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Effect of *SULT2B1* Genetic Polymorphisms on the Sulfation of Dehydroepiandrosterone and Pregnenolone by SULT2B1b Allozymes

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Abstract

Pregnenolone and dehydroepiandrosterone (DHEA) are hydroxysteroids that serve as biosynthetic precursors for steroid hormones in human body. SULT2B1b has been reported to be critically involved in the sulfation of pregnenolone and DHEA, particularly in the sex steroid-responsive tissues. The current study was designed to investigate the impact of the genetic polymorphisms of *SULT2B1* on the sulfation of DHEA and pregnenolone by SULT2B1b allozymes. Ten SULT2B1b allozymes previously prepared were shown to exhibit differential sulfating activities toward DHEA and pregnenolone in comparison to the wild-type enzyme. Kinetic studies revealed further significant changes in their substrate-binding affinity and catalytic activity toward DHEA and pregnenolone. Taken together, these results indicated clearly a profound effect of *SULT2B1* genetic polymorphisms on the sulfating activity of SULT2B1b allozymes toward DHEA and pregnenolone, which may have implications in inter-individual variations in the homeostasis of these two important steroid precursors.

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Keywords

SULT; SULT2B1b; single nucleotide polymorphisms; DHEA; pregnenolone

1. Introduction

Dehydroepiandrosterone (DHEA), the main biosynthetic precursor of sex steroids (Schiffer et al., 2018), is synthesized mainly in the adrenal glands and, to a lower extent, in ovaries and testes (Labrie, 2010). In the adrenal glands, cholesterol is first transformed to pregnenolone under the action of cytochrome P450scc (Schiffer et al., 2018). The conversion of cholesterol to pregnenolone is the rate limiting step in steroid hormone biosynthesis pathway (Neunzig and Bernhardt, 2014). Pregnenolone then serves as a precursor for glucocorticoids, mineralocorticoids, and DHEA (Neunzig and Bernhardt, 2014). In peripheral tissues, the adrenal secreted DHEA acts as a precursor for estrogen and androgen hormones through a process called intracrinology (Schiffer et al., 2018; Neunzig and Bernhardt, 2014), which involves the intracellular formation, inactivation, and action of sex steroids (Labrie, 2010). Sulfation of DHEA forming DHEA-S limits the amount of DHEA that is available for the biosynthesis of androgen hormones (Noordam et al., 2009). In the body, the sulfation of DHEA and pregnenolone has been shown to be mediated by the cytosolic sulfotransferase (SULT) enzymes, particularly SULT2A1, SULT2B1a, and SULT2B1b (Falany and Rohn-Glowacki, 2013). It is noted that in addition to serving as steroid hormones precursors, DHEA, pregnenolone, and their sulfated metabolites synthesized independently in the nervous system are considered neurosteroids that act as neuromodulators (Vallee et al., 2001).

The cytosolic sulfotransferases (SULTs) are a group of phase II conjugation enzymes that are involved in the homeostasis and detoxification of numerous exogenous and xenobiotic compounds (Falany, 1997). The SULTs catalyze the transfer of a sulfonate group from 3’-phosphoadenosine 5’-phosphosulfate (PAPS) to the hydroxyl or amino group of acceptor compounds, leading to their increased hydrophilicity and facilitated urinary and biliary excretion from the body (Falany and Roth, 1993; Weinshilboum and Otterness, 1994). In humans, there are 13 distinct SULTs that are classified into four gene families, designated SULT1, SULT2, SULT4, and SULT6 (Glatt et al., 2001; Freimuth et al., 2004). SULT2 family is previously known as the hydroxysteroid sulfotransferase family and consists of three isoforms: SULT2A1 (previously called a DHEA sulfotransferase), SULT2B1a (a pregnenolone sulfotransferase), and SULT2B1b (a cholesterol sulfotransferase) (Falany and Rohn-Glowacki, 2013). SULT2B1a and SULT2B1b isoforms are coded by the same gene, designated *SULT2B1*, and are generated as a result of alternative initiation and splicing, leading to the formation of the two isoforms with distinct N-terminal regions (Her et al., 1998). Compared with other known SULT enzymes, the two SULT2B1 isoforms carry a unique carboxy-terminal extension of about 53 amino acid residues (He and Falany, 2006). A previous study using SULT2B1b demonstrated that this carboxy-terminal extension influenced the thermostability, kinetic properties, subcellular localization, and immunogenicity, as well as posttranslational modification by phosphorylation (He and Falany, 2006). While the three SULT2 isoforms display overlapping substrate specificity

toward different hydroxysteroids such as DHEA and pregnenolone, they exhibit tissue-specific distribution (Falany et al., 2006; Geese and Raftogianis, 2001; Otterness and Weinshilboum, 1994; Thomae et al., 2002). For example, SULT2A1 is expressed mainly in the liver, adrenal glands, and intestine (Otterness and Weinshilboum, 1994; Thomae et al., 2002), whereas SULT2B1b is highly expressed in the placenta, prostate, breast, endometrium, ovary, uterus, small intestine, colon, lung, platelet, brain, and skin (Falany et al., 2006; Geese and Raftogianis, 2001). In contrast, no expression of SULT2B1a protein was detected in any of the tissues examined (Falany and Rohn-Glowacki, 2013). SULT2B1b thus is more likely the main enzyme responsible for the sulfation of DHEA and pregnenolone in steroid-responsive tissues as well as in the brain. *SULT2B1* genetic polymorphisms have been reported (Hyland et al., 2013, Levesque et al., 2014, Mostaghel 2013, Yang et al., 2013, Hu et al., 2015, Vickman et al., 2016, Chen et al., 2016; Ji et al., 2007). It is an interesting question whether *SULT2B1* missense coding SNPs (cSNPs) may influence the sulfating activity of the resulting SULT2B1b allozymes and thus affect steroid-related physiology and pathology in different individuals.

In this study, ten SULT2B1b allozymes, previously prepared by site-directed mutagenesis in conjunction with bacterial expression using the pGEX gene fusion system, were examined for sulfating activities toward DHEA and pregnenolone. Kinetic parameters of SULT2B1b allozymes in mediating the sulfation of DHEA and pregnenolone were determined to delineate their differential substrate affinity and catalytic activity toward DHEA and pregnenolone in comparison to the wild-type enzyme.

2. Materials and Methods

2.1. Materials.

Pregnenolone, DHEA, adenosine 5′-triphosphate (ATP), dimethyl sulfoxide (DMSO), N-2-hydroxylpiperazine-N′-2-ethanesulfonic acid (HEPES), dithiothreitol (DTT), and Trizma base, were products of Sigma-Aldrich (St. Louis, MO, USA). PrimeSTAR® Max DNA polymerase was a product of Takara Bio (Mountain View, CA, USA). Oligonucleotide primers were synthesized by Eurofins Genomics (Louisville, KY, USA). Cellulose TLC plates and Ultrafree-MC 5000 NMWL filter units were from EMD Millipore (Billerica, MA, USA). Ecolume liquid scintillation cocktail was a product of MP Biomedicals, LLC. (Irvine, CA, USA). Carrier-free sodium [^{35}S]sulfate was from American Radiolabeled Chemicals (St. Louis, MO, USA). PAP[^{35}S] was synthesized using recombinant human bifunctional PAPS synthase based on a previously established procedure (Yanagisawa et al., 1998). All other chemicals were of the highest grade commercially available.

2.2. Preparation of human SULT2B1 allozymes.

As described previously, SNP databases located at the U.S. National Center for Biotechnology Information (NCBI), the Ensembl Variation database, and the Universal Protein Resource (UniProt), were systematically searched. Ten *SULT2B1* coding SNPs (cSNPs) were selected based on the location and chemical nature of the amino acid variations in coded SULT2B1b allozymes (Alherz et al., 2018). The designated names and SNP ID numbers of these 10 cSNPs are: SULT2B1bPro69Ala reference SNP

(rs777924668), SULT2B1b-Gly72Val (rs746398875), SULT2B1b-Thr73Met (rs527454384), SULT2B1b-Arg147His (rs777140014), SULT2B1b-Asp191Asn (rs16982158), SULT2B1b-Arg230His (rs16982169), SULT2B1b-Ser244Thr (rs765224593), SULT2B1b-Arg274Gln (rs762765702), SULT2B1b-Gly276Val (rs774212320), and SULT2B1b-Pro345Leu (rs17842463) (Table 1). The corresponding cDNAs were generated by site-directed mutagenesis, and the recombinant SULT2B1b allozymes were expressed using pGEX-4T-2 prokaryotic expression vectors and affinity purified and cleaved from the Glutathione S-transferases fusion proteins as described previously (Alherz et al., 2018).

2.3. Sulfotransferase assay.

To quantify the sulfating activity of the recombinant SULT2B1b allozymes, PAP[^{35}S] was used as the sulfate donor. The standard assay mixture, with a final volume of 20 μL, contained 50 mM HEPES buffer (pH 7.4), 1 mM DTT, 14 μM PAP[^{35}S] (14.4 Ci/mmol), 0.5 μg of wild-type or SULT2B1b allozyme, and DHEA or pregnenolone (dissolved in DMSO at 10 times the final concentration in the assay mixture) as a substrate. The final concentration of DMSO in the assay mixture was thus 10% (volume/volume). A control with DMSO alone was installed in parallel. The reaction was performed for 10 min at 37°C and stopped by incubating the reaction mixture for 3 min at 100°C. To analyze the production of [^{35}S]sulfated DHEA or pregnenolone, 1 μL of the final reaction mixture was spotted on a cellulose TLC plate, followed by TLC using a solvent system containing n-butanol: isopropanol: formic acid: water in a ratio of 3:1:1:1 (by volume). The [^{35}S]sulfated DHEA or pregnenolone spot was located by autoradiography, cut out from the TLC plate, and eluted with 0.5 ml H_2O. The [^{35}S]radioactivity of the eluate was quantified using a liquid scintillation counter as described previously (Hui and Liu, 2015). To determine the kinetic parameters of individual SULT2B1b allozymes, varying concentrations of DHEA or pregnenolone were used, based on the same procedure described above. To determine the K_m for PAPS, varying concentrations of PAPS (ranging 0.1 to 50 μM), with 50 μM of DHEA or 10 μM of pregnenolone as substrate, were tested.

