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Structural rearrangement of SULT2A1: effects on dehydroepiandrosterone and raloxifene sulfation

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Abstract

Background—Human cytosolic sulfotransferase (SULT) 2A1 is a major hepatic isoform and sulfates hydroxyl groups in structurally diverse sterols and xenobiotics. SULT2A1 crystal structures resolved in the presence and absence of 3',5'-diphosphoadenosine (PAP) or dehydroepiandrosterone (DHEA) suggest a significant rearrangement of the peptide that forms the surface of the active site in the presence of PAP.

Materials and methods—Molecular modeling was used to examine the effects of the rearrangement in SULT2A1 associated with 3'-phosphoadenosine 5'-phosphosulfate (PAPS) binding on the binding of DHEA and raloxifene. The kinetics of DHEA and raloxifene sulfation was analyzed to investigate the effects of the rearrangement on SULT2A1 activity.

Results—Molecular models indicate that DHEA is able to bind to SULT2A1 in both conformations (open, without PAP; closed, with PAP) in a catalytic configuration, whereas raloxifene bound in a catalytic conformation only in the open structure. Raloxifene did not bind in the smaller, closed substrate binding pocket. Kinetic analysis of DHEA sulfation was consistent with a random Bi-Bi reaction mechanism, whereas raloxifene sulfation was more indicative of an ordered reaction mechanism with raloxifene binding first. Initial burst kinetics with DHEA yielded similar results after preincubation of SULT2A1 with DHEA or PAPS. Preincubation of SULT2A1 with raloxifene showed a burst of raloxifene sulfate formation with the addition of PAPS. In contrast, little raloxifene sulfate was formed if SULT2A1 was preincubated with PAPS and the reaction initiated with raloxifene.

Conclusions—The structural rearrangements in SULT2A1 caused by PAPS binding can alter the sulfation mechanism and kinetics of different substrates.

Keywords

dehydroepiandrosterone; drug metabolism; molecular modeling; raloxifene; sulfation; sulfotransferase; SULT2A1

Introduction

A major aspect of drug and xenobiotic metabolism is the broad range of compounds that are metabolized. Conjugation of drugs or compounds, such as steroids, with a sulfonate group generally results in a charged metabolite that lacks biological activity and is readily excreted in urine or bile (1). As drug metabolizing enzymes, several of the human cytosolic sulfotransferases (SULTs) have broad substrate reactivity and are capable of conjugating structurally diverse compounds. Our understanding of the structural basis for substrate recognition and catalysis in these enzymes is only beginning to be developed. The structures of most of the major human SULT isoforms have been resolved, although many were crystallized with bound 3',5'-diphosphoadenosine (PAP), a product of the reaction (2–6). The SULT structures determined without PAP generally lack definition of a major peptide that forms the surface of the PAPS and substrate binding pockets (2, 3, 7, 8). Only SULT2A1 has been determined with high resolution with an acceptor substrate bound (dehydroepiandrosterone, DHEA) and without bound PAP (9). Comparison of the structures of SULT2A1 with and without PAP bound indicates that a significant rearrangement in the active site occurs in response to PAPS binding (2, 5, 9). The volume of the active site is decreased approximately 60% upon PAP binding, suggesting significant changes in substrate accessibility and orientation that might be reflected in catalytic activity.

SULT2A1 is one of the most abundant SULTs in human liver and is considered a SULT2 family member because of its role in DHEA sulfation in the reticular layer of the adrenals (10) and bile acid sulfation in the liver (11). SULT2A1 has broad substrate reactivity and is capable of conjugating both 3 α - and 3 β -hydroxysteroids, both the 3- and 17-hydroxyls of β -estradiol and many sterol-related compounds and drugs (2, 12, 13). The effect of the structural rearrangement in SULT2A1 in the presence of PAPS on the binding and kinetics of the sulfation of known substrates has not been well investigated. Dehydroepiandrosterone (DHEA) and raloxifene (Figure 1) are both sulfated by SULT2A1 (13, 14). DHEA is a planar 3 β -hydroxysteroid and is considered the major physiological substrate of SULT2A1 (10). Raloxifene possesses a benzothiophene moiety and a phenolic ring as well as a long side-chain; SULT2A1 conjugates only the benzothiophene hydroxyl group (13). The differences in structure between DHEA and raloxifene suggest that there might be significant differences in binding characteristics and kinetics in the different conformations of the SULT2A1 active site. In this report, the binding of DHEA and raloxifene in the active site of SULT2A1 with and without PAPS bound were modeled and the predictions of the models were consistent with kinetics and reactivity.

