Sulfuryl Transfer: The Catalytic Mechanism of Human Estrogen Sulfotransferase*

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Estrogen sulfotransferase (EST) catalyzes the transfer of the sulfuryl group from 3′-phosphoadenosine 5′-phosphosulfate (PAPS) to 17β-estradiol (E2). The sulfation of E2 prevents it from binding to, and thereby activating, the estrogen receptor. The regulation of EST appears to be causally linked to tumorigenesis in the breast and endometrium. In this study, recombinant human EST is characterized, and the catalytic mechanism of the transfer reaction is investigated in ligand binding and initial rate experiments. The native enzyme is a dimer of 35-kDa subunits. The apparent equilibrium constant for transfer to E2 is $(4.5 \pm 0.2) \times 10^9$ at pH 6.3 and $T = 25 \pm 2$ °C. Initial rate studies provide the kinetic constants for the reaction and suggest a sequential mechanism. E2 is a partial substrate inhibitor ($K_i = 80 \pm 5$ mM). The binding of two E2 per EST subunit suggests that a partial inhibition occurs through binding at an allosteric site. In addition to providing the dissociation constants for the ligand-enzyme complexes, binding studies demonstrate that each substrate binds independently to the enzyme and that both the E-PAP-E$_S$S and E-PAP-E$_S$ dead-end complexes form. These results strongly suggest a Random Bi Bi mechanism with two dead-end complexes.

Transferring the sulfuryl group (-SO$_3^-$) from activated sulfate, or PAPS, to a given metabolic recipient typically switches “on” or “off” or otherwise modifies the function of that metabolite (1–6). The extent of sulfation of a given metabolite is determined by the regulated expression of two enzyme classes: the sulfotransferases, which transfer the sulfuryl group, and the sulfatases, which remove it. PAPS appears to be the sole donor of the sulfuryl group from 3′-phosphoadenosine 5′-phosphosulfate (PAPS) to 17β-estradiol (E2). The sulfation of E2 prevents it from binding to, and thereby activating, the estrogen receptor. The regulation of EST appears to be causally linked to tumorigenesis in the breast and endometrium. In this study, recombinant human EST is characterized, and the catalytic mechanism of the transfer reaction is investigated in ligand binding and initial rate experiments. The native enzyme is a dimer of 35-kDa subunits. The apparent equilibrium constant for transfer to E2 is $(4.5 \pm 0.2) \times 10^9$ at pH 6.3 and $T = 25 \pm 2$ °C. Initial rate studies provide the kinetic constants for the reaction and suggest a sequential mechanism. E2 is a partial substrate inhibitor ($K_i = 80 \pm 5$ mM). The binding of two E2 per EST subunit suggests that a partial inhibition occurs through binding at an allosteric site. In addition to providing the dissociation constants for the ligand-enzyme complexes, binding studies demonstrate that each substrate binds independently to the enzyme and that both the E-PAP-E$_S$S and E-PAP-E$_S$ dead-end complexes form. These results strongly suggest a Random Bi Bi mechanism with two dead-end complexes.

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‡ The abbreviations used are: PAPS, 3′-phosphoadenosine 5′-phosphosulfate; PAP, adenosine 3′,5′-diphosphate; ER, estrogen receptor; E$_Z$, 17β-estradiol; E$_S$, 17β-estradiol 3-sulfate; EST, estrogen sulfotransferase; DTT, dithiothreitol.
pelleted at 27,000 × g for 60 min, and the concentration of protein, determined by the method of Bradford (16), was adjusted to 3.0 mg/ml using 5.0 mM KPO4 (pH 7.4).

The protein solution was loaded onto a 100-ml bed (3.6 × 17 cm) of amyllose resin and eluted with 5.0 mM KPO4 (pH 7.4). The column was equilibrated and run in 50 mM KCl, pH 8.0 at 4 °C. Bio-Rad gel filtration standards were used to calibrate the column. The apparent native molecular mass of EST was 62 ± 2.3 kDa.

