peptides and their exact depsipeptide analogues at nearly equal rates despite the inherently more labile character of the esters (Bruice & Benkovic, 1966). If product release is rate-determing for the depsipeptide, then cleavage of the scissile bond may occur as a rapid early step in catalysis. The rate of completing turnover would then be determined by the subsequent slower product release step that could occur at rates comparable to those for peptide cleavage.

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Detection of a Transient Enzyme–Steroid Complex during Active-Site-Directed Irreversible Inhibition of 3-Oxo-Δ^5-steroid Isomerase†

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ABSTRACT: The reaction of the active-site-directed irreversible inhibitor (17*S*)-spiro[estra-1,3,5(10),6,8-pentaene-17,2′-oxiran]-3-ol (**5**β) with 3-oxo-Δ^5-steroid isomerase has been monitored by repetitive scanning ultraviolet spectroscopy of a solution of **5**β plus isomerase against a blank containing only **5**β. Upon initial mixing of **5**β with the isomerase an absorbance maximum at ca. 250 nm appears. With time, this peak decreases and is replaced with a new peak near 280 nm. These results directly demonstrate the existence of a transient enzyme–steroid intermediate in the inactivation reaction. The ultraviolet spectrum suggests that the steroid in the transient complex resembles the ionized phenol, while the phenolic group in the irreversibly bound complex is un-ionized. These spectral studies support our previous proposal that there are two enzyme–steroid complexes that are related by a 180° rotation about an axis perpendicular to the plane of the steroid nucleus. This hypothesis offers an explanation for the reaction of 17β-oxiranes with the same residue (Asp-38) that is thought to be involved in the catalytic mechanism. Two new oxiranes, (17*S*)-spiro[estra-1,3,5(10)-triene-17,2′-oxiran]-3β-ol (**6**β) and (17*S*)-spiro[5α-androstane-17,2′-oxiran]-3-one (**8**β), were also found to be potent active-site-directed irreversible inhibitors of the isomerase (k_3/K_I = 31 M^{-1} s^{-1} and 340 M^{-1} s^{-1}, respectively). The relationship of these results to the nature of the active site of the isomerase is discussed.

Active-site-directed irreversible inhibitors of steroid-metabolizing enzymes and affinity labels of steroid receptors have yielded significant insights into the molecular mechanism of biologically important processes (Gronemeyer, 1985; Katzenellenbogen, 1977, 1984; Benisek et al., 1982; Batzold et al., 1976). Fundamental to the design and utilization of such compounds is the existence of a high-affinity stereospecific steroid–protein interaction. However, there is recent evidence that several steroid-transforming enzymes may bind steroids in more than one orientation (Sweet & Samant, 1980; Bevins et al., 1980; Strickler et al., 1980; Adams & McDonald, 1981; Kayser et al., 1983; Waxman et al., 1983). Clearly, consideration must be given to the existence of multiple binding modes in the interpretation of the nature of binding of both substrates and inhibitors to the active site. In addition, it is possible that multiple binding modes of natural ligands may be biologically significant.

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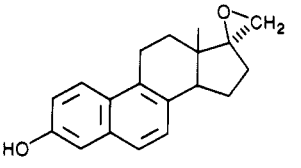
In our studies of 3-oxo-Δ^5-steroid isomerase (EC 5.3.3.1, steroid Δ-isomerase), we have proposed that at least two binding modes exist for steroid binding at the active site (Bevins et al., 1980; Kayser et al., 1983; Bantia et al., 1985). This conclusion is based on three observations: (1) Both 3β- and 17β-oxiranyl steroids are affinity labels that inactivate the isomerase, apparently by the same mechanism (Pollack et al., 1979; Bevins et al., 1980, 1984; Kayser et al., 1983; Bantia et al., 1985); the second-order rate constants at pH 7 for inactivation by a series of 17β-oxiranes (85–300 M^{-1} s^{-1}; Bevins et al., 1980) are comparable to the rate constants for the 3β-oxiranes (67–230 M^{-1} s^{-1}; Pollack et al., 1979, 1986); inactivation by both 3β- and 17β-oxiranes occurs by stoichiometric covalent bond formation between the steroid and an enzyme carboxylate residue which attacks from the α-face of the steroid (Kayser et al., 1983; Bevins et al., 1984; Bantia et al., 1985). (2) Covalent modification of Asp-38 accompanies the enzyme inactivation for both affinity labeling by 17β-oxiranes and photoaffinity labeling by 3-oxo-4-estren-17β-yl acetate (Martyr & Benisek, 1975; Ogez et al., 1977; Hearne & Benisek, 1985). In the latter, case, Asp-38 is modified by a steroid whose reactive component is in the A ring, whereas the 17β-oxiranes modify the same amino acid by reaction with a reactive residue in the D ring. (The site of covalent attachment for the 3β-oxiranes has not yet been determined.) (3) A first-order decay in the fluorescence emission at 420 nm occurs upon incubation of the isomerase with the affinity label (17*S*)-spiro[estra-1,3,5(10),6,8-pentaene-17,2′-oxiran]-3-ol (**5**β) at a rate in agreement with the rate of enzyme inactivation (Bevins et al., 1980). Since the fluorescence emission at this wavelength is negligible both for enzyme alone and for **5**β alone (Wang et al., 1963), this result supports the existence of at least two enzyme–steroid complexes—one that has a significant fluorescence emission at 420 nm and one that does not.