2.4. Statistical analysis.

GraphPad Prism® v 6.0 software was used for calculating the kinetic constants, K_m, V_{max}, K_{cat}, and K_{cat}/K_m based on Michaelis-Menten kinetics using non-linear regression. To determine statistical differences between the wild-type SULT2B1b and individual SULT2B1b allozymes, one-way ANOVA was used for inter-group comparison, followed by Dunnett's test, with p-value < 0.05 considered being statistically significant.

2.5. Rotamer analysis of single point mutations.

Side-chain conformation of a mutated amino acid residue was simulated using the Dunbrack backbone-dependent rotamer library (Dunbrack, 2002). The structure of SULT2B1b, resolved with pregnenolone and PAP, (Protein Data Bank code: 1Q20), was referred to as the wild-type. Hydrophobic and hydrogen-binding interactions of the point-mutated amino acid residue with other residues, pregnenolone, or PAP, were also simulated by Find Clashes/Contacts tool in USCF Chimera software (Pettersen et al., 2004).

2.6. Substrate-binding simulation and molecular dynamics simulation analysis.

The molecular simulations for the docking of PAPS and pregnenolone into the substrate-binding sites were carried out with the crystal structure of SULT2B1b in complex with PAP and pregnenolone (1Q20) as a template. PAP in the complex was removed and PAPS was docked into the PAPS-binding site of SULT2B1b structure with amino acid substitution for each allozyme using AutoDock Vina (Trott and Olson, 2010). Pregnenolone in the complex was removed and docked into the substrate-binding site of SULT2B1b structure with substitution for each allozyme using AutoDock Vina. Molecular dynamics simulation of individual SULT2B1 allozymes with PAP and pregnenolone was performed using MD/Ensemble analysis tool in conjunction with Amber parameter of USCF Chimera software (Pettersen et al, 2004).

3. Results

3.1. Characterization of the DHEA-sulfating activity of purified human SULT2B1b allozymes.

The sulfating activity of the recombinant SULT2B1b allozymes toward DHEA was examined. Three of the ten tested SULT2B1b allozymes (SULT2B1b-Gly72Val, SULT2B1b-Arg147His, and SULT2B1b-Gly276Val) showed no detectable activity, whereas the other seven SULT2B1b allozymes displayed differential sulfating activity toward DHEA (Fig. 1). Among these seven SULT2B1b allozymes, SULT2B1b-Arg274Gln showed the greatest decrease in DHEA-sulfating activity, with a 27-fold reduction compared with the SULT2B1b-wt. Of the other six allozymes, four (SULT2B1b-Asp191Asn, SULT2B1b-Arg230His, SULT2B1b-Ser244Thr, and SULT2B1b-Pro345Leu) displayed a considerable decrease (1.7-fold or greater) in DHEA-sulfating activity, while the other two (SULT2B1b-Pro69Ala and SULT2B1b-Thr73Met) exhibited a much greater (more than 8-fold) decrease in DHEA-sulfating activity compared with the wild-type enzyme.

To investigate further the effects of genetic polymorphisms on the DHEA-sulfating activity of SULT2B1b allozymes, kinetic experiments were performed using varying concentrations of DHEA as a substrate. The sulfation of DHEA appeared to follow the Michaelis-Menten kinetics (cf. Fig 2). The determined kinetic constants, K_m, V_{max}, K_{cat}, and K_{cat}/K_m, for the wild-type and SULT2B1b allozymes are compiled in Table 2. Of the seven SULT2B1b allozymes examined, two (SULT2B1b-Thr73Met and SULT2B1b-Arg274Gln) showed dramatic increases in K_m value (at least 5 times) compared with SULT2B1b-wt, indicating that the amino acid changes resulted in decreased DHEA binding affinity. All tested SULT2B1b allozymes exhibited lower catalytic activity (V_{max}) compared with the wild-type enzyme. Among them, SULT2B1b-Asp191Asn displayed the smallest decrease (a 12.5% reduction) in the catalytic activity, while SULT2B1b-Pro345Leu and SULT2B1b-Arg230His showed more than 37% decrease in V_{max} compared with the wild-type enzyme. In contrast, the other four allozymes exhibited much greater reduction. SULT2B1b-Ser244Thr and SULT2B1b-Thr73Met showed a more than 50% decrease in V_{max}, whereas SULT2B1b-Pro69Ala and SULT2B1b-Arg274Gln exhibited a more than 87% decrease in V_{max}, compared with SULT2B1b-wt. Consequently, the catalytic efficiency as reflected by k_{cat}/k_m was significantly lower for all seven SULT2B1b allozymes. Four of them, SULT2B1b-

Asp191Asn, SULT2B1b-Arg230His, SULT2B1b-Ser244Thr, and SULT2B1b-Pro345Leu, showed more than 24% decrease compared with the wild-type (SULT2B1b-wt), while the other three (SULT2B1b-Pro69Ala, SULT2B1b-Thr73Met, and SULT2B1b-Arg274Gln) showed more dramatic reduction (up to 90%) compared with the wild-type enzyme.

To investigate the binding affinity of the allozymes with the co-substrate (PAPS), the K_m values for PAPS of SULT2B1b allozymes were determined with DHEA as substrate. Of all SULT2B1b allozymes tested, only SULT2B1b-Arg274Gln showed a significantly higher (with a 20-fold increase) K_m value compared to the wild-type SULT2B1b.

3.2. Characterization of the pregnenolone-sulfating activity of purified human SULT2B1b allozymes.

In addition to DHEA sulfation, previous studies have shown that SULT2B1b can also sulfate pregnenolone (Falany and Rohn-Glowacki, 2013). In an initial experiment, the pregnenolone-sulfating activity of SULT2B1b allozymes was examined using 10 μM of pregnenolone as substrate. Of the ten SULT2B1b allozymes analyzed, three (SULT2B1b-Gly72Val, SULT2B1b-Arg147His, and SULT2B1b-Gly276Val) showed no detectable sulfating activity. The other seven SULT2B1b allozymes exhibited differential and significantly lower sulfating activity toward pregnenolone (Fig. 3). Compared with SULT2B1b-wt, four of them (SULT2B1b-Asp191Asn, SULT2B1b-Arg230His, SULT2B1b-Ser244Thr, and SULT2B1b-Pro345Leu) displayed a more than 1.2-fold decrease in pregnenolone-sulfating activity, while two (SULT2B1b-Pro69Ala and SULT2B1b-Thr73Met) showed a much greater decrease (by more than 5-fold) in pregnenolone-sulfating activity. Notably, SULT2B1b-Arg274Gln displayed the lowest (being greater than 35-fold lower) pregnenolone-sulfating activity compared with the wild-type enzyme.

To analyze further the effect of genetic polymorphisms on the pregnenolone-sulfating activity of SULT2B1b allozymes, kinetic experiments were performed using varying concentrations of pregnenolone as substrates. As shown in Fig. 4, pregnenolone sulfation appeared to follow the Michaelis-Menten kinetics. Kinetic constants determined for the wild-type and SULT2B1b allozymes are compiled in Table 3. Of the seven SULT2B1b allozymes analyzed, SULT2B1b-Thr73Met and SULT2B1b-Arg274Gln displayed 1.6 and 2.8-fold increase in K_m value, respectively. All seven SULT2B1b allozymes examined showed a significant reduction in V_{max} compared with that of SULT2B1b-wt. SULT2B1b-Pro69Ala and SULT2B1b-Arg274Gln exhibited the greatest reduction (a more than 91% decrease) in the catalytic activity toward pregnenolone compared with SULT2B1b-wt. Of the other five SULT2B1b allozymes, three (SULT2B1b-Asp191Asn, SULT2B1b-Arg230His, and SULT2B1b-Pro345Leu) displayed a greater than 16% decrease in V_{max}, while two (SULT2B1b-Thr73Met and SULT2B1b-Ser244Thr) showed a more than 58% decrease in V_{max} compared with the wild-type. Based on these results, four (SULT2B1b-Pro69Ala, SULT2B1b-Thr73Met, SULT2B1b-Ser244Thr, and SULT2B1b-Arg274Gln) showed a significant reduction in the catalytic efficiency (k_{cat}/k_m).Three (SULT2B1b-Pro69Ala, SULT2B1b-Thr73Met, and SULT2B1b-Arg274Gln) showed a more than 79% decrease in the catalytic efficiency compared with the wild-type (SULT2B1b-wt), while SULT2B1b-Ser244Thr showed a smaller decrease (40%) in catalytic efficiency as compared with the

wild-type. K_m values for PAPS of the SULT2B1b allozymes in mediating the sulfation of pregnenolone were also determined. As shown in Table 3, of the seven SULT2B1b allozymes, two (SULT2B1b-Pro69Ala and SULT2B1b-Arg274Gln) showed higher (2.8 and 18-fold, respectively) K_m values, compared to SULT2B1b-wt.

3.3. Structure simulation and analysis of the single amino acid mutations.

Effects of the amino acid substitution on the interaction with pregnenolone and PAP were investigated using simulated structures of SULT2B1b allozymes with corresponding amino acid substitutions (Fig. 6). Among the nine substitutions, seven substitutions (Pro69Ala, Gly72Val, Thr73Met, Arg147His, Ser244Thr, Arg174Gln, Gly276Val) were found to affect the interaction with PAP. In particular, Thr73Met was found to have altered interaction with pregnenolone as well as PAP. In contrast, Asp191Asn and Arg230His appeared to have no direct effect on the interaction with pregnenolone and PAP. Further simulation analyses were performed to predict the binding energy of SULT2B1b allozymes with pregnenolone and PAPS (Fig. 7). The docking simulation analyses suggested that substitution of Arg274 and Gly276 to Gln and Val, respectively, reduced the binding affinity with PAPS. In addition, molecular dynamics simulations were carried out to gain insight into the dynamic effects of individual amino acid substitutions on the overall structure and interaction with substrates (Fig. 8). Representative structures of most clustered group in the total 1000 structures were aligned with that of the wild-type. Most of the SULT2B1b allozyme structures were found to be similar with that of the wild-type (data not shown). The representative conformations of Pro69Ala, Thr73Met, and Arg147His are shown in Fig. 8. Interestingly, conformation of segment-2 of Arg147His appeared clearly different, which suggested that substitution of Arg147 by His may affect the interaction with PAPS. Detail observations for the simulation analyses are further elaborated in the Discussion section.

