Molecular Recognition in Biological Systems: Phosphate Esters vs Sulfate Esters and the Mechanism of Action of Steroid Sulfatases

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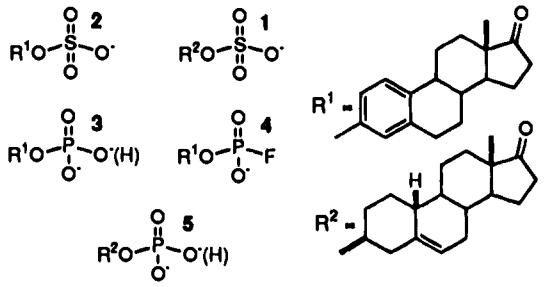
The enzyme-catalyzed desulfation of steroids is a transformation that plays an important role in biological processes as diverse as fertilization,[1] breast cancer[2,3] and cholesterol transport and metabolism.[4,5] This communication describes the development of potent inhibitors of steroid sulfatase. A proposed binding mode for these inhibitors based on a transition state analogy (or alternatively a ground state bisubstrate mimic) is then incorporated into a model for the mechanism of action of this enzyme.

Steroid sulfation is one of the most common of all forms of steroid conjugation. With the exception of cholesterol, dehydroepiandrosterone sulfate (**1**) is the most abundant of all plasma steroids.[6,7] In addition, estrone sulfate (**2**) is the most abundant of all the estrogens.[8] *In vivo*, steroid sulfatases are responsible for the deconjugation of these steroids, an action that is normally required before the steroid can execute its proper biological function. Steroid sulfatase activity is commonly found in a variety of tissue types, but it is particularly prevalent in placenta[6] and in breast tumors.[3,9–11] Because breast tumors show high levels of estrone sulfatase activity and a substantial percentage of breast tumors are estrogen dependent, the sulfatase expressed in breast tumors has become a target for chemotherapeutic intervention in breast cancer.[12,13] However, relatively little is known about the mechanism of action of this group of enzymes. Indeed, sulfatases as a whole remain the most poorly understood of all the major classes of hydrolytic enzymes.

We were curious about the enzyme's ability to differentiate between charged species with geometries closely related to sulfate esters, such as phosphate esters or their derivatives. Accordingly, we synthesized and assayed phosphorylated steroids **3**–**5**.[14]

As shown in Table 1, phosphorylated compounds **3**–**5** are all good inhibitors of steroid sulfatase, binding with an affinity that is nearly the same as or better than that of the substrate, estrone sulfate. However, in no case was hydrolysis of the inhibitors observed. Apparently, while the enzyme cannot distinguish between the phosphoryl group and the sulfuryl group



with respect to binding, it can easily distinguish between the two with respect to catalysis. Notably, the enzyme binds steroid phosphates substantially better than it binds the natural substrate, estrone sulfate. On the basis of theoretical studies[15] and crystallographic comparisons of sulfate- and phosphate-binding proteins[16,17] such a pattern of binding was totally unexpected.

To determine which form of the phosphate ester (the mono- or dianion) binds most tightly to the sulfatase, we measured the K_i as a function of pH. As shown in Table 1, the K_i for phosphate **3** drops with decreasing pH. Although the K_m for substrate also varies over this pH range, the change is rather modest. However, the change in binding of estrone phosphate varies by a factor of about 300. The limiting K_i value of 52.3 μM at high pH reflects the intrinsically poor binding of the phosphate dianion to the enzyme. In fact, this inhibition is probably due largely to residual amounts of estrone phosphate monoanion.[19] Apparently, the monoanions of steroidal phosphates bind to the sulfatase with exceptional affinity, since the

Table 1. K_i's for Compounds **2**–**6**[a]

compd	pH	K_i or K_m (μM)	K_m (mM) *p*-acetylphenyl sulfate
2	6.0	1.5 ± 0.3	
2	7.5	3.5 ± 0.4	
3	6.0	0.17 ± 0.01	0.7 ± 0.1
3	6.5	0.34 ± 0.09	0.6 ± 0.1
3	7.0	0.89 ± 0.15	0.7 ± 0.1
3	7.5	5.0 ± 0.7	1.1 ± 0.2
3	8.0	15.0 ± 1.1	2.6 ± 0.1
3	8.5	52.3 ± 1.1	8.8 ± 0.3
4[b]	6.0	14.7	
4	7.5	13.7 ± 1.6	
5[b]	6.0	0.14	
5[b]	7.5	2.0	

[a] Lineweaver–Burk plots were used to obtain K_m of steroid sulfatase for either estrone sulfate or *p*-acetylphenyl sulfate. The slopes of Lineweaver–Burk plots, generated from initial rates of *p*-acetylphenyl sulfate hydrolysis in the presence and absence of the inhibitors listed above, were plotted as a function of inhibitor concentration to determine K_i. The reaction mixture consisted of 0.2 M BIS-Tris propane–acetate buffer, pH as indicated, 0.2 mM Triton X-100, the appropriate concentration of substrate and inhibitor, and 3.5–7.1 μg of protein. The amount of protein for reactions with **2** was 0.7–1.4 μg of protein. The reaction was incubated at 37 °C and quenched into 1 M NaOH. The enzyme source was steroid sulfatase partially purified from human placenta by ion-exchange chromatography using a DEAE-cellulose column,[18] the specific activity was (110–242) × 10^{-3} units/(mg of protein) (nmol of hydrolyzed *p*-acetylphenyl sulfate min^{-1} (mg of protein)$^{-1}$). Each K_i and K_m value represents the mean of at least two independent determinations. [b] Data is the result of one determination.