5β

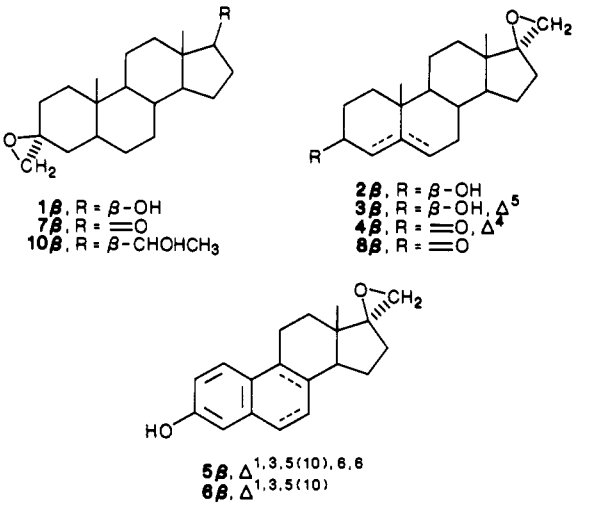
We present here repetitive scanning ultraviolet spectroscopic measurements obtained during the inactivation of the isomerase by **5**β. By using this technique, we were able to detect a transient enzyme–steroid intermediate whose spectral characteristics closely resemble those of the corresponding phenolate ion. These results clearly demonstrate the existence of two enzyme–steroid complexes and are consistent with our "backward binding" model of inactivation. In addition, we report kinetic parameters for the inactivation of the isomerase by two new oxirane irreversible inhibitors.

Materials and Methods

Materials. The preparation of (17*S*)-spiro[estra-1,3,5-(10),6,8-pentaene-17,2′-oxiran]-3-ol (**5**β) and the isolation and purification of the isomerase have been described previously (Bevins et al., 1980; Kayser et al., 1983). The specific activity of the isomerase used in this work was 47 000 units/mg. Water was double-distilled, and all other reagents were the highest available grade. All steroids not described were purchased from Sigma.

(*17S*)-*Spiro*[*estra-1,3,5(10)-triene-17,2′-oxiran*]-*3-ol* (**6**β) (see Chart I) was prepared by using the procedure of Cook et al. (1968): mp 183–185 °C (lit. mp 181–183 °C; Cook et al., 1968).

Chart I



(*17S*)-*Spiro*[*5α-androstane-17,2′-oxiran*]-*3*-one (**8**β) was synthesized by oxidation of (17*S*)-spiro[5α-androstane-17,2′-oxiran]-3β-ol (**2**β; Bevins et al., 1980) by using a 2-fold excess of pyridinium chlorochromate (Corey & Suggs, 1975). Silica gel chromatography (methylene chloride/hexane, 3:1) and recrystallization from hexane gave a product that was homogeneous by high-performance liquid chromatography (HPLC) (μBondapak C_{18}): yield, 25%; mp 179.5–181.5 °C; NMR ($CDCl_3$) δ 0.91 (18-CH_3), 1.03 (19-CH_3), 2.75 (AB pattern, J = 5 Hz, oxirane CH_2). Anal.