Materials and methods

DHEA and raloxifene were purchased from Sigma Chemical Co. (St. Louis, MO, USA). 3'-Phosphoadenosine 5'-phosphosulfate (PAPS) was obtained from Dr. Sanford Singer (University of Dayton, Dayton, OH, USA). [³⁵S]PAPS (2.2Ci/mmol) and [1,2,6,7-³H(N)]DHEA (60 Ci/mmol) were purchased from Perkin-Elmer (Waltham, MA, USA). Silica gel thin-layer chromatography (TLC) plates (250 μ m) were obtained from Whatman Inc. (Clifton, NJ, USA). All other chemicals were of reagent grade and purchased from Fisher Scientific (Norcross, GA, USA). The Molecular Operating Environment programs were operated with a license provided by Chemical Computing Group (Montreal, Quebec, Canada).

SULT2A1 assays

SULT2A1 was expressed in *Escherichia coli* DH5 α using a pKK233-2 vector to generate the native form of the enzyme and purified using DEAE-Sepharose chromatography as described previously (12, 15). DHEA sulfation activity was assayed using [³H]DHEA and a

chloroform extraction procedure to separate [^3H]DHEA and [^3H]DHEA-sulfate as described previously (14). Raloxifene sulfation was assayed using [^{35}S]PAPS as described by Falany and Falany (13). Briefly, raloxifene and [^{35}S]PAPS at the desired concentrations in 50 mM Tris pH 7.4 with 5 mM MgCl_2 were warmed to 37°C for 2 min prior to initiation of the reaction with the addition 0.7 μg of SULT2A1 to a final volume of 125 μL . Reactions were terminated by chloroform extraction, then 50 μL of the aqueous phase was spotted onto a TLC plate. The TLC plates were developed in methylene chloride:methanol:ammonium hydroxide (85:15:5), followed by identification of raloxifene-[^{35}S]sulfate by autoradiography. The raloxifene-[^{35}S]sulfate bands were scraped into scintillation fluid and quantified by scintillation spectroscopy.

Molecular modeling

Analysis of protein structure, ligand docking experiments, calculation of binding free energy (BFE) and similar structural manipulations were carried out using the MOE programs. MOE contains an integrated package of programs for molecular and protein modeling, ligand interactions, bioinformatics and cheminformatics in scientific vector language. The PDB files for DHEA and raloxifene were generated using LigX (16). PDB file 1J99 was used for the structure of SULT2A1 resolved in the presence of DHEA with a resolution of 1.99 angstrom ($\text{\AA}$) (9). PDB file 1EFH was used for the structure of SULT2A1 determined with PAP bound at a resolution of 2.4 $\text{\AA}$ (6). The SULT2A1 and ligand PDB files were protonated using Protonate 3D (17, 18), then Triangle Matcher (19, 20) was used to orient the ligands in the active site based on charge groups and spatial fit. Triangle Matcher performs a random walk with the ligand in the active site to define the optimal binding orientations. The orientation is scored by BFE using both London dG (21) and Affinity (22) that are designed to function at physiological pH values and salt concentrations. Binding orientations were then refined using FlexX (23). FlexX functions as a Monte Carlo program to generate 10^6 orientations for each initial placement established by Triangle Matcher by fixing a point around which to vary the orientation. The orientations are then scored using London dG and Affinity to define the lowest energy conformation (BFE).