**Extinction Coefficient**—The extinction coefficient of EST was determined gravimetrically at 280 nm, the \( \lambda_{\text{max}} \), for the enzyme. EST was dialyzed at 4 °C for 8 h and then overnight against 10 mM NaH2PO4 (pH 6.3), 1.0 mM KCl, and 0.10 mM DTT. The absorbance of the dialyzed EST was determined in triplicate at 280 nm in a masked 1-cm path length cuvette. Dialyzed enzyme or dialysis buffer (200 ± 1.6 μM) was added to an aluminum weight boat and dried under vacuum and over P2O5 to a constant weight (<40 h). Triplicate samples of the dialyzed enzyme and buffer were each weighed three times. The absorbance at 280 nm was divided by the concentration of enzyme to calculate the extinction coefficient; \( \varepsilon_{280} = 1.7 ± 0.1 \times 10^{4} \text{ M}^{-1} \cdot \text{cm}^{-1} \).

**Equilibrium Constant**—The equilibrium constant for the EST reaction was determined at pH 6.3 and \( T = 22 ± 2 ^\circ \text{C} \). The measurements were performed in duplicate at each of the following three sets of initial concentrations of E2S, [5-32P]PAP, and EST, respectively: 100 μM, 240 nm, and 8.0 mM; 200 μM, 240 nm, and 8.0 mM; and 500 μM, 630 nm, and 32 nm. The progress curve for each reaction was determined, and the equilibrium constant was calculated from the reactant concentrations in the stationary phase of the progress curve. The buffer was the same as that used in the initial rate experiments. The concentration of product formed in each reaction was at least 10 times the enzyme active-site concentration. The equilibrium constant was \( (4.5 ± 0.2) \times 10^{3} \).

**Divalent Cation Activation**—The initial rate of the forward reaction was studied as a function of MgCl2 concentration. The assays were performed as described below under "Initial Rate Studies of the Forward Reaction." The conditions of these experiments were as follows: 1.0 mM EST, 10 mM E2, 6.0 mM PAPS, 50 mM KPO4 (pH 6.3), 1.0 mM DTT, and 10% (v/v) glycerol at \( T = 25 ± 2 ^\circ \text{C} \). The MgCl2 concentration was varied in 2.0 mM increments from 0 to 11 mM. EDTA was added to 2.0 mM in the experiments at zero MgCl2 to ensure that the observed activity was not caused by trace divalent cations in the buffer.

**Initial Rate Studies of the Forward Reaction**—The reactions were initiated by adding 50 μl of enzyme to 200 μl of a buffered solution containing varying concentrations of E2 and PAPS. The buffer used in these experiments contained 50 mM KPO4 (pH 6.3), 7.0 mM MgCl2, 1.0 mM DTT, and 10% (v/v) glycerol. The reaction was quenched by the addition of 150 μl of 10 mM KCl. Controls were run to ensure that the KOH did not hydrolyze the E2S produced. 5.0 ml of CHCl3 was then added, and the solution was vortexed for 15 s. 800 μl of the aqueous phase was removed and counted. Velocities were determined in duplicate under each of the 16 conditions defined by a 4 × 4 matrix of substrate concentrations. The concentration of EST was 0.10 mM in all of the experiments. The concentrations of PAPS were 12, 18, 34, and 900 mM ranging from 0.2 to 5 times its \( K_{\text{m}} \). The concentrations of E2 (2.0, 2.8, 4.8, and 15.5 mM) ranged from 0.4 to 3 times its \( K_{\text{m}} \). Partial substrate inhibition by E2 is negligible at these E2 concentrations. The velocities were measured under initial rate conditions since the consumption of the concentration-limiting substrate was <7% of its end point in all cases. The data were fit to the hyperbolic model using the program developed by Cleland (17).

