Substrate Gating Confers Steroid Specificity to Estrogen Sulfotransferase*

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Estrogen sulfotransferase (EST) exhibits a high substrate specificity and catalytic efficiency toward estrogens such as estradiol (E2) but insignificant ability to sulfate hydroxysteroids such as dehydroepiandrosterone (DHEA). To provide the structural basis for this estrogen specificity, we mutated amino acid residues that constitute the substrate-binding site of EST. Among these mutants, only Tyr-81 decreased E2 and increased DHEA sulfotransferase activities. Substitution for Tyr-81 by smaller hydrophobic residues increased $K_m(E2)$ for E2 activity, whereas the $k_{cat}(E2)$ remained relatively constant. The Y81L mutant exhibited the same DHEA activity as wild-type hydroxysteroid sulfotransferase, for which $K_m(DHEA)$ remained relatively constant, and $k_{cat}(DHEA)$ was markedly increased. The side chain of Tyr-81 is directed at the A-ring of the E2 molecule in the substrate-binding pocket of EST, constituting a steric gate with Phe-142 sandwiching E2 from the opposite side. The present mutagenesis study indicates that the 3$\beta$-hydroxyl group of the DHEA molecule is excluded from the catalytic site of EST through steric hindrance of Tyr-81 with the C-19 methyl group of DHEA. Thus, this stricture-like gating caused by steric hindrance appears to be a structural principle for conferring estrogen specificity to EST.

Steroid hormones are sulfated by enzymes called cytosolic sulfotransferases that transfer sulfuryl groups to the substrates from the ubiquitous cofactor 3$\beta$-phosphoadenosine 5$\beta$-phosphosulfate (PAPS).1 Steroid sulfates are stored as inactive hormones that can be reactivated by steroid sulfatases, providing a mechanism for regulating endocrine homeostasis (1). Thus, as an alternative to aromatase inhibitors, the steroid sulfation/desulfation system is currently considered as a drug target for hormone-sensitive diseases such as certain types of breast cancer (2–5). Steroid sulfotransferases are subgrouped based on their substrate preferences, estrogen sulfotransferase (EST) and hydroxysteroid sulfotransferase (HST). Characteristically, EST displays a far greater specificity and efficiency to sulfate E2 than HST does to sulfate DHEA. The underlying structural principle for EST to manifest such high estrogen preferences remains a question of major interest.

In the last 10 years, more than 30 cytosolic sulfotransferase cDNAs have been cloned from bacteria, plant, and mammals including humans (6). The amino acid sequence comparisons of these sulfotransferases suggest a conserved sequence motif for the putative PAPS-binding site (6, 7). An empirical method has been introduced to identify residues that are involved in catalysis and/or in determining substrate specificity for sulfotransferases (8, 9). A bacterially expressed EST that was originally cloned from a mouse testis cDNA library (10) sulfates preferentially E2 (11). The crystal structures of murine EST complexed with inactive cofactor 3$\beta$-phosphoadenosine 5$\beta$-phosphate and E2 or vanadate have been solved (12, 13). These structures have revealed the PAPS-binding motif that is conserved in all cytosolic sulfotransferases. The transition state model mimicked by the EST-PAP-vanadate structure suggests that sulfotransferase reaction proceeds in an in-line sulfuryl transfer. This structure-based mechanism is consistent with a Random Bi Bi mechanism proposed previously based on enzyme kinetic analyses (14, 15). Moreover, these structural motifs and reaction mechanisms are also conserved in a group of Golgi membrane sulfotransferases that sulfate high molecular weight substrates such as carbohydrates, polysaccharides, and proteins (16–18). Thus, EST structures have provided an excellent basis to investigate the underlying principles that determine the substrate specificity of EST and other sulfotransferases.