Results

Comparison of the available SULT2A1 structures indicates that PAP binding results in a structural rearrangement in the active site. Figure 2 shows a comparison of the SULT2A1 structures resolved in the presence of PAP (PDB 1EFH) and DHEA (PDB 1J99). The structural similarity is high throughout most of the sequence; however, there is an alteration in the structures in the region encoded by amino acids 228–253. This peptide sequence forms the exterior surface of both the PAPS and substrate binding sites. There is a significant inward displacement of this peptide in the PAP-containing structure resulting in a decrease in the volume of the binding pocket from approximately $2775\text{\AA}^3$ to $1140\text{\AA}^3$. Comparison of the two structures suggests that PAPS binding results in rearrangement in the active site of SULT2A1 resulting in a significant decrease in the volume of the binding pocket in comparison to the structure with only DHEA bound. The structure of SULT2A1 without either PAP or substrate bound has not been reported, and thus any rearrangements occurring with DHEA binding alone cannot be discerned.

The structural rearrangement of the active site of SULT2A1 observed in the presence of PAP suggests that there might be a differential binding of substrates in the enzyme in the “open” conformation compared with the “closed” conformation. To investigate this possibility, DHEA, a classical SULT2A1 substrate (14), and raloxifene, a non-steroidal substrate (13, 24), were docked into the active site of SULT2A1 in the open and closed conformations. Figure 3A and B show that DHEA can readily orient in the substrate binding pocket in open and closed structures, respectively. In both models, the $3\beta\text{-OH}$ of DHEA is in

a catalytically appropriate position relative to the active site His99 and PAPS. The BFEs of DHEA in the open and closed structures were 14.4 kJ and 13.9 kJ, respectively. These models suggest that binding and sulfation of DHEA are not significantly altered in the open and closed conformations of SULT2A1.

Figure 3C shows the lowest BFE configuration of raloxifene in the open conformation of SULT2A1. The BFE is 10.1 kJ and the benzothiophene hydroxyl that is sulfated by SULT2A1 (13) is oriented close to the active site His99 residue in an apparent catalytically competent position. It should be noted that Leu233 in the flexible peptide is positioned approximately 11 Å from raloxifene in this orientation. Figure 3D displays the position of raloxifene with the lowest BFE (8.1 kJ) in the closed conformation of SULT2A1. The side chain of raloxifene is situated in the active site close to PAPS and His99. Both the benzothiophene and phenolic hydroxyls are positioned outside the binding pocket indicating that sulfation apparently does not occur. Leu233 has moved approximately 11 Å relative to PAPS in the closed conformation compared with the open conformation and blocks the entry of the ring systems of raloxifene into the binding pocket. The modeling studies suggest that DHEA can bind in a catalytically efficient orientation in both the open and closed conformations of SULT2A1 although with different BFEs. In contrast, to be efficiently sulfated, raloxifene needs to bind to the open conformation of SULT2A1 prior to PAPS binding. If SULT2A1 binds PAPS prior to raloxifene, the rearrangement of the enzyme decreases the size of the substrate binding pocket and prevents raloxifene from binding in an active configuration.

To investigate the possibility that the order of substrate binding might affect the ability of substrates to be conjugated by SULT2A1, the kinetics of DHEA and raloxifene sulfation were evaluated. The kinetic properties of SULT2A1 were determined using assays containing four different concentrations of PAPS and four different concentrations of either DHEA or raloxifene. Concentrations of DHEA were selected to avoid significant substrate inhibition (14). Substrate inhibition has not been observed during the sulfation of raloxifene with SULT2A1 (24). Figure 4A shows the $1/V$ vs. $1/\text{DHEA}$ plots, and Figure 4B displays the replots of the slopes and intercepts for the DHEA sulfation assays. The K_m value for PAPS was 0.2 μM and the K_m value for DHEA was 1.9 μM . A V_{max} value of 17.8 nmol/min/mg was calculated for DHEA sulfation. The lines in Figure 4A intercept on the axis suggesting there is little synergy between DHEA and PAPS binding.

Figure 4C shows the $1/V$ vs. $1/\text{raloxifene}$ plots, and Figure 4D shows the associated replots of the slopes and intercepts obtained from the raloxifene sulfation assays. The K_m value for PAPS in raloxifene sulfation was 0.9 μM and the K_m value for raloxifene was 1.3 μM . A V_{max} value of 4.3 nmol/min/mg was calculated for raloxifene sulfation. The lines in Figure 4C intercepted in quadrant 1 between the x - and y -axes indicative of a synergistic interaction occurring between PAPS binding and raloxifene binding.