**Stabilizing EST Activity**—In the absence of glycerol, the activity (i.e. the initial rate of PAP and E2S synthesis) of EST is stable for several days at 70 °C, and the half-life of the activity is ~2 h at 25 °C. Glycerol (10% (v/v)) prevents detecta-
ble deterioration of the activity over 3 h at 25 °C. The addition of E2 to EST in glycerol causes a rapid loss of activity (t1/2 < 30 min). This E2-induced inactivation was prevented by the addition of DTT. At 1.0 mM DTT, the activity was not affected at a saturating concentration of E2 over 4 h at 25 °C. Thus, it appears that the binding of E2 potentiates an inactivating oxidation reaction that is suppressed by DTT.

**Optimizing Turnover**—Most enzyme-catalyzed transfer reactions involving nucleotides require divalent cations. In these cases, the cations are often directly bound to the polyphosphate chain of the nucleotide. The mechanism of this activation appears to be due predominantly to the entropy reduction associated with the positioning of functional groups for reaction (20). It is interesting that while sulfotransferases catalyze transfer reactions that, in many ways, resemble phosphoryl oxidation reaction that is suppressed by DTT.

A plot of the initial rate of the forward EST reaction versus [MgCl2] is bell-shaped with a maximum at 7.0 mM MgCl2 (see “Experimental Procedures”). The initial rate of the forward reaction was determined with the equilibrium binding studies for PAPS, causing the concentration of the reactive form(s) of the enzyme to decrease, increasing the concentration of PAPS would drive the initial rates, at inhibitory

**Figure 1.** An initial rate study of the synthesis of PAP and E2. The initial rate of PAP and E2 synthesis is shown as a function of PAPS and E2 concentrations. The PAPS concentration was varied between 0.20 and 5.0 times its K_m (59 nM). The E2 concentration was varied between 0.4 and 3 times its K_a (5.2 nM). The E2 concentration range was sufficiently low that inhibition by E2 was negligible. Each point represents the average of two independent determinations. The lines through the points represent the best fits to the data. The experiments were performed at 25 ± 2 °C. For the experimental protocol, see “Experimental Procedures.”

**Table 1**

| Kinetic constants | K_a | K_m | k_cat |
|-------------------|-----|-----|-------|
| PAPS              | 155 (±35)nM | 59 (±13) |       |
| E2                | 13 (±4) | 5 (±0.8) |       |
| PAP               | 38 (±0.8) |       |       |

* The numbers in parentheses refer to a single S.D. unit.

**Equilibrium Constant**—To evaluate the energetics associated with transferring the sulfuryl group between PAPS and E2 and to aid in the design of initial rate experiments, the equilibrium constant for the EST reaction was determined at pH 6.3 and T = 25 ± 2 °C (the conditions of the initial rate studies). The equilibrium constants were calculated from reactant concentrations in the stationary phases of reaction progress curves constructed in duplicate at three different sets of E2S and PAPS concentrations (see “Experimental Procedures”). Controls were run to ensure that the EST activity did not change during the experiments. The equilibrium constant is (4.5 ± 0.2) × 10^3. The ΔG° associated with this K_eq is ~5.0 kcal/mol. It should be emphasized this apparent equilibrium constant does not explicitly include the proton and divalent cation dependences.

**Initial Rate Study of the Forward Reaction**—To determine the kinetic constants for the forward reaction and to obtain a preliminary assessment of the order of substrate binding, a classical initial rate study of the forward reaction was performed. The initial rate was determined as a function of both E2 and PAPS concentrations (see “Experimental Procedures”). The results of this study are shown in Fig. 1. The pattern of the data is indicative of a sequential mechanism (one in which both substrates must bind to the enzyme before product is released). However, it does not rule out a ping-pong mechanism with an unstable enzyme intermediate. An equilibrium ordered mechanism is ruled out by the fact that the lines through the data of the 1/V versus 1/[E2] and 1/V versus 1/[PAPS] (not shown) plots do not intersect on the 1/V axis (22). The kinetic constants obtained from this study are compiled in Table 1. k_cat (1.3 ± 0.08 s⁻¹) and the K_m for PAPS (59 ± 13 nM) are similar to those measured for other sulfotransferases (23, 24). The K_m for E2 is comparable to the in vivo concentration of E2 (1 nM (25), suggesting that the enzyme is optimized to perform at the physiological concentration of E2.

