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# Estrogen Sulfotransferase – a Half-site Reactive Enzyme

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## Abstract

Estrogen sulfotransferase (EST) regulates the biological activity of estradiol – sulfation of this important signalling molecule inactivates its estrogen-receptor-binding activity. EST is a cytosolic sulfotransferase – one of family of structurally well conserved enzymes that exhibit broad, overlapping substrate specificities and that together comprise a robust catalytic network designed to handle the many substrates present in the cell. We have discovered recently that EST is a half-site enzyme – only one of the subunits of the dimer is capable of forming product during turnover. Thus, during the catalytic cycle a molecular "decision" is made that couples the silencing one of the subunits to the activity of the other. The discovery of this remarkable behaviour and the implications of the EST mechanism for the sulfotransferase field are the subject of this article.

## Introduction

Estrogen sulfotransferase (EC 2.8.2.4), a dimer of identical 35 kDa protomers, catalyzes the transfer of the sulfuryl moiety (-$SO_3$) of PAPS (3'-phosphoadenosine 5'-phosphosulfate) to the 3'-hydroxyl of 17$\beta$-estradiol ($E_2$), to form $E_2S$ (mechanism I).

$$E_2 + PAPS^{4-} \rightleftarrows E_2S^{-} + PAP^{4-} + H^{+} \qquad \text{(I)}$$

The presence of the sulfuryl-group prevents estradiol from binding the estrogen receptor at physiologically relevant concentrations [1]. The group is removed hydrolytically by sulfatases, which regenerate $E_2$, and the coordinated actions of the sulfotransferase and sulfatase balance the sulfated and non-sulfated forms of $E_2$ to meet the metabolic requirements of the cell.

It is generally agreed that sulfation/desulfation cycles are responsible for regulating a large, diverse set of metabolites and cellular processes including steroid hormones [2, 3], signalling peptides [4 – 7], heparin [8, 9], haemostasis [10 – 12], lymph circulation [13] and numerous drugs and xenobiotics [14, 15].

Approximately ten distinct sulfotransferases have been identified in the cytosols of mammalian cells [16, 17]. These enzymes typically exhibit a broad substrate specificity that is centred on particular molecular traits of non-nucleotidyl substrate (or acceptor), and the enzyme common names reference the metabolite for which the enzyme is believed to exhibit its highest affinity. The substrates specificities of these enzymes often overlap, producing a redundant and robust metabolic network designed to manage the sulfation requirements of the organism.

## A Suggestion of Half-site Reactivity

In characterizing the rates and equilibria of reactions occurring on the surface of the recombinant, human estrogen sulfotransferase (EST) during its catalytic cycle, we discovered that the enzyme produces a presteady-state burst of the product [18]. Such behaviour reveals that a rate-determining step(s) occurs after the product has formed on the enzyme; however, it gives no indication as to where in the product release branch of the catalytic cycle that step(s) occurs. The amplitude of the burst corresponds to precisely one equivalent of product formed per dimer, suggesting for the first time that the enzyme might be half-site reactive (Fig. 1).

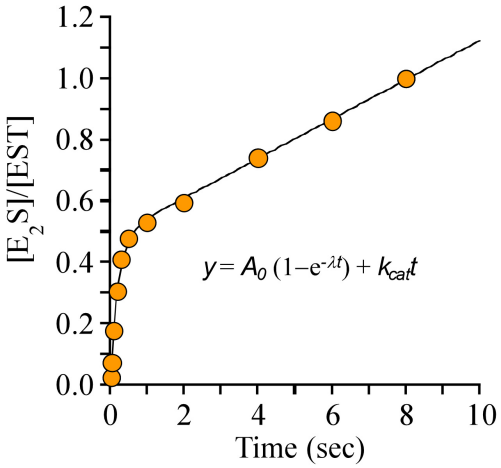


**Figure 1. A burst of $E_2S$ formation**. $E_2S$ synthesis was initiated by rapidly mixing a solution containing EST (1.0 µM, dimer), [$^3$H]$E_2$ (3.6 µM, 720 x $K_m$, SA = 90 Ci/mmol), glycerol (10% v/v), $MgCl_2$ (7.0 mM), DTT (1.0 mM), and 50 mM $KPO_4$ (pH 6.3) with a solution of equal volume that was identical except that it lacked EST and $E_2$, and contained PAPS (18.0 µM, 305 x $K_m$). Reactions were quenched by rapidly mixing the reacting solutions with an equal volume of HCl (0.66 M). [$^3$H]$E_2$ was extracted from the quenched mixture using $CCl_4$ and [$^3$H]$E_2S$, which remained in the aqueous phase, was counted. All solutions were equilibrated at 25 (± 2) °C prior to mixing. Reactions were performed in triplicate and averaged. The smooth curve represents the best-fit of the averaged data to the equation: $A_0 (1-e^{-\lambda t}) + k_{cat}t$.