For the present study, we have employed the structural information for EST to select residues for mutagenesis. EST transfers the 5$\beta$-sulfate of PAPS to the phenolic hydroxyl at 3-position of E2. The E2 molecule is accommodated horizontally in the cylindrical hydrophobic pocket, in which the hydroxyl group of E2 is inserted deep into the active site, whereas the 17$\beta$-hydroxyl group is directed outward near the pocket entrance. Vertical sectioning of the pocket has revealed a gate-like structure that positions the E2 molecule. Key residues in this region were mutated in order to examine whether they act as a gate in determining the substrate specificity of EST. EST and its mutants were expressed in Escherichia coli cells, purified, and used to determine selectively their $K_m$ and $k_{cat}$ values using E2, DHEA, and androstenediol as substrates. We herein describe experimental considerations that lead us to propose that tyrosine at position 81 is the key residue that confers substrate specificity of EST for E2 over DHEA.

EXPERIMENTAL PROCEDURES

Materials—[1,2,6,7-3H]Dehydroepiandrosterone (60 Ci/mmol), [2,4,6,7-3H]Estradiol (72 Ci/mmol), [7-3H]Pregnenolone (25 Ci/mmol), and [1,2-3H]Androstenediol (42 Ci/mmol) were purchased from NEN Life Science Products. AD was obtained from Steraloids, and E2, DHEA, and PREG were obtained from Sigma.

Site-directed Mutagenesis and Bacterial Expression of the Mutated
FIG. 1. Substrate binding pocket of EST displaying residues that have been mutated in this study. Residues in cyan belong to section I; residues in yellow belong to section II, and those in magenta correspond to section III. Sections I, II, and III are labeled based on residues that surround the region D-, B/C-, and A-rings of E2, respectively. This figure was created using Molscript (24).

Enzymes—Site-directed mutagenesis, expression, and purification were performed as described previously (13). Briefly, a QuickChange kit (Stratagene) was used to introduce mutations into EST sequence in pGEX-4T3 plasmid by polymerase chain reaction, and mutations were verified by DNA sequencing. Mutated enzymes were expressed as fusion proteins with glutathione S-transferase, applied on glutathione-Sepharose (Amersham Pharmacia Biotech) and subsequently eluted by thrombin cleavage (Sigma).

Sulfotransferase Assay and Kinetic Analysis—Sulfotransferase activity was assayed as described previously (10). The reaction mixture (200 μl of 100 mM Tris-HCl buffer, pH 8.0, and 100 μM PAPS) was extracted with 2 volumes of dichloromethane, and an aliquot of the aqueous phase was used for scintillation counting. Km and kcat values were calculated by nonlinear regression analysis using either a constant amount of radioactive substrate (in total concentrations of 3.5, 7.0, 13.9, 17.8, 25.6, 41.3, 72.5, 135, 260, and 510 nM) or constant ratio of labeled to unlabeled ligand (in total concentrations of 0.25, 0.5, 1.0, 2.0, 4.0, and 8 μM). For cases where sulfotransferase activity was inhibited by a high concentration of substrate, the data were processed using a model previously published to determine Km and kcat values (15). Protein concentration was determined spectrophotometrically using an A280 nm,1 mg/ml of 1.6 for EST and 2.2 for HST. Apparent molecular masses of 35,000 and 30,000 were used to calculate kcat values for EST and HST, respectively.

RESULTS AND DISCUSSION

Substrate-binding Site of EST—EST binds the hydrophobic substrate E2 tightly within the van der Waals surface of the hydrophobic substrate binding pocket (12). Fig. 1 depicts residues constituting the surface of the substrate pocket. Arg-23, Asn-86, Ser-148, and Met-247 are positioned below the D-ring of the E2 molecule. This group of residues forms the entrance to the pocket, with the side chain of Asn-86 hydrogen-bonded to the 17β-hydroxyl group of the section I group (Fig. 1). On section II, Phe-24, Cys-84, Ile-146, and Tyr-149 reside near the B/C-rings of the E2 molecule, whereas the side chains of Phe-24 and Cys-84 are directed toward the C-18 methyl group. Residues Tyr-81, Phe-142, and Tyr-240 are located around the A-ring of the E2 molecule on section III, with the side chains of Tyr-81 and Phe-142 forming the narrowest channel of the pocket (Fig. 1). Many of these residues were mutated to examine their role in determining the substrate specificity of EST.