**Initial Rate Study of the Reverse Reaction**—The K_m for PAP and k_cat for the reverse reaction were determined in an initial rate study at a saturating (920 × K_m) concentration of E2S (250 μM). Controls were run to ensure that E2S did not inhibit the velocity at this concentration. The K_m for PAP was 38 ± 0.8 nM, and k_cat was 0.16 ± 0.0013 min⁻¹ (Table 1). The experimental protocol is described under “Experimental Procedures.” Given the technical obstacles associated with the unfavorable equilibrium constant for the reverse reaction and the relatively high K_m for E2S, the order of substrate addition for the reverse reaction was determined with the equilibrium binding studies described below.

**Partial Substrate Inhibition by E2**—The initial rate data shown in Fig. 2 demonstrate that E2 inhibits the forward reaction. The fact that the velocity decreases to a plateau, rather than to zero, means that one or more of the kinetic parameters for the reaction are being titrated from one value to another as E2 adds to the enzyme. The inhibition experiment (Fig. 2, ●) was performed at a fixed near-saturating concentration of PAPS (600 nM, 10 × K_m). If the inhibition were due solely to an increase in the K_m for PAPS, causing the concentration of the reactive form(s) of the enzyme to decrease, increasing the concentration of PAPS would drive the initial rates, at inhibitory
concentrations of E$_2$, back to the uninhibited levels. This, in fact, does not occur. Increasing the concentration of PAPS 10-fold (Fig. 2, □) had no significant effect on the initial rates at high E$_2$ concentrations. Thus, it is $k_{cat}$ that is affected by the binding of E$_2$ in these experiments.

The $K_i$ for E$_2$ was evaluated by fitting the data shown in Fig. 2 to the algebra that describes the kinetic behavior of the model shown in Fig. 3. In the model, all of the enzyme forms are saturated with PAPS. The kinetic constants $V_1$ and $K_m$ for E$_2$ were obtained from the initial rate study of the forward reaction (Table I). $V_2$ was set at 0.18 nM/min, which is slightly below the plateau shown in Fig. 2. The data were fit to the following equation: $v = V_1\left(1 + (V_2/[E_2]/V_1K_i)\right)/(1 + K_m/[E_2] + [E_2]/K_i)$ (26). This equation assumes that substrate binding is at equilibrium, which is plausible given the very low turnover of the enzyme. The value of $K_i$ that provides the best fit to the data is $80 \pm 6$ nM. It should be mentioned that the inhibition model was used to select the E$_2$ concentrations used in the initial rate studies (Fig. 1) such that inhibition by E$_2$ was negligible.

Equilibrium Binding Studies—The excitation and emission wavelength maxima for EST are 275 and 340 nm, respectively. The fine structure and $\lambda_{max}$ of the emission spectrum do not change significantly when substrates bind to EST; however, the intensity decreases 30–50% depending on the ligand. These ligand-dependent decreases in the quantum yield of EST provide excellent experimental handles to determine both the equilibrium constants and stoichiometry of the enzyme-ligand interactions.

The stoichiometry of the enzyme-ligand interactions was determined in fluorescence titration experiments in which the concentration of enzyme active sites was $>15 \times K_i$. At these enzyme concentrations, the binding isotherms have two linear regions. They are linear in the substrate concentration range 0–0.4 [enzyme] because virtually all of the substrate is bound to the enzyme in this range, and they are linear (with a slope of zero) at saturating substrate concentrations. The intersection of the lines extrapolated from these linear regions corresponds, on the [ligand]/[E] axis, to the stoichiometry of the ligand-enzyme complex. The results of these studies for each of the EST substrates are depicted in Fig. 4 (A–D). The stoichiometries of the ligand-enzyme complexes are 1:1 for all of the ligands except E$_2$, which has a stoichiometry of 1:0.5.