4. Discussion

In humans, DHEA and its sulfate ester, DHEA-S, are known to be the most abundant steroids in circulation (Falany and Rohn-Glowacki, 2013). DHEA is synthesized and secreted mainly from the adrenal glands and, to a lower extent, in brain, gonads, and skin (Davis et al., 2011). DHEA has been proposed to be effective in reducing cardiovascular risk, alleviating insulin resistance, stimulating endothelial proliferation, and improving memory and cognitive function (Labrie, 2010; Traish et al., 2011). More importantly, DHEA is a major precursor for the biosynthesis of sex steroid hormones (Schiffer et al., 2018). In peripheral tissues, the adrenally secreted DHEA is taken up and converted to estrogens and androgens via a process known as intracrinology (Schiffer et al., 2018). SULT2B1b has been shown to be capable of sulfating specifically 3β-hydroxysteroids including cholesterol, DHEA, and pregnenolone, and is highly expressed in steroid hormone-responsive tissues such as prostate, breast, placenta, and endometrium (Falany et al., 2006). Genetic polymorphisms of *SULT2B1* have been correlated with the progression and proliferation of several different types of cancer including prostate cancer, esophageal squamous cell carcinoma, hepatocellular carcinoma, gastric cancer, and colorectal cancer (Hyland et al., 2013; Levesque et al., 2014; Mostaghel, 2013; Yang et al., 2013; Hu et al., 2015; Vickman et al., 2016; Chen et al., 2016). SULT2B1b mRNA has been detected in a number of cancerous

human tissues and cell lines including prostate adenocarcinoma cells, LNCaP prostate adenocarcinoma cells, as well as T47D and MCF-7 breast cancer cell lines (He and Falany, 2007). While the exact role of SULT2B1b in different type of cancers has not been fully elucidated, studies have suggested that its involvement in the sulfation of hydroxysteroids may limit their availability for the sex steroids biosynthesis and their capacity to bind to corresponding androgen receptors (He and Falany, 2007; Seo et al., 2013). Interestingly, the down-regulation of SULT2B1b in prostate cancer has been proposed to be the reason behind prostate cancer progression due to the lack of the protective effect of SULT2B1b in decreasing steroid hormone precursors like DHEA (He and Falany, 2007; Seo et al., 2013).

The current study was designed to investigate the functional relevance of *SULT2B1* cSNPs on the sulfating activity of the resulting SULT2B1b allozymes toward two major steroids precursors, DHEA and pregnenolone. Ten SULT2B1b allozymes, previously prepared via site-directed mutagenesis and bacterial expression using the pGEX gene fusion system (Alherz et al., 2018), were analyzed. (It is noted that while several of the *SULT2B1* cSNPs studied exhibit very low allele frequencies (cf. Table 1), one of them that causes an Arg274Gln substitution (SNP ID: rs762765702) has been linked to autosomal-recessive congenital ichthyosis, a genetic skin disorder (Heinz et al., 2017). The functional relevance of other *SULT2B1* cSNPs studied still awaits clarification from epidemiological studies.) As shown in the Results section, three of the ten allozymes (SULT2B1b-Gly72Val, SULT2B1b-Arg147His, and SULT2B1b-Gly276Val) showed no detectable activity with both DHEA and pregnenolone. The other seven allozymes (SULT2B1b-Pro69Ala, SULT2B1b-Thr73Met, SULT2B1b-Asp191Asn, SULT2B1b-Arg230His, SULT2B1b-Ser244Thr, SULT2B1b-Arg274Gln, and SULT2B1b-Pro345Leu) exhibited significant decrease in their sulfating activity toward DHEA and pregnenolone. It should be pointed out that the sulfating activity of SULT2B1b-Asp191Asn, SULT2B1b-Arg230His, and SULT2B1b-Pro345Leu in this study were noticeably lower than the sulfating activity of the same allozymes previously reported using DHEA as a substrate (Ji et al., 2007). It is possible that this discrepancy could have been due to the use of different enzyme preparation (purified recombinant enzymes vs. enzymes expressed in COS-1 cells (Ji et al., 2007)). Subsequent kinetic analysis showed that amino acid variations in the seven allozymes caused significant decrease in the catalytic activity and efficiency toward DHEA, whereas with pregnenolone, only four allozymes (SULT2B1b-Pro69Ala, SULT2B1b-Thr73Met, SULT2B1b-Ser244Thr, SULT2B1b-Arg274Gln, and SULT2B1b-Pro345Leu) showed significant decrease in the K_{cat}/K_m. These results indicate clearly that SULT2B1b cSNPs indeed have great effect on the enzyme function. It is interesting to note that the amino acid substitution in SULT2B1b-Pro345Leu occurs in a proline- and serine-rich carboxyl terminal region of the SULT2B1b molecule, which has been shown to be subjected to phosphorylation on serine residue(s) (He and Falany, 2006). Whether the phosphorylation status of the enzyme may affect the kinetic properties of SULT2B1b-Pro345Leu, as well as other SULT2B1b allozymes, will be an intriguing issue for future investigation.

The crystal structure of human SULT2B1b determined previously has revealed a number of structural elements that are critical to the functioning of the enzyme (Lee et al., 2003). In relation to the interaction with the co-substrate, PAPS, the elements include a 3’-phosphate-binding region, a 5’-phosphosulphate-binding (PSB) loop, and a PAP adenine-binding

region (Lee et al., 2003). The PSB loop is composed of the conserved amino acid sequence ^{67}TYPKSGT^{73}, of which the amino acid residues Lys70, Ser71, Gly72, and Thr73, as well as Thr74, are involved in binding the 5'-phosphate of PAPS. The amino acid residues Arg274, Lys275, Gly276, Arg147, and Ser155 are involved in binding the 3'-phosphate of PAPS. Moreover, Ser244, Tyr210, Trp75, and Phe246 are involved in the interaction with the adenine group of the PAPS molecule (Lee et al., 2003). It is noted that seven (SULT2B1b-Pro69Ala, SULT2B1b-Gly72Val, SULT2B1b-Thr73Met, SULT2B1b-Arg147His, SULT2B1b-Ser244Thr, SULT2B1b-Arg274Gln, and SULT2B1b-Gly276Val) of the ten SULT2B1b allozymes examined in this study contain amino acid variations in the PAP/PAPS-binding pocket (Fig. 5) (Lee et al., 2003). Of these seven SULT2B1b allozymes, the amino acid changes in three (SULT2B1b-Gly72Val, SULT2B1b-Arg147His, and SULT2B1b-Gly276Val) were found to exert the most drastic effects, leading to the complete loss of sulfating activity toward DHEA or pregnenolone. For SULT2B 1b-Gly72Val and SULT2B1b-Gly276Val, Gly72 and Gly276 in the wild-type enzyme have been proposed to form hydrogen-bonding with the O4P and O2P phosphate oxygens of the co-substrate, PAPS, respectively (Fig. 6). Replacement of these glycine residues, which have more conformational flexibility, with valine residues, which carry a bulkier side chain, may place more conformational restriction and interfere with the interaction, and thus the binding, with PAPS. Furthermore, the replacement of glycine with valine may affect the interaction of Gly276 and Phe246, leading to the alteration of the PAPS orientation and less effective catalysis. In the case of SULT2B1b-Arg147His, the substitution of the arginine residue with histidine may also affect the hydrogen-bonding to the oxygen atom of the 3'phosphate of the PAPS and thus the loss of the sulfating activity (Lee et al., 2003; Betts and Russell, 2003). These three substitutions, therefore, may directly affect the interaction with PAPS rather than with substrate. In contrast to the three SULT2B1b allozymes mentioned above, the amino acid changes in SULT2B1 b-Pro69Ala, SULT2B1 b-Thr73Met, SULT2B 1b-Ser244Thr, and SULT2B1b-Arg274Gln caused only differential decreases in the sulfating activity. Among them, SULT2B1b-Ser244Thr resulted with the smallest reductions (24% and 40%, respectively) in the catalytic efficiency toward DHEA and pregnenolone, compared with the wild-type enzyme. As shown in Fig. 6 (E), the substitution of serine with threonine may enable hydrogen bonding with the a14-helix, possibly affecting the loop-3 structure. These relatively small decreases in sulfating activity could have been due to the replacement of the serine residue with a highly similar amino acid residue, threonine, which also carries a hydroxyl group in the side chain (Betts and Russell, 2003). On the other hand, the amino acid changes in SULT2B1b-Pro69Ala, SULT2B1b-Thr73Met, and SULT2B1b-Arg274Gln caused more dramatic differences in the sulfating activity. For SULT2B1b-Pro69Ala, the substitution of proline, which has no direct interaction with PAPS in the wild-type enzyme, with alanine may affect the interaction between Pro69 and His125 and Phe186 that interact with the substrate, leading to less effective catalysis and substrate recognition. Conformational change of the substrate binding pocket in SULT2B1b-Pro69Ala may be the reason behind the dramatic decrease (with a more than 79% and 90%, respectively) in the catalytic efficiency toward both pregnenolone and DHEA and compared to the wild-type. In the case of SULT2B1b-Thr73Met, the decrease in the sulfating activity was due to the replacement of the threonine residue that carries a polar side chain with methionine that carries a hydrophobic side chain, which might have disrupted the hydrogen bonding with the