Screening of Mutants—Mutated ESTs were expressed, purified, and their sulfotransferase activities determined using E2 and DHEA as substrates (Table I). Mutations of residues on section I affected E2 sulfotransferase activity only slightly. Even the N86A mutant that abolishes a hydrogen bond of Asn-86 to the 17β-hydroxyl group essentially retained wild-type EST activity. Mutation of Ser-148 to Ala displayed the most noticeable decrease in activity (5-fold). None of these mutant enzymes increased DHEA sulfotransferase activity. Because of its close proximity to the C-18 methyl group, Phe-24 on section II was mutated to a smaller amino acid Ala, whereas Cys-84 was mutated to a larger amino acid to fill the space between its side chain and the C-18 methyl group. These mutants (F24A, C84F, and C84Y), however, exhibited substrate specificity similar to that of wild-type EST. Mutations of the other residues in section II (Ile-146 and Tyr-149 that sandwich the C-ring from the opposite side of the pocket) displayed as high E2 sulfotransferase activity and as low DHEA sulfotransferase activity as did wild-type EST. The most profound increase of DHEA activity occurred with a Tyr-81 mutant in section I; the Y81A mutant exhibited approximately 8-fold higher DHEA sulfotransferase activity but decreased E2 sulfotransferase activity by 3-fold (Table II). On the other hand, the mutation of Phe-142 to Leu (F142L) decreased E2 activity 30-fold but did not confer activity toward DHEA. In addition, mutations of tyrosine at position 240 did not affect the activities of wild-type EST.

Kinetic Analysis of Tyr-81 and Phe-142 Mutants—Since Y81L was the only mutant that significantly increased DHEA sulfotransferase activity, further mutational analysis was conducted with Y81L, Y81F, and Y81A. First, we determined Km and kcat values of wild-type EST for E2 and DHEA sulfotransferase activities (Table II). General Km(E2) and kcat(E2) values were on the order of 10 nM and 10 s⁻¹ x 10⁸ M⁻¹ s⁻¹, respectively, whereas those for DHEA were the 1 μM and 1 s⁻¹ x 10⁸ M⁻¹ s⁻¹, respectively. Decreasing the side chain size by substituting Tyr-81 with Phe, Leu, and Ala, respectively, increased the Km(E2) values up to 10-fold. The kcat(E2) values for these mutants, on the other hand, remained relatively constant, although Y81L decreased kcat(E2) about 2.5-fold. Removing the hydroxyl group from Tyr-81 by substituting it with Phe resulted in more than 10-fold higher kcat(DHEA) for EST with Km(DHEA) only 2.5-fold lower. Placing Leu at position 81 (Y81L) resulted in the high kcat(DHEA) and Km(DHEA) values similar to those observed with Y81F. The kcat(DHEA) of Y81A was 35-fold higher than that of wild-type EST, with both having similar Km(DHEA) values. These results are consistent with the hypothesis that as the size of residue 81 becomes smaller, EST acquires higher DHEA and lower E2 sulfotransferase activities.

The highest DHEA sulfotransferase activity, however, is achieved with the Y81L mutant. Moreover, kcat is the primary factor for altering DHEA sulfotransferase activity, whereas Km defines mainly E2 sulfotransferase activity. The kcat(DHEA) and Km(DHEA) values of Y81L were practically identical to those of wild-type HST. As a result, the catalytic power (Km(DHEA)/kcat(DHEA) ratio) of EST to sulfate DHEA can be strengthened to...
levels equal to that for HST by a single amino acid mutation at position 81.