**Fig. 2.** Partial substrate inhibition by E$_2$. The initial rate of E$_2$S and PAP synthesis was determined as a function of E$_2$ concentration. The experimental conditions are described under "Experimental Procedures." ○, data obtained at EST and PAPS concentrations of 1.0 and 600 nM, respectively; □, velocities determined at 6.0 $\mu$m PAPS. Each velocity is the average of at least two independent determinations. The solid line passing through the points represents the initial rate profile predicted by the best fit model.

**Fig. 3.** Kinetic model for partial substrate inhibition by E$_2$. $K_m$ was obtained from Table I; $V_1$ was calculated from $k_{cat}$ (Table I), and $V_2$ was set at 0.18 min$^{-1}$, slightly below the initial rate in the plateau of Fig. 2. The algebra that describes the kinetic behavior of this model was used to obtain a best fit value for $K_i$.

**Fig. 4.** Stoichiometry of ligand binding. The stoichiometries were determined by monitoring the change in fluorescent intensity of the enzyme as a function of ligand concentration at enzyme active-site concentrations $>14 \times K_i$. II$_0$ is the ratio of the fluorescence intensity of the enzyme at a given ligand concentration to that in the absence of ligand. The straight lines through the points were fit by eye. Each point represents the average of at least two independent determinations. The PAP, PAPS, E$_2$S, and E$_2$ titrations are shown in A–D, respectively. The EST concentrations used in the experiments associated with A–D were 1.5, 2.0, 2.0, and 1.0 $\mu$m, respectively.
The Catalytic Mechanism of Human Estrogen Sulfotransferase

2.1. The binding of two E₂ to each EST subunit strongly suggests that one of the binding sites is the catalytic site, whereas the other is the allosteric site that regulates the turnover of the enzyme (Fig. 3). Notwithstanding the possibility that these fluorescence experiments monitor the formation of nonproductive complexes, the independent binding of each of the EST substrates demonstrates that the mechanism of the enzyme is random sequential.

The equilibrium constants for the formation of enzyme-substrate complexes were determined by fluorescence titrations in which the enzyme concentration was held fixed between 0.4 and 5 × K_d. The binary complex binding constants were determined by fitting the data shown in Fig. 5 (A, C, and D) to a single-site binding model. The K_d values for the E-PAPS, E-PAP, and E-E₂-S complexes are 27 ± 0.3, 30 ± 0.3, and 271 ± 49 nM, respectively (Table II). The data associated with the binding of E₂ (Fig. 5B) are equally well fit using either a single-site model (K_d = 26 ± 2 nM; shown in Fig. 5B) or a two-site model that assumes no interaction energy (27) in which the dissociation constants are within an order of magnitude of one another and symmetrically disposed about the best fit K_d predicted by the single-site model.

The formation of the ternary dead-end complexes was also investigated using fluorescence titrations (Fig. 5, E and F). The binding of PAPS to the E-E₂-S complex and of PAP to the E-E₂ complex was monitored at a saturating concentration of E₂S or E₂. The data clearly demonstrate that both dead-end complexes form. The nucleotide dissociation constants for the E-PAPS-E₂S and E-PAP-E₂ complexes are 20 ± 2.8 and 22 ± 1.7 nM, respectively. Comparison of the binary and dead-end dissociation constants for PAPS and PAP reveals a slight binding synergism between the ligands in the ternary complex (Table II). These results corroborate the dead-end complexes implicated by earlier product inhibition studies with arylsulfotransferase (24) and strongly suggest that the mechanism of EST is Random Bi Bi with two dead-end complexes.

Conclusions—Initial rate and ligand binding experiments have been used to investigate the catalytic mechanism of EST. The kinetic parameters for the mechanism were determined from the initial rate studies, which also suggested that the mechanism is sequential. Ligand binding studies were used to determine the equilibrium constants and stoichiometries of the enzyme-substrate interactions. The binding studies demonstrated that each of the substrates can bind independently to the enzyme and that two dead-end complexes can form. These results strongly suggest a Random Bi Bi mechanism with two dead-end complexes. The initial rate experiments revealed that E₂ is a partial substrate inhibitor of the reaction with a K_i of 80 ± 5 nM. The mechanism of the inhibition is partially delineated by the stoichiometry studies, which show that the enzyme contains two E₂-binding sites/catalytic subunit, suggesting that the enzyme harbors an allosteric E₂-binding site.