The modeling and kinetic studies indicate that DHEA and raloxifene might be binding in sufficiently different orientations to the open and closed forms of SULT2A1 so as to alter the ability of the enzyme to sulfate the two substrates. To investigate whether DHEA and raloxifene bind differentially in the open and closed conformations of SULT2A1, the enzyme was preincubated in either substrate or PAPS, and then the reactions were initiated with the addition of the other substrate. The reaction was sampled every 10 s to detect the initial formation of sulfated products. Figure 5A shows that preincubation of SULT2A1 in either PAPS or DHEA did not result in a significantly different rate of DHEA sulfation in the first 2 min of the reaction. In contrast, Figure 5B demonstrates that preincubation of SULT2A1 with raloxifene allowed for the initial rapid generation of raloxifene sulfate, whereas preincubation in PAPS resulted in a 30-delay in the detection of raloxifene-sulfate

formation. The delay in the formation of raloxifene sulfate is consistent with the inability of SULT2A1 in the closed conformation to bind raloxifene in a catalytically competent orientation.

Figure 5 indicates that DHEA might be efficiently sulfated when added to the reaction either before or after PAPS; however, if PAPS is added before raloxifene then raloxifene sulfation is very slow. To investigate whether the order of addition of raloxifene and PAPS to SULT2A1 affected the initial formation of raloxifene sulfate, the effect of the amount of SULT2A1 on the initial rates of raloxifene sulfation was investigated. Figure 6 shows that with increasing amounts of SULT2A1 in the reaction there was a concentration-dependent increase in raloxifene sulfation when the enzyme was preincubated with raloxifene and the reactions started with PAPS. The amount of raloxifene sulfate formed in the initial reactions when raloxifene is preincubated with SULT2A1 is equivalent to the amounts of enzyme present. When SULT2A1 was preincubated with PAPS and the reactions started with raloxifene, there was a 20-fold lower amount of raloxifene sulfate formed in the first 10–30 s.

Discussion

The cytosolic SULTs as a family have a role in the sulfonation of a wide range of compounds consistent with their function in drug and xenobiotic metabolism. Understanding the structural aspects of substrate recognition in the SULT family and the kinetic properties related to the conjugation of structurally different compounds is important in appreciating the physiological functions of sulfation. The structures of most of the human cytosolic SULT isoforms have been reported (2–9). Among these structures, relatively complete high resolution structures of an isoform in the presence of substrate without PAP have been reported only for SULT2A1 (9). For SULT isoforms, other than SULT2A1, with structures resolved without PAP/PAPS, the regions analogous to aa 228–253 in SULT2A1 are not well defined (2). This region forms the outer surface of both the PAPS and substrate binding pockets (Figure 2). The lack of resolution in these SULT structures is apparently related to the flexibility of this region.

In the structures of SULT2A1 with PAP bound compared with those with only DHEA bound, the 228–253 peptide rearranges so that the volume of the binding pockets is approximately 60% smaller. PAPS binding rather than DHEA binding seems to be the stimulus for the rearrangement. DHEA is a classical SULT2A1 substrate and, as a planar 3 β -hydroxysteroid, is capable of fitting into the substrate binding site in both the closed and open configurations with similar BFEs. Kinetic analysis of DHEA sulfation indicates that little observed synergy exists between DHEA and PAPS binding. These data are consistent with a previous report that SULT2A1 (6) demonstrates random order kinetics with DHEA (14). DHEA, therefore, represents those compounds, including planar hydroxysteroids, that bind efficiently in both the open and closed conformations of SULT2A1.

Structurally, raloxifene is a larger compound than DHEA and possesses a long side chain (Figure 1). Also, the benzothiophene and phenolic ring systems are also positioned perpendicular to each other in the energy-minimized structure predicted with LigX indicating that it is larger in volume than DHEA. Raloxifene is sulfonated only at the benzothiophene hydroxyl by SULT2A1 (13). In the open SULT2A1 configuration, raloxifene bound with a BFE of 10.1 kJ with the benzothiophene hydroxyl in an appropriate site for sulfonyl transfer (Figure 3C). However, in SULT2A1 with PAP/PAPS bound, the repositioning of the 223–253 peptide resulted in Leu 233 and 234 blocking the access of raloxifene to the active site. The model with the lowest BFE had the inert side chain adjacent to PAPS and the active site His99 (Figure 3D). The raloxifene ring structures including the

benzothiophene hydroxyl were not positioned inside the binding pocket. The 5-fold increased K_m value for PAPS determined with raloxifene sulfation compared with DHEA sulfation might result from the interference of the PAPS driven rearrangement when raloxifene is bound in the substrate binding pocket. The structure of SULT2A1 with raloxifene bound has not been reported.