In the x-ray crystal structure of EST complexed with E2 and PAP (12), Phe-142 and Tyr-81 form a narrow channel to the substrate pocket that sandwiches the E2 molecule. Although the mutation of Phe-142 to Leu did not increase the DHEA sulfotransferase activity of EST, it decreased profoundly E2 sulfotransferase activity (Table I). To examine further the role of Phe-142 in determining the substrate specificity of EST, we constructed an additional Phe-142 mutant and also a double mutant in which both Tyr-81 and Phe-142 were mutated simultaneously. F142L exhibited a 40-fold higher $K_m$(E2) for E2 sulfotransferase activity than wild-type EST, with the same $k_{cat}$(E2) value. Both $K_m$(DHEA) and $k_{cat}$(DHEA) of F142L for DHEA sulfotransferase activity remained at levels similar to those of wild-type EST. Finally, we mutated Phe-142 to Ala (F142A) to make the side chain size smaller. This mutation resulted in a 100-fold decrease of the catalytic power ($k_{cat}$(E2)/$K_m$(E2)) of EST to sulfate E2, caused primarily by a marked increase of $K_m$(E2). F142A abolished completely DHEA sulfotransferase activity. As expected, the F142L/Y81L double mutant showed little catalytic power for either E2 or DHEA sulfotransferase activity. In the double mutant, the change in $K_m$ for E2 sulfotransferase activity was determined by Leu-142, whereas the alteration of $k_{cat}$ was dictated by Leu-81. Similarly, Leu-81 and Leu-142 defined the $k_{cat}$ and $K_m$ values for DHEA sulfotransferase activity, respectively. These observations for the F142L/Y81L mutant are consistent with the manner in which $K_m$ and $k_{cat}$ were defined in the corresponding single mutants.

**Androstenediol Sulfotransferase Activity**—One of the main structural differences of DHEA from E2 is the presence of the C-19 methyl group. We might infer that the presence of the C-19 methyl group may prevent DHEA from becoming an efficient substrate of EST. For testing this inference, we used steroid androstenediol (AD), which contains the C-19 methyl group as a substrate. AD differs from DHEA by only the hydroxyl group at the C-17 position. As expected, wild-type EST exhibited little ability to sulfate AD, whereas wild-type HST sulfated AD efficiently (Table III). The Y81L mutant of EST exhibited markedly increased AD sulfotransferase activity, with a 4-fold lower $K_m$ and a 5-fold higher $k_{cat}$ for AD sulfotransferase activity compared with wild-type EST. Based on the $k_{cat}/K_m$ ratio, the Y81L mutation elevated the AD sulfotransferase activity to 30% of the level observed in wild-type HST. The F142L mutant had virtually no AD activity; the $K_m$ and $k_{cat}$ values were practically identical to wild-type EST. In addition to AD, pregnenolone (PREG) was also sulfated by the Y81L mutant but not by the wild-type and F142L enzymes (data not shown). PREG is another steroid having the C-19 methyl group. Thus, these results provided further evidence that EST utilizes steric hindrance by the C-19 methyl group of steroids with Tyr-81 to confer substrate specificity.

### Table II

| $K_m$(E2) | $k_{cat}$(E2) | $K_m$(DHEA) | $k_{cat}$(DHEA) | $K_m$(DHEA)/$k_{cat}$(DHEA) |
|-----------|--------------|-------------|-----------------|-----------------------------|
| EST wild  | 25 ± 1       | 30 ± 22     | 1234 ± 967      | 4569 ± 75                   |
| Y81L*     | 92 ± 40      | 8 ± 1       | 104 ± 59        | 957 ± 30                    |
| Y81L      | 235 ± 19     | 18 ± 3      | 77 ± 5          | 3026 ± 639                  |
| F142L     | 812 ± 197    | 39 ± 21     | 46 ± 15         | 3127 ± 639                  |
| F142A     | 1200 ± 404   | 15 ± 3      | 12 ± 1          | ND                          |
| Y81L/F142L| 735 ± 0      | 1 ± 0       | 1 ± 0           | 1073 ± 99                   |
| HST wild  | 2000 ± 163   | 5 ± 3       | 2 ± 2           | 993 ± 391                   |

*S* These values were calculated using an substrate inhibition-applying model (15).

*ND,* not determined.