O4P oxygen atom of the 5'-phosphate of the PAPS (Lee et al., 2003; Betts and Russell, 2003). Moreover, the substitution of the threonine residue with methionine may affect the hydrogen bond formation between Thr73 and Thr67, Trp103, and His125, leading to less effective catalysis and substrate recognition. For SULT2B1b-Arg274Gln, the substitution of arginine with glutamine may alter the interaction between O3P and O1P oxygen atoms of the PAPS molecule with the positively charged nitrogen atom of arginine, as well as the interaction of the Arginine274 and both Leu217 and His158, causing a dramatic decrease in the sulfating activity toward DHEA or pregnenolone. Interestingly, SULT2B1b-Arg274Gln has recently been linked to autosomal-recessive congenital ichthyosis, a genetic skin disorder (Heinz et al., 2017). On the other hand, amino acid variations in SULT2B1b-Asp191Asn and SULT2B1b-Arg230His allozymes seem not to affect the interaction of the amino acid residues at these locations and the surrounding residues, which could be the reason for the smaller effect of the amino acid variation in theses allozymes compared to the wild-type SULT2B1b. Therefore, it appears that most of the allozymes that showed the decrease in sulfating activity may have altered interactions with PAPS due to substitutions of the corresponding residues. Docking simulation analyses of the docking of PAPS into the active site of SULT2B1b allozymes suggested the low binding affinity of Arg274Gln and Gly276Val with PAPS (Fig. 7). Substitution of those residues may affect the superposition of PAPS in the structure as predicted by docking simulation analysis (Fig 7B).The superpositions of PAPS docked into Arg274Gln and Gly276Val allozymes were also shifted in comparison with the superposition of PAPS docked into the wild-type enzyme. Other allozymes showed similar binding affinity with PAPS and superposition of PAPS docked into the active site of respective allozymes (Fig. 7A). Among allozymes tested in this study, the substitution of Pro63 and Thr73 with Ala and Met, respectively, may affect the interaction with substrate. It should be noted, however, that rotamer analysis of the substituted residues was performed by fixing the backbone structure. Potential effects of the substitutions on the overall structure and the interaction with substrate can not be ruled out. Molecular dynamic analysis was performed to further investigate the effect of the substitutions in the overall structure of SULT2B1b (Fig. 8). For Pro69Ala allozyme, superposition of pregnenolone differed from that of the wild-type enzyme (Fig. 8A). For Thr73Met allozyme, the location of His125 was slightly shifted toward substrate side (Fig. 8B). These two observations may be (one of) the reasons for the dramatic decrease in their catalytic activity. Interestingly, segment-2 conformation of Arg147His allozyme was highly differed from that of the wild-type, which may affect the stable binding of PAPS (Fig. 8C). It has been reported that PAPS binding shifts the conformation of the SULT molecule from a "closed state" to an "open state", leading to alteration in the substrate-binding profiles (Tibbs et al., 2015). In the case of SULT2A1, PAPS binding has been reported to restrict the docking of raloxifene to the active site, while having no effect on the docking of DHEA (Cook et al., 2012). Although the structure of SULT2B1b without PAP has not been resolved, PAPS binding, like with other SULTs, may change the conformation of SULT2B1b, leading to alteration in the binding of DHEA or pregnenolone. The structure of SULT2B1b in an "open state", if available, may provide the detailed effects of the substitution on the catalytic activity of SULT2B1b with DHEA, pregnenolone, or other substrates. It should be noted that SULT2B1b exhibits the extended N-terminus and C-terminus, whose structures remain to be resolved (Fuda et al., 2002, Lee et al., 2002).

Previous studies also showed that N-terminal sequence in SULTs may play an important role in the substrate entry (Fuda et al., 2002, Lee et al., 2002). Further structure analysis may eventually resolve the conformation of N- and C-terminal sequences in SULT2B1b.

To conclude, the current study demonstrated clearly the differential sulfating activity of human SULT2B1b allozymes toward DHEA and pregnenolone. Kinetic analysis revealed further the differences in substrate affinity and catalytic activity between different SULT2B1b allozymes. These results showed unambiguously the effect of human *SULT2B1* genetic polymorphisms on the enzymatic characteristics of the resulting SULT2B1b allozymes. Further studies are warranted in order to clarify the impact of *SULT2B1* genetic polymorphisms on steroid metabolism, as well as the risk for SULT2B1b-associated disorders such as colorectal cancer and autosomal-recessive congenital ichthyosis.

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Abbreviations:

DHEA	dehydroepiandrosterone
PAPS	3’-phosphoadenosine 5’-phosphosulfate
SULT	cytosolic sulfotransferase
TLC	thin-layer chromatography

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Highlights

- Ten SULT2B1b allozymes, corresponding to *SULT2B1b* single nucleotide polymorphisms selected based on the locations and potential importance of resulting amino acid substitutions, were bacterially expressed and affinity-purified.
- Pregnenolone- and dehydroepiandrosterone- sulfating activities of SULT2B1b allozymes, in comparison with the wild-type enzyme, were analyzed.
- Kinetic constants of SULT2B1b allozymes, as well as the wild-type enzyme, in mediating the sulfation of Pregnenolone or dehydroepiandrosterone were determined.

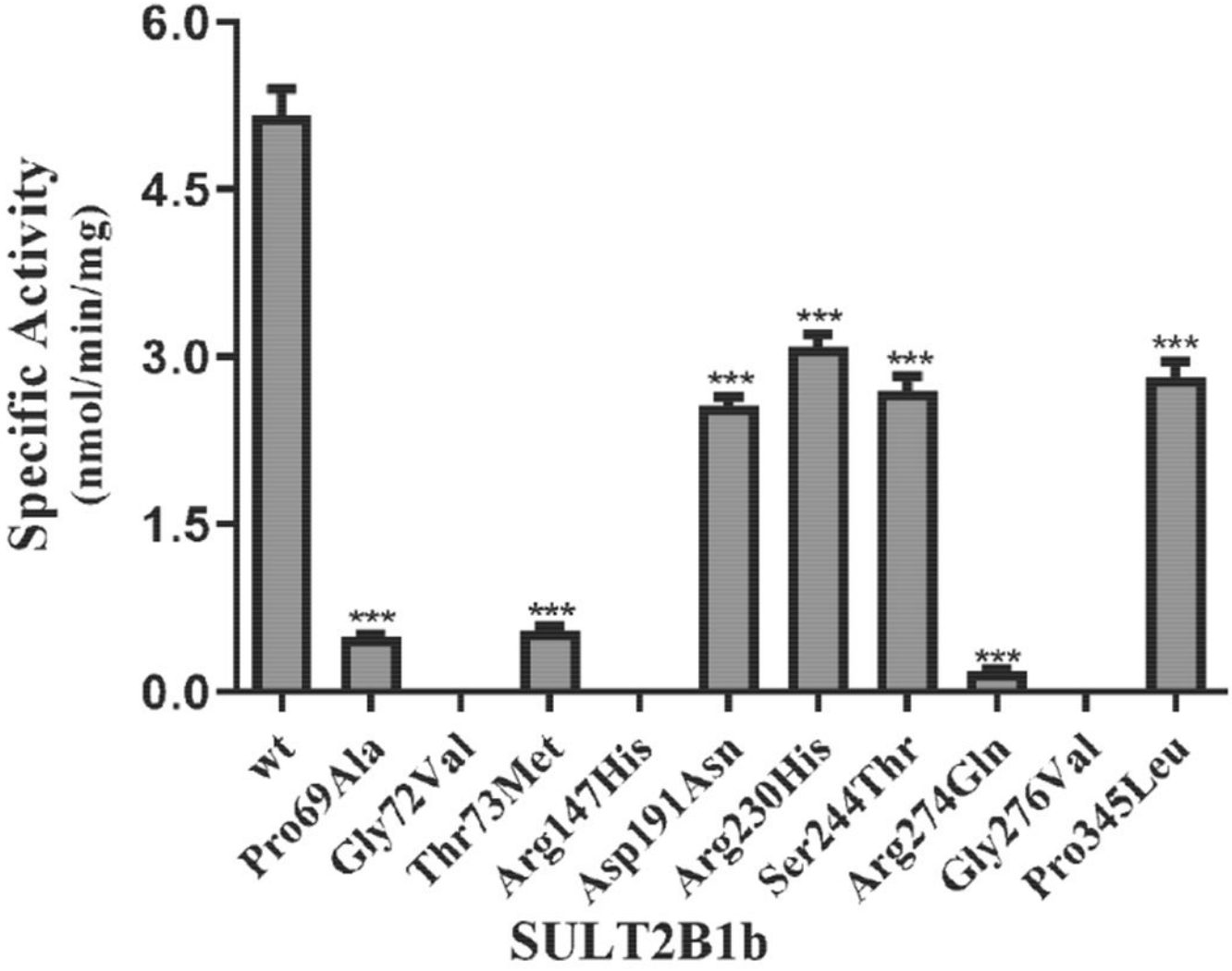


Fig. 1. Specific activity of the human SULT2B1b allozymes toward DHEA.

Concentration of DHEA used in the enzymatic assays was 50 μM. Data shown represent mean ± standard deviation derived from three separate determinations. One-way ANOVA was performed followed by Dunnett's post hoc analysis. *** Statistical significant $p<0.001$ from SULT2B1b-wt. Three allozymes, SULT2B1b-Gly72Val, SULT2B1b-Arg147His, and SULT2B1b-Gly276Val, showed no detectable activity.