The structural changes caused by PAPS binding alter the sulfation of raloxifene significantly compared with DHEA because it inhibits the binding of raloxifene to the enzyme in a catalytically competent orientation. When DHEA and raloxifene sulfation assays were initiated with either PAPS or substrate, no difference was observed in DHEA sulfation (Figure 5A). This is in accordance with DHEA binding to both the open and closed SULT2A1 conformations with similar efficiencies. However, preincubation in PAPS resulted in a 20–30 s lag in the detection of raloxifene sulfate formation (Figure 5B). This lag apparently results from the inability of raloxifene to efficiently bind the closed SULT2A1•PAPS complex. It is possible that a small percentage of the SULT2A1•PAPS complex is in the open conformation at any given time and can bind raloxifene. Alternatively, the bound PAPS might need to disassociate from the enzyme to generate the open conformation of the enzyme and allow efficient raloxifene binding. If the enzyme is preloaded with raloxifene, then an initial rapid production of raloxifene sulfate occurs. The rate of raloxifene sulfation slows from the initial burst to a constant rate suggesting that a significant portion of the initially formed raloxifene sulfate might be slowly released from the enzyme.

Although it is a substrate for SULT2A1, raloxifene binds most efficiently when PAPS is not bound to the enzyme and the SULT2A1•PAPS complex is not in the closed conformation. When increasing amounts of SULT2A1 were incubated with PAPS there was little initial raloxifene sulfate formed when raloxifene was added. This suggests that the majority of the SULT2A1•PAPS complex is in the closed conformation and does not bind raloxifene efficiently. The rapid formation of raloxifene sulfate in the initial rate reactions when SULT2A1 was preincubated with raloxifene was dependent on the amount of SULT2A1 in the reaction (Figure 6). This is consistent with a rapid sulfation of the bound raloxifene and a slower release of the raloxifene sulfate from the enzyme. In both situations, the reaction reaches a similar steady-state reaction rate after approximately 2 min.

Both ordered and random reaction mechanisms have been reported for the cytosolic SULTs (25–28). As described with DHEA and raloxifene, the analysis of the reaction mechanism might also need to take into account the structure of the substrate and the effect of PAPS binding on substrate recognition and the reaction rate. PAPS-stimulated rearrangement of the active site might also have other effects on activity. Lu et al. (8) recently reported that DHEA is capable of binding in the open configuration of SULT2A1 in an alternative orientation and that this might be associated with the substrate inhibition reported with DHEA sulfation by SULT2A1. The substrate binding pocket of SULT2A1 in the open configuration is large enough to allow binding of at least two DHEA molecules. The closed conformation of SULT2A1 limits binding of DHEA to a single molecule in a catalytic orientation. The observation that the DHEA sulfation rate becomes constant at high DHEA concentrations during substrate inhibition might reflect equilibrium between the open configuration with DHEA bound in an inhibitory orientation and the active closed configuration of SULT2A1 binding DHEA in the active orientation. Similarly, SULT1E1 and SULT1A1 that also show substrate inhibition during the conjugation of β -estradiol and p-nitrophenol, respectively, have been reported to bind two substrate molecules (27, 29). In the modeling studies, binding of DHEA in the alternative orientation in the open conformation was not observed with a BFE similar to the active binding orientation. Models

with two raloxifene molecules in the open conformation SULT2A1 active site were not predicted owing to the size of the molecule.