Half-site reactivity is a curious phenomenon in which seeming identical subunits are locked into a relationship in which only one out of every two subunits of a multi-subunit enzyme is capable of forming product. Thus, at some point in the catalytic cycle, a "molecular decision" is made to assign reactivity to a specific subunit – a decision almost certainly rooted in a structural asymmetry that allosterically links the behaviours of the two active sites to one another.

## Confirmation of Half-Site Reactivity

While the observed burst amplitude is suggestive of half-site behaviour, it is equally consistent with a mechanism in which, for example, the equilibrium constant governing interconversion of the central complexes of the enzyme (the *internal equilibrium constant*, or $K_{eq\ int}$) is equal to 1, and the release of product is sufficiently slow to allow the interconversion to approach equilibrium.

A burst amplitude provides a stoichiometry, i.e. [product formed]/[active sites occupied]. Assuming a half-site mechanism, the value of 0.52/dimer requires that $K_{eq\ int}$ be considerably larger than one (≥ ~10) on the subunit that turns over – where this is not the case, the

amplitude would be less. The value of 0.52 was determined under conditions where both active sites of the dimer are saturated. Under conditions in which only one of the subunits of the dimer has substrate bound, virtually all of the bound substrate is expected to be converted to the product due to the favourable internal energetics of the active subunit, and the stoichiometry (normalized to bound active sites) will approach ~1.0. On the contrary, the full-site model predicts a stoichiometry of 0.5 regardless of the occupancy of dimer active sites (due to the restriction that $K_{eq\ int} = 1$). Thus, the mechanisms can be distinguished on this basis.

Capitalizing on these differences to ascertain the operative mechanism, the stoichiometry was determined as a function of occupancy of the EST dimer. Occupancy was controlled by varying the $[EST]_{active\ site}/[E_2]$ ratio at a fixed concentration of $E_2$ (1.0 µM) – PAPS is fixed and saturating in all cases. At the lowest [EST] (1.0 µM), the majority of dimers have both active sites occupied, and either mechanism predicts an amplitude of 0.5 (which is what is observed, Figure 2). At the highest [EST] (12 µM), simple statistics (i.e., independent subunits) predict that 17% of the dimers will have both of their active sites occupied; 83% will have only one. At this condition, the half-site mechanism yields a maximum amplitude of ~91%; whereas, the full-site model yields 5%. Figure 2 reveals how the amplitude varies as a function of [EST], and compares the experimental data (▲) to the result predicted for the half-site (●) and full-site (●) mechanisms. The experimental data follow the predictions of the half-site model well, and differ sharply from the expectations of the full-site mechanism. Thus it appears that EST is a half-site enzyme.

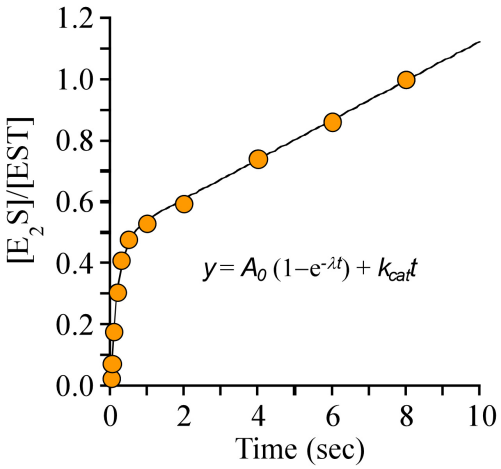


**Figure 2. Confirmation of half-sites reactivity**. Reactions were initiated by mixing a solution containing PAPS (16 µM), glycerol (10% v/v), $MgCl_2$ (7.0 mM), DTT (1.0 mM), and 50 mM $KPO_4$ (pH 6.3) with an equal volume of an identical solution that did not contain PAPS, but did contain $[^3H]E_2$ (2.0 µM) and various concentrations of EST (2.0, 3.0, 4.0, 6.0, 8.0, 16.0, 24.0 µM – monomer concentrations). The plotted $E_2S$ concentrations represent the $E_2S$ formed in reactions quenched at 0.30 s – at this

time-point, the burst is essentially complete and steady-state turnover contributes only slightly to overall product formation (see Figure 1). Triangles (▲) represent experimentally determined [$E_2S$]; red circles (●) represent [$E_2S$] predicted using a half-site reactivity model in which $K_{eq\ int} >> 1.0$; tan circles (●) represent [$E_2S$] predicted using a full-site reactivity model with $K_{eq\ int} \sim 1.0$. Experimental values were determined in triplicate and averaged. All solutions were equilibrated at 25 (± 2) °C prior to mixing.

An important question in the sulfotransferase field is how, confronted with the considerable array of substrate options provided by the cell, a given sulfotransferase selects one substrate over another. Given a simple mechanism in which substrate (S) binds to enzyme (E) and is converted to a product that is released slowly enough to allow both binding and the ES – EP interconversion to approach equilibrium, $K_m$ is approximated by the product of the equilibria for these two steps. Such mechanisms provide a flexible, two-stage selection process that offers certain benefits to the cell. For example, it offers the opportunity to provide tight overall binding while maintaining binding at the first step that is sufficiently weak to allow the active site to sample numerous substrates during selection.