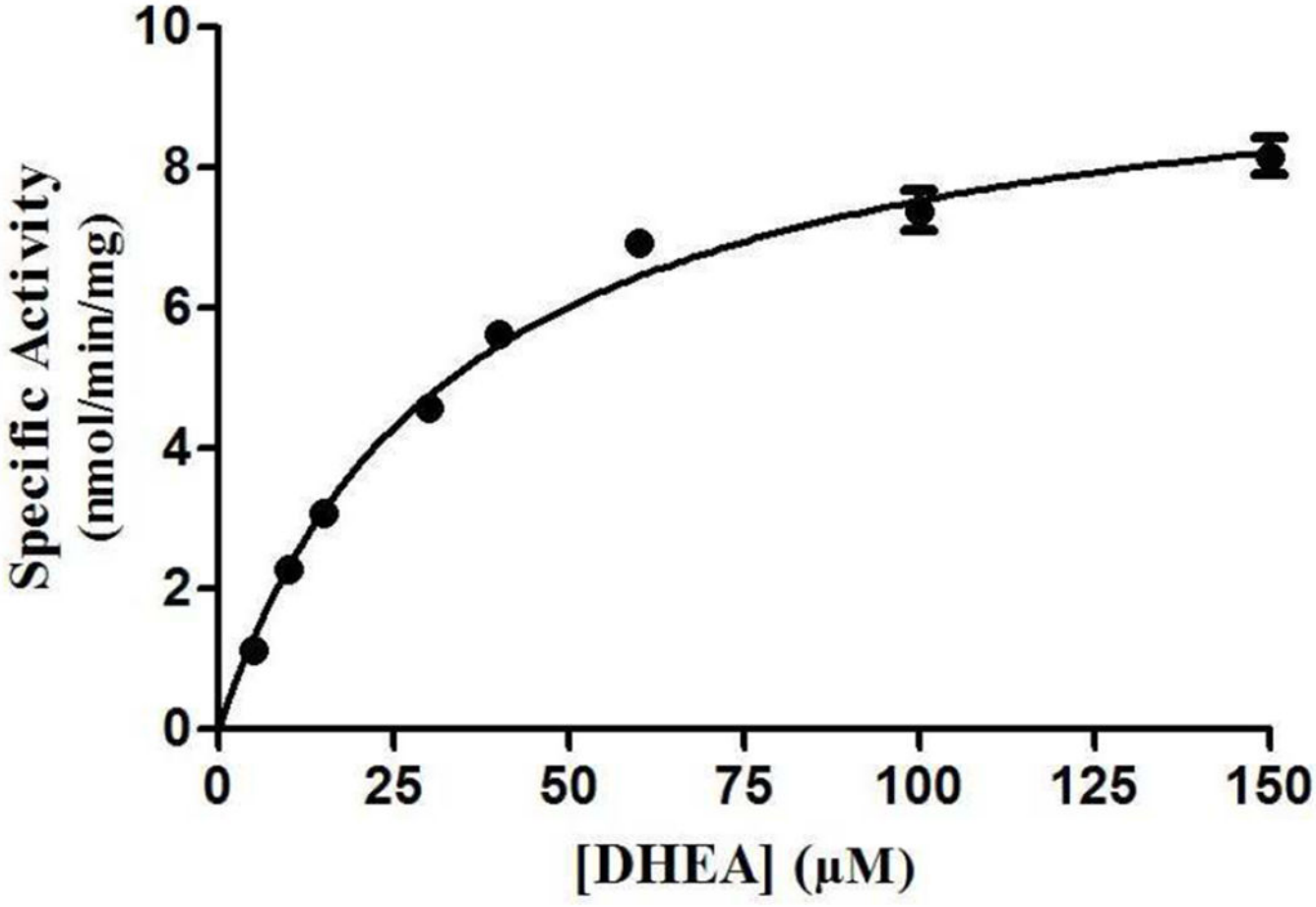


Fig. 2. Kinetic analysis of the sulfation of DHEA by human wild-type SULT2B1b.

The figure shows the saturation curve analysis of the sulfation of DHEA. The fitting curve was generated based on Michaelis-Menten kinetics. Data shown represent calculated mean ± standard deviation derived from three separate experiments.

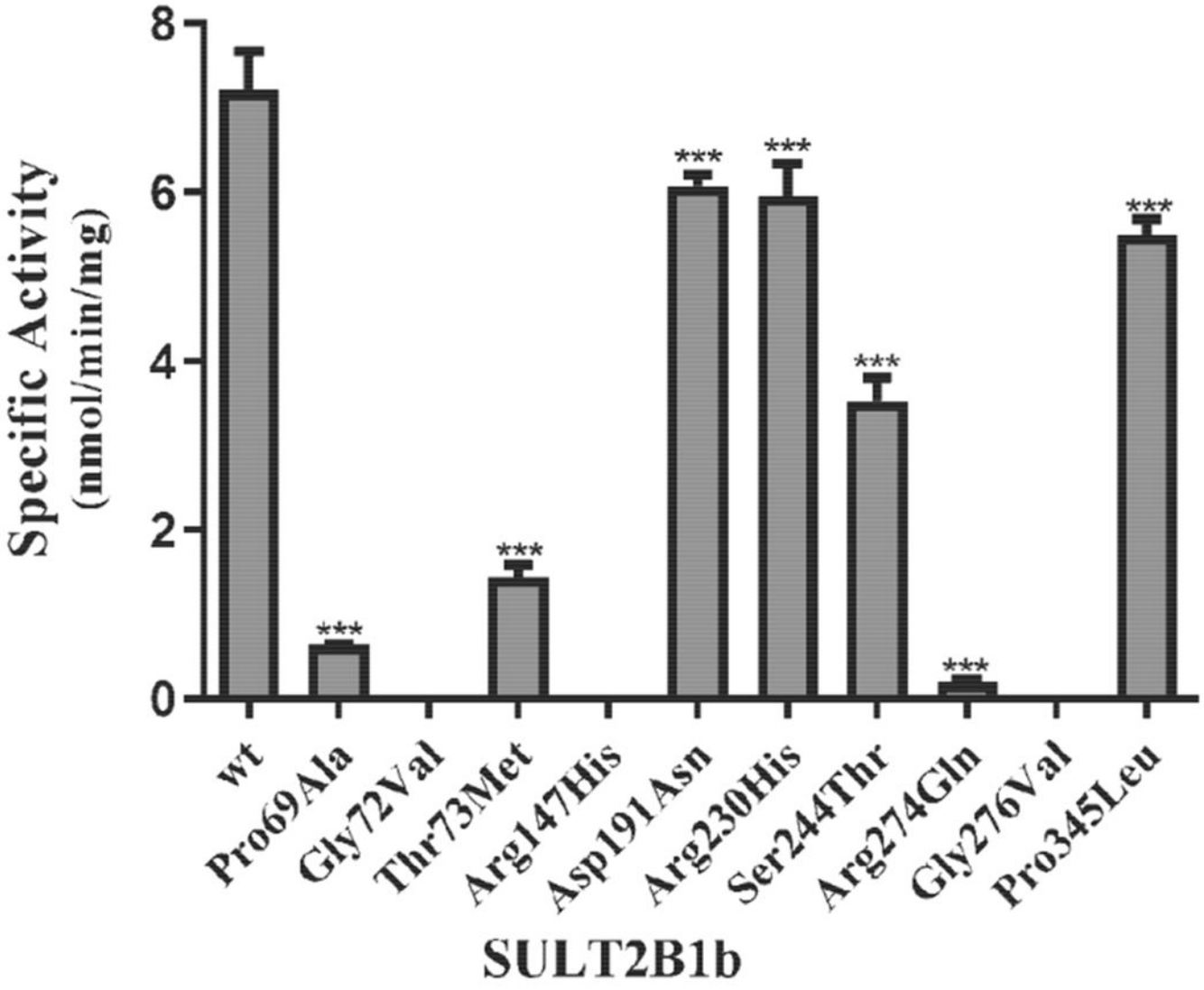


Fig. 3. Specific activity of the human SULT2B1b allozymes toward pregnenolone.

Concentration of pregnenolone used in the enzymatic assays was 10 μM. Data shown represent mean ± standard deviation derived from three separate determinations. One-way ANOVA was performed followed by Dunnett's post hoc analysis. *** Statistical significant $p<0.001$ from SULT2B1b-wt. Three allozymes, SULT2B1b-Gly72Val, SULT2B1b-Arg147His, and SULT2B1b-Gly276Val, showed no detectable activity.