Spectroscopic Measurements. A solution of **5**β (7.28 μM) in 0.03 M potassium phosphate buffer (pH 7.0, 0.98 mL) containing 1.7% MeOH (v/v) at 27.1 °C was blanked in a Cary Model 16 recording spectrophotometer against a second solution of identical composition. The base line (machine auto base line zeroing) was then recorded. At time zero, 1.2 nmol of isomerase in 20 μL of 0.01 M potassium phosphate buffer (pH 7) was added to the sample solution and thoroughly mixed. An equivalent volume of the potassium phosphate buffer was added to the blank solution. At various time intervals, the absorbance spectrum of the reaction solution vs. the blank solution was recorded (0.05 nm/s scan rate). In the interim between scans, the absorbance difference at 250 nm was also recorded at various times. At 270 s, concentrations of free enzyme, reversible enzyme–**5**β complex, and covalent enzyme–**5**β complex were calculated by using the known values of the kinetic constants for inactivation (Bevins et al., 1980).

Kinetics of Inactivation. The determination of the time and concentration dependence of inactivation by oxiranes has been previously described (Bevins et al., 1980; Pollack et al., 1979).

Results

The ultraviolet spectrum of a solution of **5**β (7.28 μM) and isomerase (1.2 μM) at pH 7.0 was scanned at various times against a blank containing only **5**β (Figure 1). The scan at 6.9 h shows an absorbance maximum near 280 nm, a slight absorbance trough at 265 nm, and a large end absorbance. This spectrum resembles that for the native isomerase, which shows λ_{max} = 277 nm and λ_{min} = 250 nm (Kawahara et al., 1962; Jarabak et al., 1969; Tivol et al., 1975). At earlier times the spectrum is more complex. At 270 s there is an absorbance maximum at ca. 250 nm; in scans at successive time intervals this absorbance becomes smaller and an isosbestic point at 242 nm becomes evident.

The decrease in the absorbance at 250 nm was also monitored as a function of time as shown in Figure 2. The decrease in the absorbance at 250 nm is first order with a rate

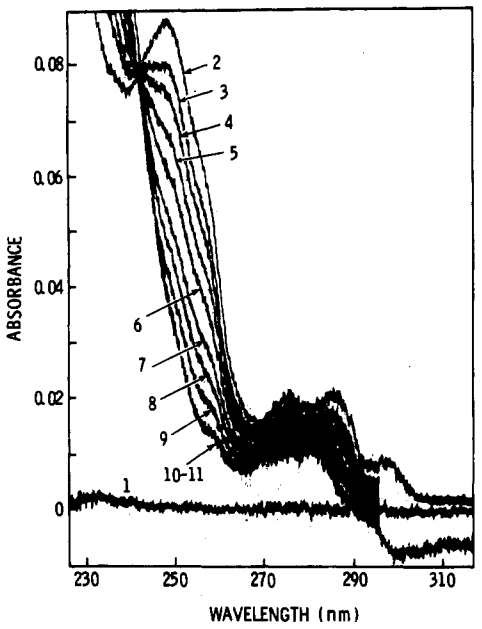


FIGURE 1: Ultraviolet absorbance changes of a solution containing (17*S*)-spiro[estra-1,3,5(10),6,8-pentaene-17,2′-oxiran]-3-ol (**5**β) and 3-oxo-Δ^5-steroid isomerase at 27 °C. A solution (0.98 mL) containing 7.43 μM **5**β, 1.73% (v/v) methanol, and 0.033 M potassium phosphate buffer (pH 7) was blanked against a solution of identical composition (scan 1). At time zero, 1.2 nmol of isomerase in 20 μL of neutral potassium phosphate buffer (0.033 M) was added to the experimental solution. An equivalent amount of phosphate buffer without enzyme was added to the blank. Spectral scans (0.5 nm/s) were initiated at the following incubation times: scan 2, 4.5 min; scan 3, 16.6 min; scan 4, 26.1 min; scan 5, 43.3 min; scan 6, 1.07 h; scan 7, 1.66 h; scan 8, 2.11 h; scan 9, 2.96 h; scan 10, 4.40 h; scan 11, 6.89 h. The path length was 1 cm.