## Selectivity and the EST Mechanism

Notwithstanding electrostatic guidance [19], the maximum microscopic rate constant for the binding of ligand to an enzyme active site is given by the diffusion on-rate constant, $\sim 8 \times 10^8\ M^{-1}s^{-1}$ [20]. However, because binding is a complex process involving multiple, sequential events to achieve docking of ligand, the docking on-rate constant is often one-to-two orders of magnitude less that the diffusion rate constant. $K_m$ for estradiol is 5 nM. If the affinity of EST for $E_2$ were determined solely at the binding step, the maximum $k_{off}$ (assuming diffusion-limited $k_{on}$) can be estimated at $\sim 4\ s^{-1}$, and the half-life of $E_2$ on the surface of EST is $\sim 0.17\ s^{-1}$. If $k_{on}$ were one-to-two orders of magnitude less than diffusion, the half-life increases to 17 s. On the other hand, if the affinity is determined in two steps, where the internal equilibrium constant is, say, 50 in favour of product, then the half-lives will range, instead, from $0.0035 - 0.35\ s^{-1}$. Hence, addition of the second step allows the system to maintain the overall affinity for substrate while allowing to enzyme to sample far greater number of substrates in a given period of time.

The active-site surface of an enzyme restructures continuously as the system moves through its catalytic cycle – R-groups are recruited and dispatched to perform reaction-stage specific functions. Whether a particular enzyme form participates in substrate selection is determined by whether it occurs before or after the system has committed to the forward reaction. For example, in the case where a single step is rate-determining, all forms that appear subsequent to the slow step will not contribute to selection, since the system cannot return from that region of the reaction coordinate – it is committed to the forward path. Thus, as product release becomes slow enough to allow both binding and surface chemistry to approach equilibrium, the product-bound forms of the enzyme become active in the selection of substrate, and $K_{eq\ int}$ can become a significant selection determinant.

While product release from EST is not slow enough to allow both binding and chemistry to reach near-equilibrium, it *is* slow enough to approach this condition and for the effects of the internal equilibrium constant to play significantly into the selection process. To appreciate how the EST mechanism can produce such effects, consider Mechanism II, which represents the binding PAPS to $E{\cdot}E_2$, followed by formation and release of products (PAPS = S, $E{\cdot}E_2$ = E).

$$E \underset{k_{off}}{\overset{[S]k_{on}}{\rightleftharpoons}} E \cdot S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} E \cdot P \xrightarrow{k_3} E + P \qquad \text{(II)}$$

$K_{m\ S}$ for this mechanism is given by Equation 1.

$$K_m = \frac{k_{off}k_2 + k_{off}k_2 + k_1k_3}{k_{on}(k_1 + k_2 + k_3)} \qquad (1)$$

For the EST mechanism, it has been demonstrated that $k_1 >> k_3$, $k_1 >> k_2$, and $k_{off} >> k_1$. Given these constraints, Equation 1 reduces to 2.

$$K_m = \frac{k_{off}}{k_{on}} \cdot \frac{k_2 + k_3}{k_1} \qquad (2)$$

It is important to realize that $(k_2 + k_3)/k_1$ represents the ratio of [E·S] to [E·P] in the steady state, $([E{\cdot}S]/[E{\cdot}P])_{ss}$. That this is so can be seen by applying the steady-state assumption to the rate of formation and disappearance of EP:

$$\frac{dEP}{dt} = [ES]k_1 = [EP](k_2 + k_3) = -\frac{dEP}{dt} \qquad (3)$$

Rearranging,

$$\left(\frac{[ES]}{[EP]}\right)_{SS} = \frac{k_2 + k_3}{k_1} \qquad (4)$$

Thus, for the EST mechanism, $K_m$ is a linear function of the steady-state, mass-ratio of the central complexes (Equation 2). Given experimental values for $k_1$, $k_2$ and $k_3$ ($4.4\ s^{-1}$, $0.070\ s^{-1}$ and $0.072\ s^{-1}$ [18]), $([E{\cdot}S]/[E{\cdot}P])_{ss}$ is calculated at 0.031, which, when multiplied by $K_d$ for PAPS binding to $E{\cdot}E_2$, yields a $K_m$ of 130 nM – a value that is in reasonable agreement with the published value of 59 nM [21].

The EST mechanism has evolved to a point where its rate constants are balanced to control the steady-state central complex mass ratio, with the result that the affinity of both $E_2$ and PAPS are enhanced ~30-fold. It is interesting to consider that changes in this balance across a series of substrates will bias substrate selection, and further, that both the substrate and product forms of the acceptor are ''scrutinized'' by the enzyme during the selection phase of the catalytic cycle. One is left to wonder to what extent similar mechanisms play important roles in the selection of substrate within the highly conserved sulfotransferase family.

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