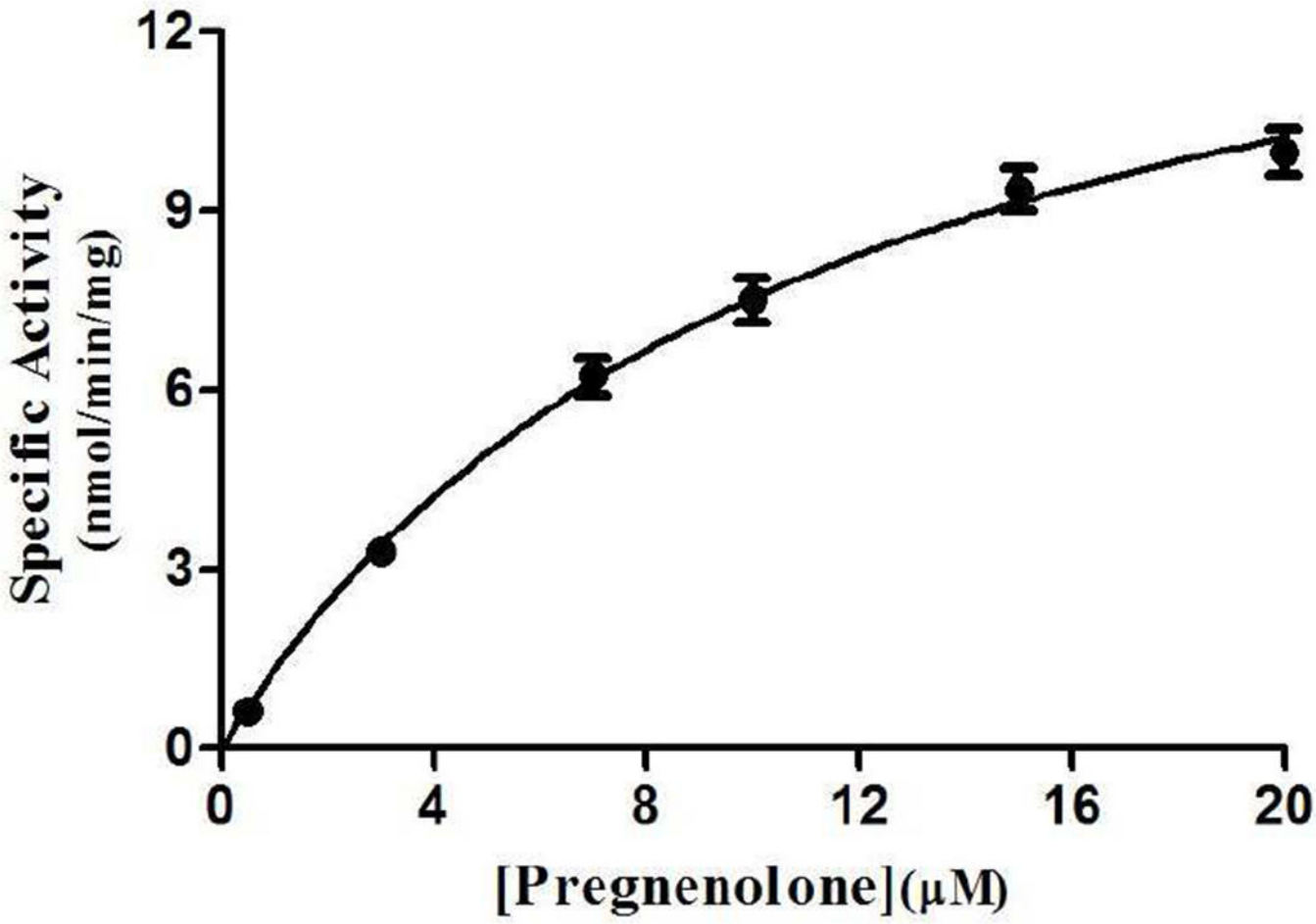
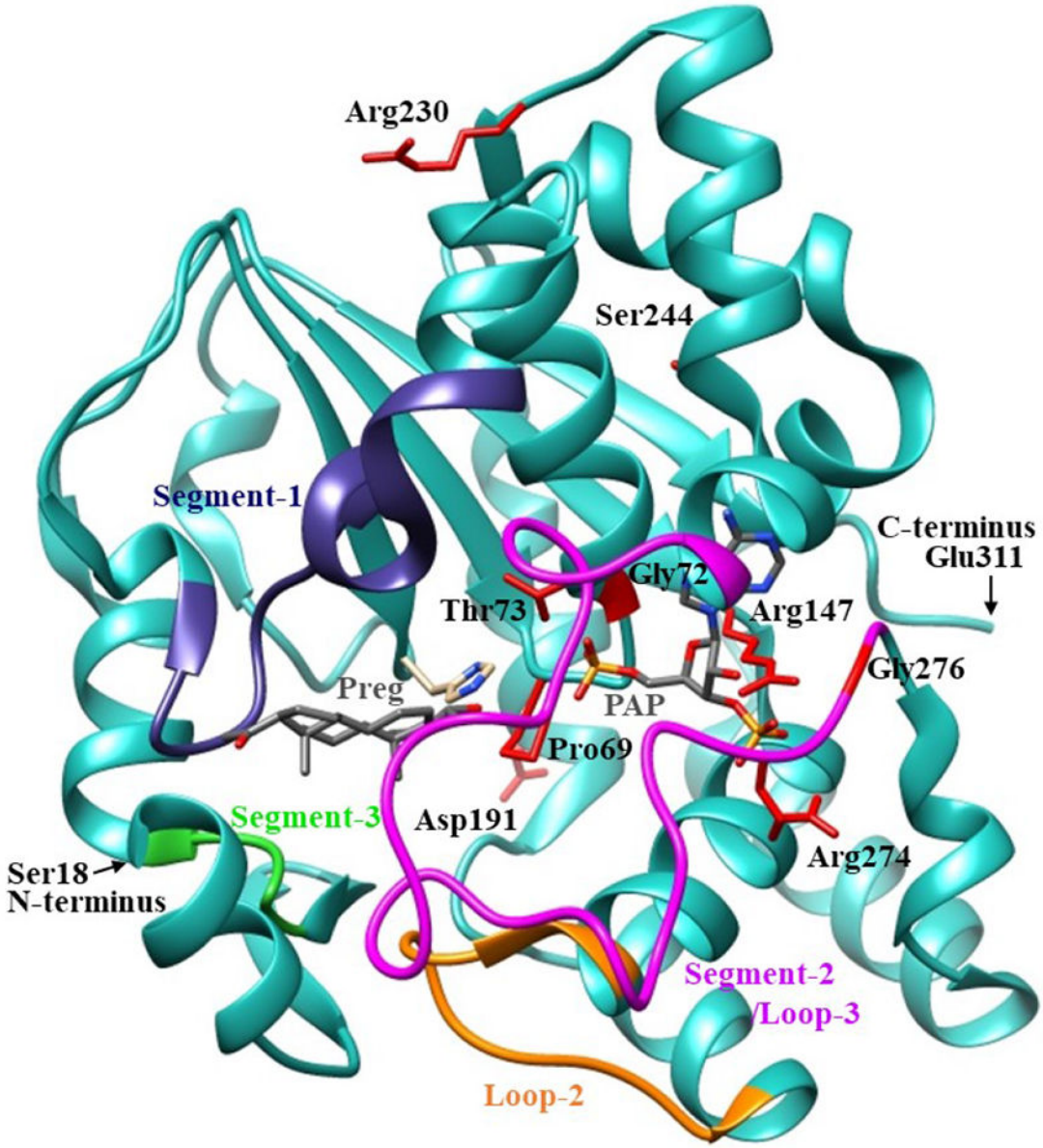


Fig. 4. Kinetic analysis of the sulfation of pregnenolone by human wild-type SULT2B1b.

The figure shows the saturation curve analysis of the sulfation of pregnenolone. The fitting curve was generated based on Michaelis-Menten kinetics. Data shown represent calculated mean ± standard deviation derived from three separate experiments.

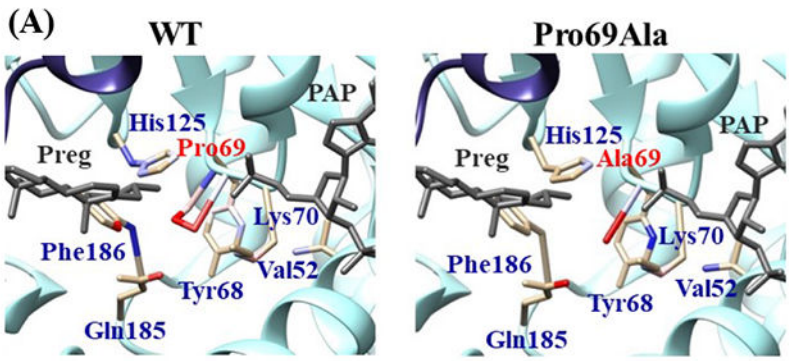


Note: Pro345 does not appear in this figure. The C-terminal region seems to be flexible so that full-size SULT2B1b can not be crystalized or resolved.

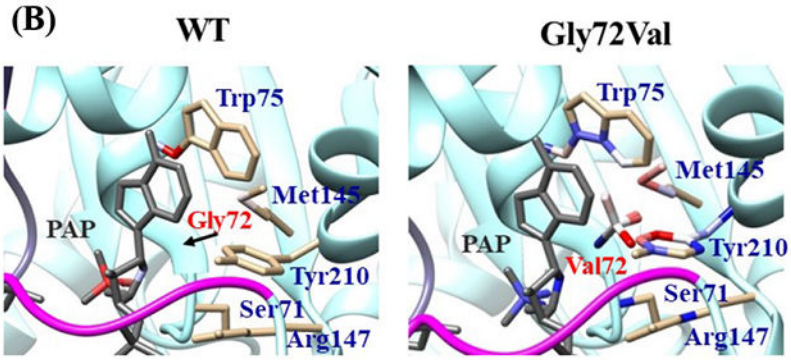
Fig. 5. Ribbon diagram of the structure of human SULT2B1b-pregnenolone-PAP complex showing the locations of amino acid residues involved in the SULT2B1 cSNPs.

The structure of SULT2B1b complexed with pregnenolone and PAP (Protein Data Bank code: 1Q20), containing the polypeptides from Ser18 (N-terminus) to Glu311 (C-terminus), was shown by ribbon diagram. Pregnenolone (Preg) and PAP molecules in the structure are shown by bond structures. Segement-1, Segment-2 (Loops 3), Segment-3, and loop-2 refer to Arg93-Gly109 (dark blue), Asn252-Ala271 (magenta color), Val41-Ser45 (Green), and Ile-Thr171 (orange color) previously reported to form a gate for substrate entry [Cook et al. 2012, Tibbs et al. 2015]. Side chains of the amino acid residues involved in the SULT2B1b

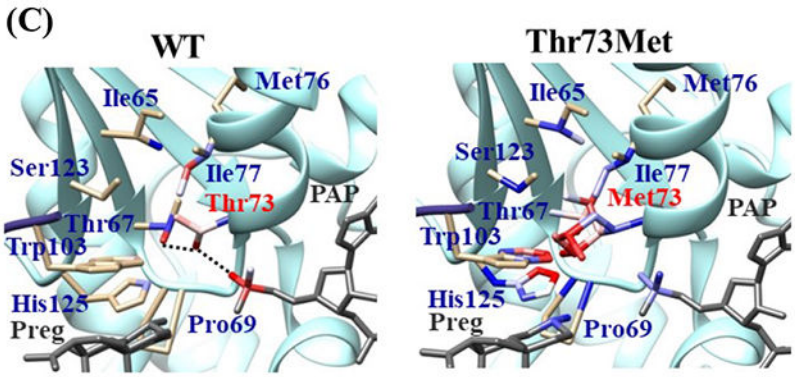
cSNPs, Pro69, Gly72, Thr73, Arg147, Asp191, Arg230, Ser244, Arg274, and Gly276 are indicated by bond structures (red color).



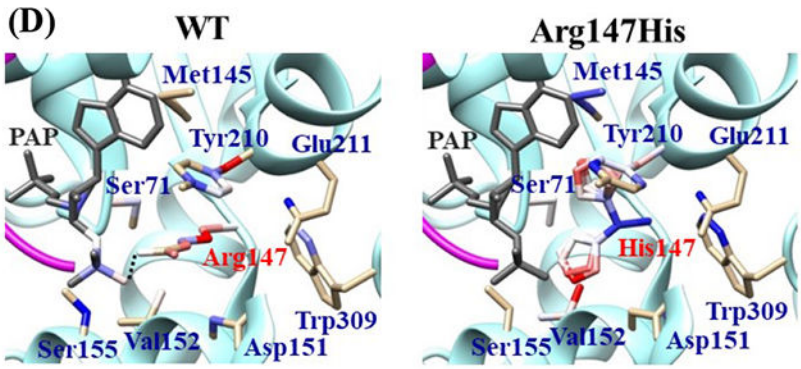
Note: Substitution of Ala for Pro69 may alter the interactions with His125 and Phe186, leading to less efficient catalysis and substrate-recognition.