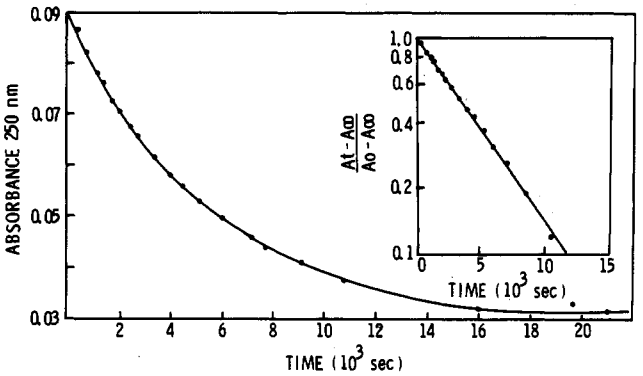


FIGURE 2: Ultraviolet absorption changes at 250 nm of a solution containing (17*S*)-spiro[estra-1,3,5(10),6,8-pentaene-17,2′-oxiran]-3-ol (**5**β) and 3-oxo-Δ^5-steroid isomerase at 27 °C. The incubation medium contained 7.28 μM **5**β, 1.2 μM isomerase, 1.7% (v/v) methanol, and 0.033 M potassium phosphate buffer (pH 7) in a final volume of 1.0 mL. The absorbance was measured against a blank containing all components except enzyme (see Materials and Methods).

constant (1.95×10^{-4} s^{-1}) in excellent agreement with the rate constant of 1.86×10^{-4} s^{-1} for inactivation of the isomerase by **5**β under the same experimental conditions (Bevins et al., 1980). A similar experiment at 1.9 μM **5**β also gave a rate constant (1.14×10^{-4} s^{-1}) indistinguishable from the rate constant for inactivation (1.17×10^{-4} s^{-1}).

In an effort to further characterize the absorbance peak observed in the scan at the early time point, the absorbance spectrum of the initial scan (at 270 s) was subtracted from the final scan (at 6.9 h). This procedure effectively removes the absorbance contribution of the native enzyme (and unbound steroid in the blank solution) and yields a difference spectrum of reversibly bound steroid *minus* the absorbance of an equivalent amount of irreversibly bound steroid (Figure 3A).[1] This spectrum bears a striking resemblance to the difference spectrum for ionized and un-ionized **5**β (Figure 3B). The major peaks in these two difference spectra have similar wavelengths (256 nm for Figure 3A and 248 nm for Figure 3B) and extinction coefficients (log ϵ = 4.76 for Figure 3A and log ϵ = 4.56 for Figure 3B). The minor peaks and isosbestic points are similarly comparable.

Table I: Kinetic Constants for Irreversible Inhibition of 3-Oxo-Δ^5-steroid Isomerase by 3β- and 17β-Oxiranes[a]

compd	K_I (μM)	k_3 (s^{-1})	k_3/k_I (M^{-1} s^{-1})	ref
1β	17 ± 3	(1.13 ± 1.9) × 10^{-3}	67 ± 4	*b*
2β	40 ± 25	(1.2 ± 0.7) × 10^{-2}	300 ± 20	*c*
3β	47 ± 12	(7.2 ± 1.9) × 10^{-3}	150 ± 20	*c*
4β	50 ± 7	(4.3 ± 0.6) × 10^{-3}	85 ± 30	*c*
5β	1.7 ± 0.3	(2.3 ± 0.2) × 10^{-4}	130 ± 20	*c*
6β	5.4 ± 1.5	(1.7 ± 0:4) × 10^{-4}	31 ± 11	*d*
7β	70 ± 35	(1.6 ± 0.7) × 10^{-2}	230 ± 20	*e*
8β	46 ± 20	(1.6 ± 0.6) × 10^{-2}	340 ± 30	*d*
10β	29	1.5×10^{-3}	52	*f*

[a] pH 7.0, 21.0 °C, 3.8% methanol for **1**β–**4**β, **7**β, and **8**β; pH 7.0, 27.0 °C, 1.7% methanol for **5**β and **6**β; pH 7.0, 25 °C for **10**β. [b] Pollack et al., 1979. [c] Bevins et al., 1980. [d] This work. [e] Pollack et al., 1986. [f] Penning, 1985.