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(19) The change in K_i of the inhibitor, over the pH range examined, approximately correlates with the change in concentration of estrone phosphate monoanion as predicted by the Henderson–Hasselbalch equation.

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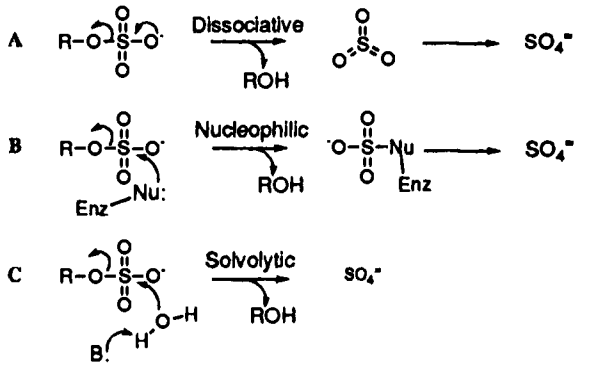
Scheme 1

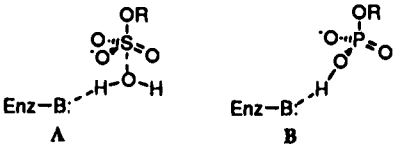


K_i for DHEA phosphate at pH 6.0 is 140 nM. Given the complete lack of structural data about this enzyme's active site, it is not immediately apparent why an enzyme that hydrolyzes sulfate esters should be inhibited so powerfully by phosphate esters. Indeed, the ability of sulfate- and phosphate-binding proteins to discriminate between the two species is well established.[16,17] Moreover, phosphatases, even those that operate on the monoanions of phosphate esters, such as prostatic acid phosphatase, are very poorly inhibited by sulfate esters,[20] indicating that these enzymes can easily distinguish between phosphates and sulfates with respect to both binding and catalysis. In the current instance, the rather striking departure from the norm may be dictated by the catalytic imperatives of the enzyme active site. In such cases, it may be instructive to seek a mechanistic rationale for the behavior of this enzyme.

Several possible mechanisms for the enzyme-catalyzed reaction are shown in Scheme 1. The enzyme-catalyzed hydrolysis might be dissociative in nature (as shown in A), proceeding via the intermediacy of SO_3. On the other hand, the reaction might be associative, proceeding via a sulfated enzyme intermediate (shown in B), or via the direct attack of a bound water molecule (shown in C). In the case of pathways B and C, the mechanism might be stepwise via a pentacoordinate intermediate, or concerted, with a single pentacoordinate transition state. Because the stereochemistry of enzyme-catalyzed sulfate group transfers to water cannot currently be ascertained, it is difficult to discriminate between these mechanistic alternatives. In this light, our inhibitor data may be useful in making such a distinction.[21]

In pathway C (Scheme 1) a putative active site base acts to deprotonate an incoming water molecule (**A** in Scheme 2). The peripheral OH group of the phosphate monoanion of **3** is

Scheme 2



probably well situated to mimic this incoming water molecule and make a hydrogen bond to the active site base (**B** in Scheme 2). Such a combination should result in a very strong hydrogen bond because of the matching of the pK_a's of the groups involved.[22] (The pH dependence of k_{cat} suggests the involvement of an active base with a pK_a of 5.8.[23] Chemical labeling studies have implicated a histidine residue as this base. The pK_a of estrone phosphate in the presence of Triton is 5.9.) The other mechanistic options outlined in Scheme 1 provide no readily apparent rationale for the powerful inhibition exhibited by **3** and **5**. This is underscored by the behavior of compound **4**. This compound contains a phosphoryl group that is singly charged (like a sulfate ester) and has only H-bond-accepting components. Unlike the phosphate ester monoanions, this compound cannot donate an H-bond to an active site residue. Therefore, it binds with a much lower affinity than the phosphate ester.

We have demonstrated a strategy for the design of tight-binding inhibitors of estrone sulfatase. It is possible to understand the inhibitory potency of these compounds by reference to a transition state or ground state bisubstrate analogy. Although the evidence presented herein is only suggestive, it is well accommodated by a mechanism involving general base promoted attack by a bound water molecule. Our results also predict that phosphonates, which exist largely as the monoanion at neutral pH, will be much better sulfatase inhibitors than sulfonates. This hypothesis is currently being tested.

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Supplementary Material Available: Dixon plots for estrone phosphate, estrone phosphofluoridate, and dehydroepiandrosterone phosphate and Lineweaver–Burk plots (12 pages). This material is contained in many libraries on microfiche, immediately follows this article in the microfilm version of the journal, can be ordered from the ACS, and can be downloaded from the Internet; see any current masthead page for ordering information and Internet access instructions.

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(21) Although caution must be exercised in choosing between mechanistic alternatives solely on the basis of inhibitor data, the alternative is to assume that the tight binding of inhibitor **3** is entirely fortuitous, something that is certainly possible, but is an ad hoc explanation of the available data.

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