### Table III

| $K_m$(AD) | $k_{cat}$(AD) | $k_{cat}$(AD)/$K_m$(AD) |
|-----------|--------------|-------------------------|
| EST wild  | 3224 ± 1091  | 0.9 ± 0.1               |
| Y81L      | 868 ± 80     | 4.9 ± 1.0               |
| F142L     | 2900 ± 211   | 1.8 ± 0.4               |
| HST wild  | 191 ± 61     | 4.3 ± 1.4               |

General Discussion—Residues Tyr-81 and Phe-142 form a stricture-like gate that dictates the binding of steroids in the substrate pocket of EST. Superposition of DHEA with E2 in the substrate binding pocket shows that the C-19 methyl group of DHEA molecule is within 2.29 Å of C-e2 of Tyr-81 (Fig. 2). Based on a characteristic alteration of $K_m$(E2) with mutagenesis of Tyr-81 and Phe-142, the gate appears to determine the binding affinity for E2 to EST. Placing a smaller residue at position 81 not only decreases E2 sulfotransferase activity but also increases DHEA sulfotransferase activity. Mutation of Phe-142 is more destructive, resulting in a profound decrease of both E2 and DHEA sulfotransferase activities of this enzyme, consistent with the fact that Phe-142 is conserved in all cytosolic sulfotransferases. Thus, the Tyr-81/Phe-142 gate is a structural factor that determines the estrogen specificity of EST.

Tyr-81 appears to be the determining factor in the alteration of the substrate specificity of EST to DHEA sulfotransferase activity. In the Tyr-81 mutants, $k_{cat}$ for DHEA sulfotransferase activity was increased markedly compared with wild-type EST. This indicates that the gate can be widened by mutation of Tyr-81, allowing for EST to accommodate DHEA deep into the substrate pocket near the catalytic center. The molecule modeled in the EST pocket shows that the C-19 methyl group makes a clashing contact (2.29 Å) with a carbon atom (C-e2) of the phenol ring of Tyr-81 but not with the phenolic hydroxyl at the 3-position (3.85 Å). Paradoxically, our present site-directed mutagenesis of Tyr-81 has suggested that the C-19 methyl group may conflict sterically with the phenol group. This apparent paradox might be explained if DHEA does not bind exactly in the same manner as the E2 molecule pictured in Fig. 1 and thus may actually have a steric interaction with the phenol group. Alternatively, the structural orientation of the Phe side chain with respect to the Tyr at this position may differ, thus allowing the Y81F mutant to accommodate DHEA. Nevertheless, it should be reiterated that placing a smaller amino acid at position 81 can widen the Tyr-81/Phe-142 gate, resulting in an increase of DHEA sulfotransferase activity in EST.

Steric hindrance by a critical residue has been proposed as an underlying principle that can regulate substrate and/or product specificities of enzymes catalyzing hydrophobic sub-
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FIG. 2. Superposition of DHEA to E2 in the EST substrate gate. E2 is in green, DHEA in blue, and the gate residues Tyr-81 and Phe-142 in magenta. The van der Waals surfaces are displayed for E2, Tyr-81, and Phe-142. The possible clashing contact that would exist if DHEA were forced to bind to wild type EST (between C-19 of DHEA and C-e2 of Tyr-81) is represented by a cyan line. This figure was created using Molscript (24).

strates. It is well known that the sizes of these residues alter the P450 specificities (Ref. 19 and references therein). For example, mutation of Phe-209 to Leu converted the substrate specificity of CYP2A5 from coumarin to a larger molecule, testosterone. Placing a large amino acid Phe at position 209 specificities. To our knowledge, the Tyr-81/Phe-142 gate of EST to an enzyme that sulfates DHEA with a high catalytic power to sulfate E2 by regulating the binding affinity of EST to E2. The gate can be widened through mutagenesis so as to accommodate DHEA deep into the catalytic core, altering EST to an enzyme that sulfates DHEA with a high catalytic efficiency. A gate-like structure in the substrate-binding pocket may be an underlying factor for enzymes catalyzing hydrophobic chemicals and thereby regulating their substrate and/or product specificities.