The kinetic parameters for inactivation of the isomerase by two other oxiranes, (17*S*)-spiro[estra-1,3,5(10)-triene-17,2′-oxiran]-3-ol (**6**β) and (17*S*)-spiro[5α-androstane-17,2′-oxiran]-3-one (**8**β), were determined at pH 7.0 (0.03 M phosphate) and 21.0 °C. Both oxiranes show time-dependent irreversible inhibition leading to complete inactivation of the enzyme. Pseudo-first-order kinetics are obeyed over 3–4 half-lives or more, and the variation of the rate constant is consistent with saturation kinetics (eq 1). Values of the

$$E + I \underset{}{\overset{K_I}{\rightleftharpoons}} E{\cdot}I \xrightarrow{k_3} E\text{–}I \text{ (inactive enzyme)} \qquad (1)$$

binding constants (K_I) and rate constants (k_3) were determined by the method of Kitz and Wilson (1962) and are given in Table I, along with those for other oxiranes of the 3β and 17β series.

Discussion

We have previously shown that **5**β and other 17β-oxiranes are potent active-site-directed irreversible inhibitors of steroid isomerase (Bevins et al., 1980). The site of covalent attachment of the 17β-oxiranes is aspartic acid-38 (Kayser et al., 1983), an amino acid thought to participate in the catalytic mechanism of the enzyme (Martyr & Benisek, 1975; Ogez et al., 1977; Benisek et al., 1980; Kayser et al., 1983; Bantia et al., 1985; Hearne & Benisek, 1985). We have proposed that 17β-oxiranes gain access to Asp-38 by binding at the active site in a backward mode, that is, with the D ring of the steroid nucleus in the A ring binding site of the enzyme (Bevins et al., 1980; Kayser et al., 1983). The two modes of binding

[1] Two assumptions are inherent in this approach. The initial scan is actually of a rather complex mixture containing 0.07 μM enzyme with **5**β irreversibly bound, 0.18 μM enzyme with no bound steroid, 0.95 μM enzyme with **5B** reversibly bound, and 6.26 μM of **5**β free in solution (calculated by using observed values of k_3 and K_I; Bevins et al., 1980). The final spectrum is of a solution containing 1.20 μM enzyme with **5**β irreversibly bound plus 6.08 μM **5**β free in solution. Our model assumes the following: (1) Free enzyme and enzyme with bound steroid (reversibly or irreversibly) have similar spectra. This assumption is not crucial since the contribution of the enzyme to the absorbance spectra is small relative to that for **5**β at wavelengths greater than 240 nm. (2) The spectrum of irreversibly bound steroid is the same as its spectrum in solution. This assumption appears to be validated by the observed results.

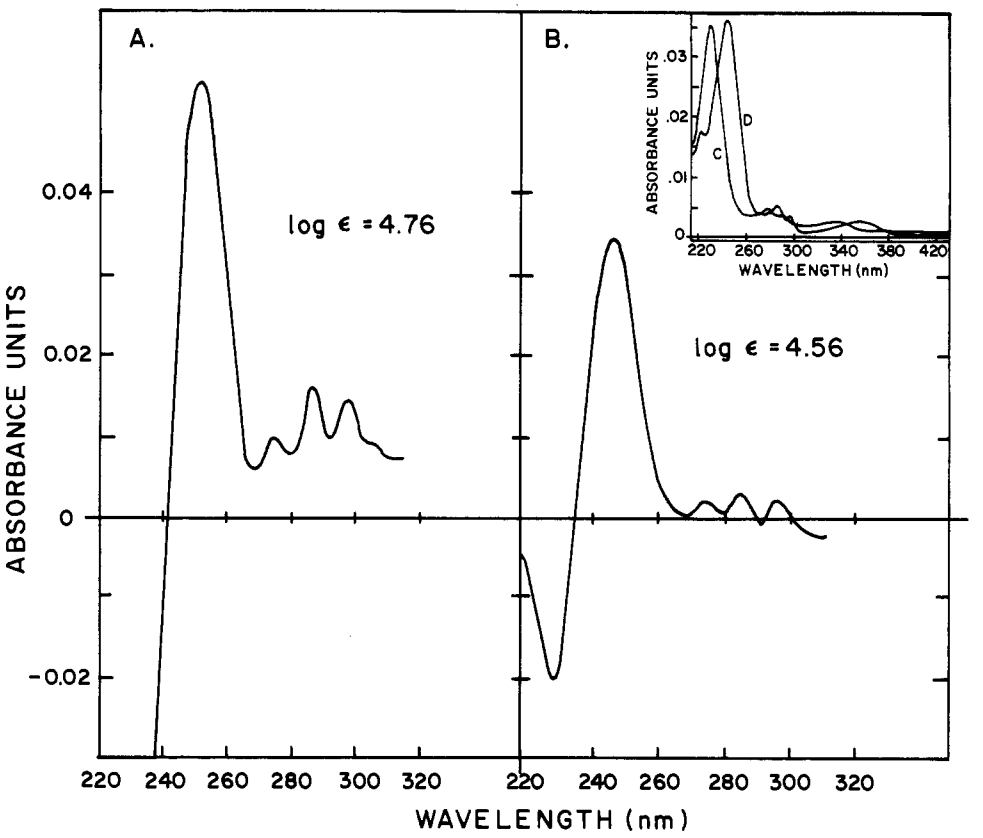
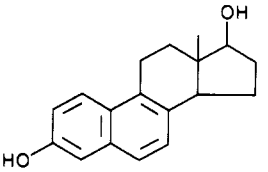