The examination of the binding and sulfation of DHEA and raloxifene by SULT2A1 indicates that significant kinetic differences and reaction rates might be observed in the conjugation of substrates by a single isoform. Understanding the changes in the enzyme active site and interactions with substrates of different structure will provide valuable insights for the prediction of sulfation in drug metabolism. Also, the changes in the SULTs caused by PAPS binding might significantly alter the observed reaction rates depending on how the reactions are performed. This will need to be considered when reaction rates and substrate reactivities are compared using different assay procedures. If the PAPS-stimulated rearrangement of the substrate binding pocket is a common feature in the SULT family, then attention must be directed to the binding of substrates to both isoform conformations.

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Figure 1.
Structures of DHEA and raloxifene.

Figure 2.

Comparison of the PAPS and substrate binding sites of SULT2A1 in structures resolved in the presence of PAP or DHEA. The blue strand depicts the structure of SULT2A1 resolved in the presence of DHEA [PDB 1J99; (9)]. The green strand shows the rearrangement of the 228–253 aa peptide in the structure of SULT2A1 with PAP bound [PDB 1EFH; (6)]. The region in both structures encompassing aa 228–253 is in ribbon. The regions of the structure with PAP bound that were similar in both structures were removed for clarity. The closed structure was resolved with PAP; however, for the modeling studies sulfonate was added to PAP in the crystal structure of SULT2A1 and the structure energy minimized to convert the PAP to PAPS. DHEA is depicted in red, PAPS in yellow, and the active site His99 in sand.

Figure 3.

Modeling of DHEA and raloxifene in the open and closed conformations of the SULT2A1 active site.

The open structure of SULT2A1 (PDB 1J99) and the closed structure (PDB 1EFH) were used in the docking studies. The closed structure was resolved in the presence of PAP; however, for the modeling studies sulfonate was added to PAP in the crystal structure of SULT2A1 and the structure energy minimized to convert the PAP to PAPS. The DHEA and raloxifene structures were initially positioned in the models based on the crystal structures and the models energy minimized using the MOE programs as described in the materials and methods section. Panel (A) shows the modeling of DHEA in the open structure of SULT2A1 and panel (B) shows the position in the closed structure. Panel (C) depicts raloxifene in the open structure of SULT2A1 and panel (D) shows raloxifene in the closed structure.

Figure 4.

Bisubstrate kinetics of DHEA and raloxifene sulfation with SULT2A1. SULT2A1 was expressed in *E. coli* and purified by DEAE-chromatography (12).

Reactions contained 0.16 μM SULT2A1 and PAPS concentrations from 0.5 to 10 μM , DHEA concentrations 0.5 to 2.0 μM or raloxifene concentrations 0.5 to 10 μM . The reactions without SULT2A1 were warmed to 37°C for 2 min and the reactions started with the addition of SULT2A1. The reactions were run for 10 min, stopped with the addition of chloroform and the sulfated products quantitated as described in the materials and methods section. Panels (A) and (B) show the plots of $1/v$ vs. $1/[\text{DHEA}]$ and the replots of the slopes or intercepts vs. $1/[\text{PAPS}]$, respectively. Panels (C) and (D) show the plots of $1/v$ vs. $1/[\text{raloxifene}]$ and the replots of the slopes or intercepts vs. $1/[\text{PAPS}]$, respectively. Each point represents the average of two reactions.

Figure 5.

Effect of pretreatment of SULT2A1 with PAPS, DHEA, or raloxifene on the initial rate of sulfate formation.

SULT2A1 was pretreated at 37°C with either PAPS (5 μ M) or either DHEA or raloxifene (2 μ M) and the reaction started with addition of the other substrate. Reactions were terminated by chloroform extraction at the times indicated. Each point presents the mean $\pm$ SD of three reactions.

Figure 6.

Effect of increasing SULT2A1 concentrations in the initial rate reactions of raloxifene sulfation.

SULT2A1 (4–16 pmol) was pretreated at 37°C with either PAPS (5 μ M) or raloxifene (2 μ M) and the reaction started with addition of the other substrate. Reactions were performed with 4 pmol (gray), 8 pmol (white), or 16 pmol (black) of SULT2A1 in a 100- μ L volume. The substrate added first is listed and the reaction started with addition of the other substrate. Reactions were terminated by chloroform extraction at the times indicated. Each bar represents the mean $\pm$ SD (n=3) of the amount of sulfated product formed.