In conclusion, we have demonstrated that the gate-like structure regulates EST specificity depending on the chemical properties of the substrates. The gate provides EST with a high catalytic power to sulfate E2 by regulating the binding affinity of EST to E2. The gate can be widened through mutagenesis so as to accommodate DHEA deep into the catalytic core, altering EST to an enzyme that sulfates DHEA with a high catalytic efficiency. A gate-like structure in the substrate-binding pocket may be an underlying factor for enzymes catalyzing hydrophobic chemicals and thereby regulating their substrate and/or product specificities.

REFERENCES

1. Hobkirk R. (1993) Trends Endocrinol. Metab. 4, 69–73
2. Evans, T. R., Rowlands, M. G., Law, M., and Coumbe, R. C. (1994) Br. J. Cancer 69, 555–561
3. Utsumi, T., Yoshimura, N., Takeuchi, S., Ando, J., Maruta, M., Meada, K., and Harada, N. (1999) Cancer Res. 59, 377–381
4. Ahmed, S., James, K., Sampson, L., and Mastrri, C. (1999) Biochem. Biophys. Res. Commun. 254, 811–815
5. Selcer, K. W., Jagannathan, S., Rhodes, M. E., and Li, P.-K. (1996) J. Steroid Biochem. Mol. Biol. 59, 83–91
6. Weinstabilien R. M., Otterness, D. M., Askoy, J. A., Wood, T. C., Her, C., and Raftognias, R. B. (1997) FASEB J. 11, 3–14
7. Varin, L., Marsolais, F., Richard, M., and Rouleau, M. (1997) FASEB J. 11, 517–525
8. Brix, L. A., Duggleby, R. G., Gasdik, A., and McManus, E. (1999) Biochem. J. 337, 337–343
9. Dujani, R., Hood, A. M., and Coughtrie, M. H. (1998) Mol. Pharmacol. 54, 942–948
10. Song, W.-C., Moore, R., McLachlan, J. A., and Negishi, M. (1995) Endocrinology 136, 2477–2484
11. Kakuta, Y., Pedersen, L. C., Chea, K., Song, W.-C., Lablanc, D., London, R., Carter, C. W., and Negishi, M. (1998) Biochem. Pharmacol. 55, 313–317
12. Kakuta, Y., Pedersen, L. G., Carter, C. W., Negishi, M., and Pedersen, L. C. (1997) Nat. Struct. Biol. 4, 964–968
13. Kakuta, Y., Petrochenko, E. V., Pedersen, L. C., and Negishi, M. (1998) J. Biol. Chem. 273, 27325–27330
14. Duffel, M. W., and Jakoby, W. B. (1981) J. Biol. Chem. 256, 11123–11127
15. Zhang, H., Varmalova, O., Vargas, F. M., Falany, C. N., and Leyh, T. S. (1998) J. Biol. Chem. 273, 10888–10892
16. Kakuta, Y., Pedersen, L. G., Pedersen, L. C., and Negishi, M. (1998) Trends Biochem. Sci. 23, 129–130
17. Sueyoshi, T., Kakuta, Y., Pedersen, L. C., Wall, F. E., Pedersen, L. G., and Negishi, M. (1998) FEBS Lett. 433, 211–214
18. Kakuta, Y., Sueyoshi, T., Negishi, M., and Pedersen, L. C. (1999) J. Biol. Chem. 274, 10673–10676
19. Negishi, M., Uno, T., Darden, T. A., Sueyoshi, T., and Pedersen, L. G. (1996) FASEB J. 10, 683–689
20. Lindberg, R., and Negishi, M. (1989) Nature 340, 632–634
21. Graham-Lorence, S., Truan, G., Peterson, J. A., Falck, J. R., Wei, S., Helvig, C., and Capdevila, J. H. (1997) J. Biol. Chem. 272, 1127–1135
22. Shioke, D. L., Leung, R., Craik, C. S., and Sigai, E. (1991) Nature 354, 149–152
23. Wyckoff, T. J. O., Lin, S., Cotter, R. J., Dotson, G. D., and Rartz, C. R. H. (1998) J. Biol. Chem. 273, 32369–32372
24. Kraulis, P. J. (1991) J. Appl. Crystallogr. 24, 946–950