FIGURE 3: Ultraviolet absorbance difference spectra. (A) Difference spectrum representative of 5β reversibly vs. 5β irreversibly bound to 3-oxo-Δ^5-steroid isomerase. The difference spectrum was generated by subtracting the final scan (6.9 h) from the initial scan (270 s) presented in Figure 1. The extinction coefficient for the major peak was calculated by using an estimate of the amount of reversibly bound 5β in the initial scan (0.98 μM) as described under Materials and Methods. (B) Calculated difference spectrum of 5β at 0.95 μM in basic (pH 12) vs. neutral (pH 7) solution. Inset: Spectra of 5β (0.73 μM) measured at pH 7 (C) and 12 (D). The path length was 1 cm for all spectra.

are thought to be related by a rotation of 180° about an axis perpendicular to the plane of the steroid nucleus, allowing the 17β-oxirane moiety to be in close proximity to the catalytically important residues of the enzyme.

Our investigation of the spectral changes of phenolic steroids during inactivation of the isomerase was designed to specifically characterize the intermediate complex proposed by our model. The choice of 5β was prompted by the observation of Wang et al. (1963) that estra-1,3,5(10),6,8-pentaene-3,17β-diol (**9**) undergoes a marked change in its ultraviolet and



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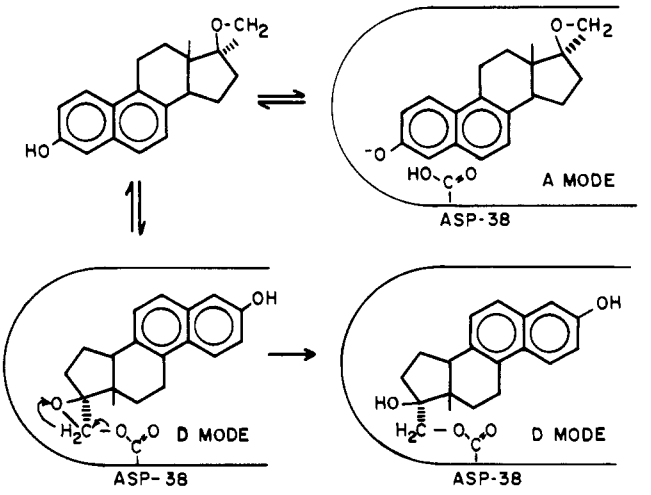
fluorescence spectra upon binding to the isomerase. The spectra of reversibly bound steroid are quite similar to those of the ionized form of **9** in aqueous solution. If 5β were to bind rapidly and reversibly to the enzyme in the same manner as **9**, then it should display similar spectral characteristics. Furthermore, if irreversible inactivation is occurring through a backward binding mode, the ultraviolet spectrum of the covalent enzyme–inhibitor complex might not show this perturbed spectrum. If this model is valid, one should observe a change in the spectral properties of a solution of 5β immediately upon addition of isomerase, reflecting the reversible binding of 5β to the enzyme. With time, the spectral properties of the solution would then change as 5β irreversibly binds to the enzyme.