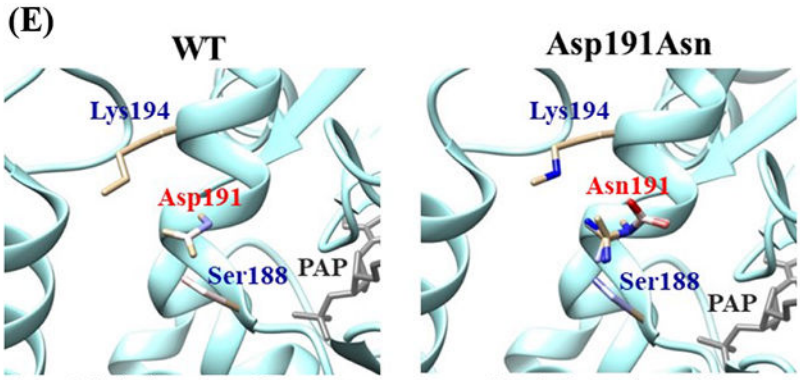
Note: Substitution of Val for Gly72 may alter the interaction with PAPS, leading to less efficient PAPS-recognition and catalysis.



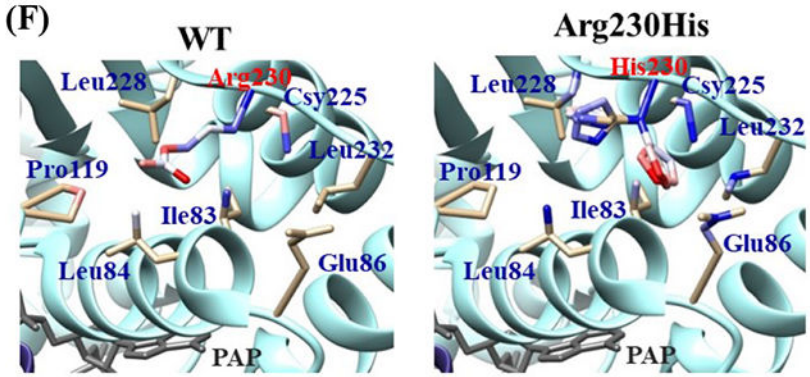
Note: Substitution of Met for Thr73 may alter the hydrogen-bonding with Thr67 and PAPS, as well as interactions with Trp103 and His125, leading to less efficient catalysis and substrate-recognition.



Note: Substitution of His for Arg147 may alter the hydrogen bond formation with PAPS, leading to less efficient PAPS-recognition and catalysis.



Note: Substitution of Asn for Asp191 seems not to alter the interactions with surrounding residues. The effect of the substitution, therefore, was not dramatic.



Note: Substitution of His for Arg230 seems not to alter the interactions with the surrounding residues. The effect of the substitution, therefore, was not dramatic.

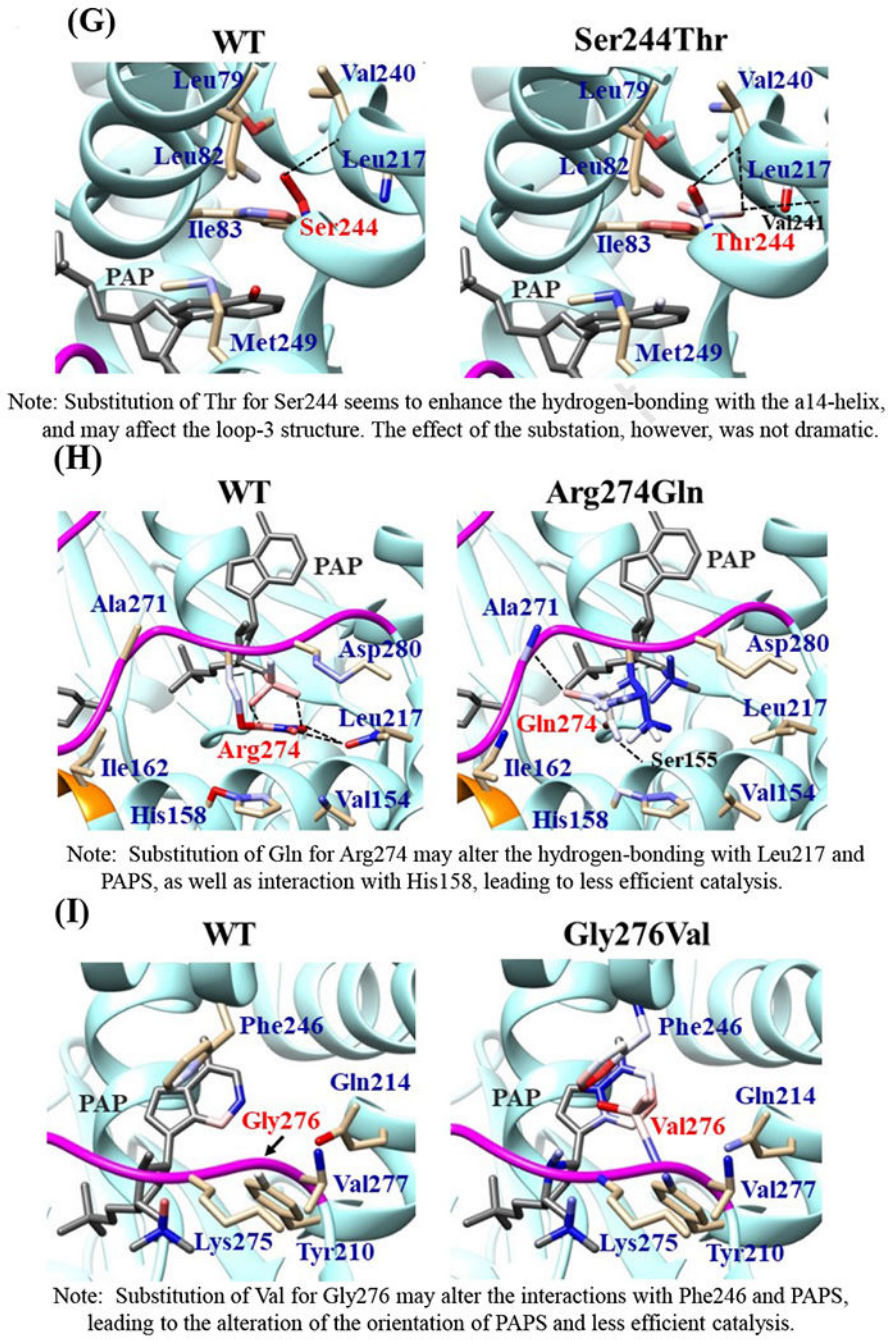


Fig. 6. Hydrophobic interaction and hydrogen bonding analyses of the SULT2B1b cSNPs.

Atoms interacting with Pro69 (A), Gly72 (B), Thr73 (C), Arg147 (D), Asp191 (E), Arg230 (F), Ser244 (G), Arg274 (H), and Gly276 (I) are colored by the blue-white-red gradient (left panels). Estimated interaction formed with Ala69 in Pro69Ala (A), Val72 in Gly72Val (B), Met73 in Thr73Met (C), His147 in Arg147His (D), Asn191 in Asp191Asn (E), His230 in Arg230His (F), Thr244 in Ser244Thr (G), and Gln274 in Arg274Gln (H), Val276 in Gly276Val (I) are colored by the blue-white-red gradient (right panels). Top five-ranked rotamers of each substituted residue are modeled using the Dunbrack backbone-dependent

rotamer library [Dunbrack 2002] and interaction was analyzed by Find Clashes/Contacts tool in a molecular modeling software, USCF Chimera software (Pettersen et al. 2004). Hydrogen bonds formed with Thr73 (C), Arg147 (D), Ser244 (E), and Arg274 (H) are shown by dashed lines (left panels). Estimated hydrogen bonds formed with Thr244 in Ser244Thr (G), Gln274 in Arg274Gln (H) are shown by dashed lines (right panels).

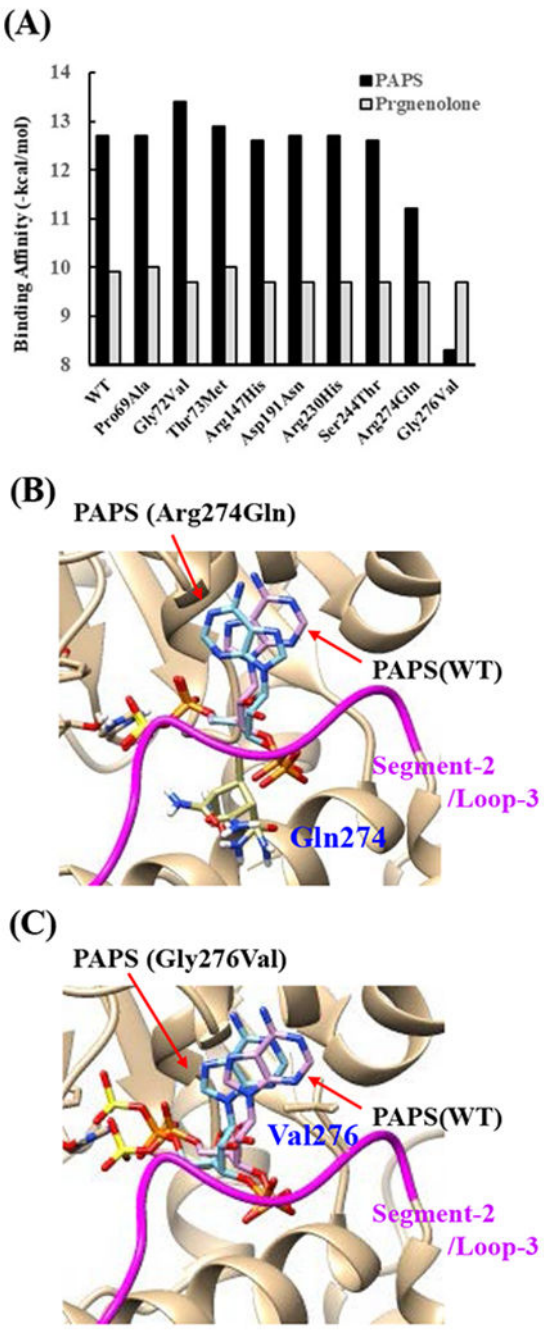


Fig. 7. Simulation analyses of the docking of PAPS and pregnenolone into the active site of SULT2B1b allozymes.