REFERENCES

1. Brand, S. J., Andersen, B. N., and Rehfeld, J. F. (1984) Nature 306, 456–458
2. Falany, C. N., Wheeler, J., Tae, S. O., and Falany, J. L. (1984) J. Steroid Biochem. Mol. Biol. 24, 369–375
3. Garay, R. P., Labaune, J. P., Mesangeau, D., Nazaret, C., Imbert, T., and Moiney, G. (1990) J. Pharmacol. Exp. Ther. 255, 415–422
4. Hennemrich, S., Bertozzi, C. R., Leffler, H., and Rosen, S. D. (1994) Biochemistry 33, 4820–4829
5. Jensen, R. T., Lemp, G. F., and Gardner, J. D. (1982) J. Biol. Chem. 257, 5554–5559
6. Merrifield, R. B., MacLusky, N. J., Picard, M. K., and Naeflin, F. (1980) Steroids 36, 1–11
7. Gorski, J., and Shymala, G. (1968) J. Biol. Chem. 244, 1097–1103
8. Pschorn-Walcher, H., and Ecker, J. (1975) Methods Enzymol. 36, 369–375
9. Unsworth, C. D., Hughes, J., and Morley, J. S. (1982) Nature 295, 519–522
10. Tagliamonte, J. A., McReynolds, L. A., and Guan, C. (1988) A. Rev. Biochem. 57, 565–573
11. Miller, J. H. (1973) Experiments in Molecular Genetics, pp. 431–433, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
12. Bradford, M. M. (1976) Anal. Biochem. 72, 248–254
13. Cleland, W. L. (1979) Methods Enzymol. 56, 105–138
14. Ward, L. D. (1985) Methods Enzymol. 117, 400–414
15. Wetlaufer, D. B. (1962) J. Biol. Chem. 237, 904–908
16. Herschlag, D., and Jencks, W. P. (1990) J. Steroid Biochem. Mol. Biol. 52, 529–539
17. Maina, C. V., Riggs, P. D., Grandeau, A. G., Slatko, B. E., Moran, L. S., Tagliamonte, J. A., McReynolds, L. A., and Guan, C. (1988) Gene (Amst.) 74, 365–373
18. Herschlag, D., and Jencks, W. P. (1990) J. Steroid Biochem. Mol. Biol. 52, 529–539
19. Kesner, J., Andersen, B. N., and Rehfeld, J. F. (1984) Nature 306, 456–458
20. Falany, C. N., Wheeler, J., Tae, S. O., and Falany, J. L. (1984) J. Steroid Biochem. Mol. Biol. 24, 369–375
21. Falany, C. N., Wheeler, J., Tae, S. O., and Falany, J. L. (1984) J. Steroid Biochem. Mol. Biol. 24, 369–375
22. Cleland, W. L. (1979) Methods Enzymol. 56, 105–138
23. Ward, L. D. (1985) Methods Enzymol. 117, 400–414
24. Wetlaufer, D. B. (1962) Adv. Protein Chem. 17, 303–390
25. Herschlag, D., and Jencks, W. P. (1990) J. Steroid Biochem. Mol. Biol. 52, 517–5179
26. Kakuta, Y., Pedersen, L. G., Carter, C. W., Negishi, M., and Pedersen, L. C. (1996) J. Biol. Chem. 271, 7645–7653
27. Duffel, M. W., and Jakoby, W. B. (1981) J. Biol. Chem. 256, 11123–11127
28. Guerrero, R., Montiel, L. R., Cekan, Z., and Diczfalusy, E. (1975) Contraception 11, 169–177
29. Fromm, H. J. (1975) Initial Rate Enzyme Kinetics, p. 145, Springer-Verlag New York Inc., New York
30. Wyman, J. J. (1964) Adv. Protein Chem. 19, 223–286