluoride (PMSF) were purchased from Fisher Scientific. Glutathione- and nickel-chelating resins were obtained from GE Healthcare. Competent Escherichia coli (BL21[DE3]) was purchased from Novagen. PAPS and PAP were synthesized and purified according to previously published protocols (67).

Computer and Software.

Molecular dynamics simulations were performed using a Parallel Quantum Solutions Q532-2670C-X58 computer. PQS Molecular Builder was purchased from Quantum Solutions. A GOLD license was obtained from the Cambridge Crystallographic Data Center. The source code for GRONingen Machine for Chemical Simulation (GROMACS) 4.5 was downloaded from www.GROMACS.org under the GROMACS General Public License (GPL).

The SULT1A3 Constructs.

SULT1A3 expression plasmid consists of the SULT1A3 coding region inserted into a triple-tag pGEX-6P vector containing an (N-terminal)-His-GST/MBP tag (68, 69). The expression vectors used for regio-specific attachment of maleimide-based labels were constructed as follows: Three single-cys mutants were created by inserting cys into the wild-type SULT 1A3 scaffold, which was not DTNB reactive, at residues E26, E151, and K234. All mutagenesis projects began with the SULT1A3 expression-plasmid and used standard PCR mutagenesis protocols (70).

The mutants used to confirm the structure of the THB binding (Y76A, D68A, and E89I) were constructed by mutating the wild-type SULT1A3 coding region.

Protein Purification.

SULT expression and purification were performed using protocols that have been described in detail previously (68, 69). The proteins (SULTs 1A3, 1A1, 1E1, 2A1, and 2B1) are ≥95% pure as judged by Coomassie-blue staining of SDS/PAGE. Protein concentrations are determined by UV absorbance $(\epsilon_{280} \text{SULT1A3} = 53.9 \text{mM}^{-1} \text{cm}^{-1})$, $(\epsilon_{280} \text{SULT2A1} = 51.9 \text{mM}^{-1} \text{cm}^{-1}), \epsilon_{280} \text{SULT1A3} = 79.5 \text{mM}^{-1} \text{cm}^{-1})$, and $(\epsilon_{280} \text{SULT2A1} = 67.6 \text{mM}^{-1} \text{cm}^{-1})$. The final protein is flash-frozen and stored at −80°C.

Covalent Tagging.

Labels (N-cyclohexylmaleimide or 3-maleimido-PROXYL) were added (20-fold excess over reactive cyste) to a solution containing enzyme (50 μM, monomer), PAP (0.50 mM), KPO$_4$ (50 mM), pH 7.4, 25 ± 2°C. PAP was added to enhance enzyme stability (43). Reactions were monitored using DTNB to measure unreacted cysteine and were considered complete only when ≥98% of the cysteine had reacted (−3 h). Following completion, the reaction mixtures were dialyzed against PAP (0.50 mM), KPO$_4$ (50 mM), pH 7.4, D$_2$O (≥95%), 4 ± 2°C.

Initial-Rate Studies of Labeled Constructs and THB Binding-Site Mutants.

Initial-rate and THB-inhibition parameters were determined for the labeled constructs and THB binding-site mutants as previously described (43). Reactions are initiated by addition of PAPS (0.50 mM, 17 x $K_a$) to a solution containing enzyme (25 mM), 1-HP (0.40 mM, −20 x $K_a$), DTT (5.0 mM), and KPO$_4$ (50 mM), pH 7.5, 25 ± 2°C. Reaction progress was monitored via sulfonation-dependent change in 1-HP fluorescence ($\lambda_{ex}$ 325 nm, $\lambda_{em}$ 370 nm (71)), and $K_m$ and $V_{max}$ are extracted from the data by progress-curve analysis (72). THB-inhibition studies used identical conditions. $K_i$ was determined from weighted least-squares fitting of initial-rate versus THB data plotted in double reciprocal space (14). Inhibition by THB for the mutants used to verify the position of the THB-binding pocket (Y76A, D68A, E89I) was not detected at THB concentrations as high as 200 μM.

NMR Measurements.

Spectra were collected, using a Bruker DRX600 spectrometer equipped with a TCI H/F-cryogenic probe at 298 K, at the following THB concentrations: 0, 5, 10, 20, and 40 (nmole/active site). Peak widths for each THB proton used in the structure determination were obtained using NMRdraw (Lorentzian peak shape) (73). At each THB concentration, a 10 proton spectrum was collected from 0 to 14 ppm, using a 0.96 acquisition time and presaturating water pulse. Each spectrum was the time average of 512 scans, with a 1.5-s delay between each scan.

Screening for Human-Metabolable SULT1A3 Allosteres (GOLD Modeling).

SULT1A3 and SULT1A1 models were constructed using available crystal structures, 2A3R (74) and 2D06 (75), respectively, as described previously (72). In silico docking was

<table>
<thead>
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<th>Enzyme</th>
<th>$K_i$, nM*</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>25 (2.6)</td>
</tr>
<tr>
<td>V77M</td>
<td>32 (3.1)</td>
</tr>
<tr>
<td>E89D, V77I</td>
<td>43 (5.0)</td>
</tr>
</tbody>
</table>

Values in parentheses indicate SD.

*Initial-rate protocol is described in Materials and Methods.
performed using GOLD (76–78). The program was configured to use flexible side chains, 2,500 generations, and an initial population of 100 random positions. Docking was limited to a 1 Å radius around the center of the catechin-binding site. Simulations were performed three times for each compound. GOLD scores (which are indicative of binding affinity) were calibrated by constructing standard curves for SULT1A1 and 1A3 that correlate the GOLD scores and experimentally determined affinities of epigallocatechin gallate, epicatechin gallate, epicatechin, and quercetin for each isoform.

**Molecular Dynamics Modeling.** SWISS-MODEL (79) was used to create a ligand-free model of SULT1A3 from the SULT1A3–PAP-Dopamine (PDB ID code 2A3R) structure (74), which was missing 27 atoms. The model was protonated at pH 7.4 and energy-minimized in GROMACS (45). GROMASS7 energy-parameter files were created for THB, PAPS, and spin-labeled cystines using Automated Topology Builder (49). The GROMASS7 field was modified in GROMACS to allow the program to recognize the spin-labeled cysteine as a canonical residue. Spin-labeled cystines were inserted by replacing residues E26, E151, and K234; PAPS was positioned at the active site using GOLD (76–78); and the system was equilibrated using GROMACS (100 ps increments) to the simulated condition: 298 K, NaCl (0.50 mM, 17 × 10−3 M), DTT (5.0 mM), KPO4 (50 mM), pH 7.5, 2 ± 2 °C. Titrations were performed in triplicate. Data were averaged and least-squares fit using a model that assumes a single binding site per monomer. THB concentration was varied from 0.10 to 20 × Kd. THB binding was not observed for the THB-binding-site mutants (Y76A, D86A, E89I) at THB concentrations as high as 200 μM.

**Equilibrium Binding Studies.** The binding of ligands to SULT1A3 was monitored via changes in the intrinsic fluorescence of the enzyme (λex 290 nm, λem 370 nm). Typical conditions were as follows: SULT (~25 m), dimer, PAP (0 or 0.50 mM, 17 × 10−3 M), DTT (5.0 mM), KPO4 (50 mM), pH 7.5, 2 ± 2 °C. Titration experiments were performed in triplicate. Data were averaged and least-squares fit using a model that assumes a single binding site per monomer. THB concentration was varied from 0.10 to 20 × Kd. THB binding was not observed for the THB-binding-site mutants (Y76A, D86A, E89I) at THB concentrations as high as 200 μM.

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