(A) Minimum binding energy of PAPS (black scale) and pregnenolone (grey scale) in the active site of SULT2B1 allozymes. Conformation of mutated amino acid residues of allozymes simulated using the Dunbrack backbone-dependent rotamer library was used for docking analyses as the template protein structures. (B) Stereo view of the PAPS docked into the active site of Arg274Gln. Superpositions of PAPS docked in Arg274Gln and wild-type are shown in blue and purple backbone, respectively. (C) Stereo view of the PAPS docked

into the active site of Gly276Val. Superpositions of PAPS docked in Gly276Val and wild-type are shown in blue and purple backbone, respectively.

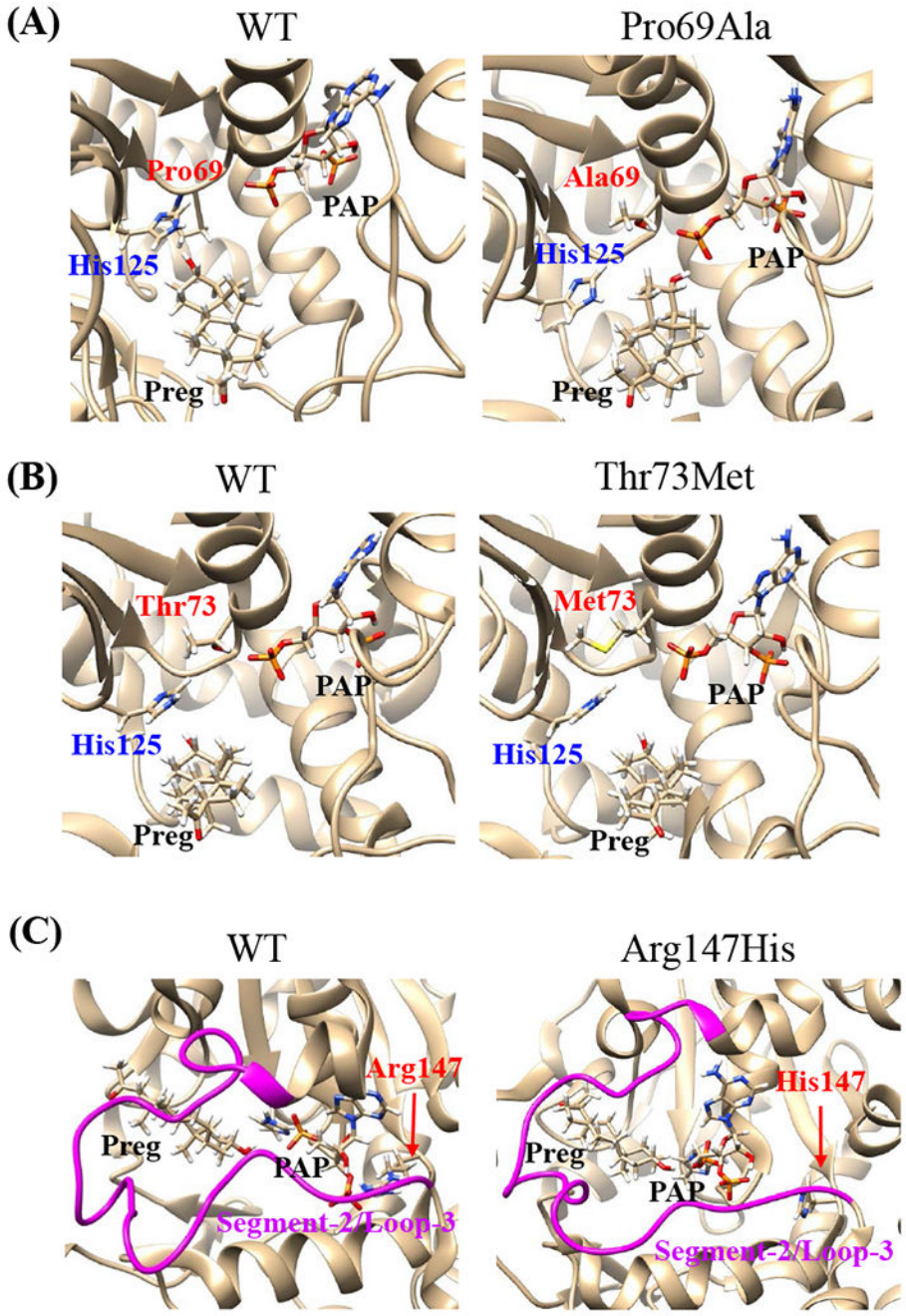


Fig. 8. Snapshots of molecular dynamics simulations of SULT2B1b cSNPs.

Snapshots of most clustered conformation of allozymes, Pro69Ala (A), Thr73Met (B), Arg147His (C), are shown in comparison with that of the wild-type. (A,B) Active site of SULT2B1b structures are shown with His125 (catalytic residue), Preg (pregnenolone), and PAP, as well as the original and substituted residues. (C) Conformations of segment-2/loop-3 structures (magenta color) are shown with Preg (pregnenolone) and PAP, as well as the original and substituted residues.

Table 1.

Allele frequencies of human SULT2B1b enzyme

SNP ID	SULT2B1b Allozyme	Allele Frequency	Reference
rs777924668	SULT2B1b-Pro69Ala	0.00001	NCBI
rs746398875	SULT2B1b-Gly72Val	0.00001	NCBI
rs527454384	SULT2B1b-Thr73Met	0.00002	NCBI
rs777140014	SULT2B1b-Arg147His	0.00001	NCBI
rs16982158	SULT2B1b-Asp191Asn	0.008 AA	Ji et al., 2007
rs16982169	SULT2B1b-Arg230His	0.008 AA	Ji et al., 2007
rs765224593	SULT2B1b-Ser244Thr	0.00006	NCBI
rs762765702	SULT2B1b-Arg274Gln	0.0001	NCBI
rs774212320	SULT2B1b-Gly276Val	0.0000	NCBI
rs17842463	SULT2B1b-Pro345Leu	0.025 CA	Ji et al., 2007

AA, African American population. CA, Caucasian American population.

Table 2.

Kinetic constants of the human SULT2B1b allozymes in catalyzing the sulfation of DHEA

Enzyme	DHEA				PAPS
	K_m (μM)	V_{max} (nmol/min/mg)	K_{cat}(s^{-1}) ×10^{-3}	K_{cat}/K_m (s^{-1} M^{-1})	K_m (μM)
SULT2B1b-wt	36 ± 3	8 ± 0.2	6 ± 0.1	167 ± 11	1.2 ± 0.2
SULT2B1b-Pro69Ala	62 ± 6	1 ± 0.0***	1 ± 0.0***	16 ± 1***	2.6 ± 0.6
SULT2B1b-Gly72Val	N.D.	N.D.	N.D.	N.D.	N.D.
SULT2B1b-Thr73Met	222 ± 24***	3 ± 01***	2 ± 0.1***	9 ± 1***	0.8 ± 0.1
SULT2B1b-Arg147His	N.D.	N.D.	N.D.	N.D.	N.D.
SULT2B1b-Asp191Asn	62 ± 8	7 ± 0.3***	5 ± 0.2***	81 ± 7***	0.5 ± 0.0
SULT2B1b-Arg230His	35 ± 4	5 ± 0.2***	3 ± 01***	86 ± 7***	1.0 ± 0.1
SULT2B1b-Ser244Thr	24 ± 4	4 ± 0.2***	3 ± 01***	127 ± 17***	0.3 ± 0.1
SULT2B1b-Arg274Gln	190 ± 25***	1 ± 0.0***	1 ± 0.0***	5 ± 1***	24.2 ± 4***
SULT2B1b-Gly276Val	N.D.	N.D.	N.D.	N.D.	N.D.
SULT2B 1b-Pro345Leu	38 ± 3	5 ± 0.3***	3 ± 0.2***	79 ± 1***	1.3 ± 0.1

Data shown represent mean ± SD derived from 3 independent experiments.

*Statistical significance from SULT2B1b-wt (***p-value<0.0001) using one-way ANOVA followed by Dunnett's post hoc analysis.

N.D. no detectable activity.

Table 3.

Kinetic constants of the human SULT2B1b allozymes in catalyzing the sulfation of pregnenolone

Enzyme	Pregnenolone				PAPS
	K_m (μM)	V_{max} (nmol/min/mg)	K_{cat} (s^{-1}) ×10^{-3}	K_{cat}/K_m (s^{-1} M^{-1})	K_m (μM)
SULT2B1b-wt	8 ± 1	12 ± 0.7	8 ± 0.5	1005 ± 64	1.3 ± 0.2
SULT2B 1b-Pro69Ala	5 ± 1	1 ± 0.0 ***	1 ± 0.0 ***	206 ± 26 ***	3.7 ± 0.5 **
SULT2B1b-Gly72Val	N.D.	N.D.	N.D.	N.D.	N.D.
SULT2B 1b-Thr73Met	13 ± 2 **	3 ± 0.2 ***	2 ± 0.1 ***	156 ± 16 ***	1.0 ± 0.1
SULT2B1b-Arg147His	N.D.	N.D.	N.D.	N.D.	N.D.
SULT2B 1b-Asp 191Asn	6 ± 1	8 ± 0.5 ***	6 ± 0.3 ***	1013 ± 100	0.7 ± 0.0
SULT2B 1b-Arg230His	7 ± 1	10 ± 0.5 ***	7 ± 0.3 **	1010 ± 102	1.3 ± 0.1
SULT2B 1b-Ser244Thr	5 ± 1	5 ± 0.7 ***	3 ± 0.5 ***	603 ± 21 ***	0.6 ± 0.1
SULT2B 1b-Arg274Gln	22 ± 2 ***	1 ± 0.1 ***	1 ± 0.1 ***	45 ± 4 ***	23.6 ± 2 ***
SULT2B1b-Gly276Val	N.D.	N.D.	N.D.	N.D.	N.D.
SULT2B 1b-Pro345Leu	8 ± 1	10 ± 0.6 ***	7 ± 0.5 **	877 ± 27	1.4 ± 0.1

Data shown represent mean ± SD derived from 3 independent experiments.

Statistical significance from SULT2B1b-wt (p-value<0.05, **p-value<0.001, ***p-value<0.0001) using one-way ANOVA followed by Dunnett's post hoc analysis.

N.D. no detectable activity.