Scheme I



By using repetitive scanning ultraviolet spectroscopy, we are now able to clearly demonstrate the formation of a transient enzyme–steroid complex. The spectral characteristics of this transient complex are remarkably similar to those of the ionized form of the phenolic steroid (Figure 3). The observed decay of this intermediate is first order, with a rate virtually identical with the rate of enzyme inactivation (Figure 2). Since it has been firmly established that Asp-38 is accessible to reactive groups located at either the A ring (Martyr & Benisek, 1975; Ogez et al., 1977; Hearne & Benisek, 1985) *or* the D ring (Kayser et al., 1983) of certain affinity labels, the results presented here are most reasonably accommodated by the existence of backward binding as diagramed in Scheme

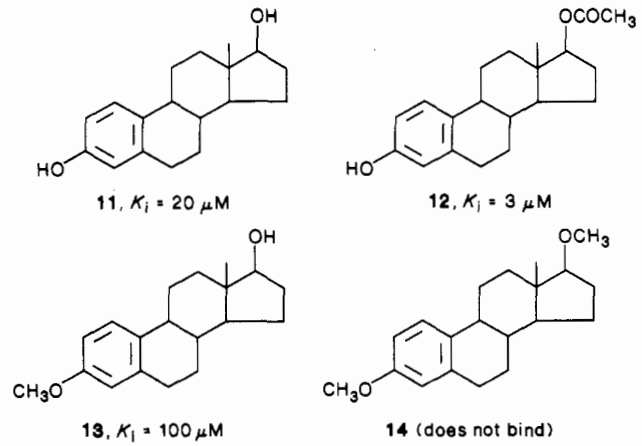
I. When bound in the A mode, **5**β is oriented in a similar manner to bound substrate during catalysis. The ionization of the phenolic hydrogen could be due to the carboxylate (Asp-38), which ultimately forms a covalent bond with the steroid.

Westbrook et al. (1984) have examined the 6-Å crystal structure of the isomerase and concluded that the steroid binding site is composed of a deep hydrophobic pit. Hearne and Benisek (1985) have concluded on the basis of their studies on the photoaffinity labeling of the isomerase by a solid-phase reagent that Asp-38 lies near the bottom of this pit. If this interpretation is correct, then the binding mode of **5**β that shows spectral properties of an ionized phenol is the one with the phenolic group in a very hydrophobic environment. Since Asp-38 is "hyperreactive" (Benisek et al., 1980), it may well be sufficiently basic to abstract a phenolic proton. The phenolate ion that is formed must therefore be solvated better in the hydrophobic pit than it would be in a completely aqueous environment, since **9** is not ionized in aqueous solution at pH 7. Possible candidates for hydrogen bond donors to the phenoxide oxygen are the carboxyl of Asp-38 (in the protonated form) and the group proposed by Malhotra and Ringold (1965) to act to polarize the 3-oxo group of the substrate in the normal catalytic reaction.

Aside from offering a reasonable explanation for the observed similarities between the inactivation by 3β- and 17β-oxiranes and for the observed spectral changes seen upon inactivation by **5**β, backward binding can explain some other interesting phenomena regarding the isomerase. Although the second-order rate constants of inactivation (k_3/K_I) are comparable for various oxiranyl inhibitors, the individual binding (K_I) and rate (k_3) constants vary widely. For example, **5**β and **3**β have almost identical k_3/K_I values (130 and 150 M^{-1} s^{-1}, respectively; Bevins et al., 1980). Yet **5**β binds tightly (K_I = 1.7 μM) and reacts slowly ($k_3 = 2.3 \times 10^{-4}$ s^{-1}), while **3**β binds 25-fold less well (K_I = 47 μM) and reacts 25-fold faster ($k_3 = 7.2 \times 10^{-3}$ s^{-1}). These results may be rationalized if one assumes that because of the presence of the planar A ring in **5**β (a structural feature that enhances binding of steroids to the isomerase; Wang et al., 1963; Weintraub et al., 1977), binding to the isomerase in the A mode (the nonproductive mode for inactivation by this compound) is preferred and is of high affinity.[2] Under saturating conditions, then, virtually all of the **5**β bound to the isomerase would be in the nonproductive mode, and the maximum rate of inhibition (k_3) would be low. Similarly, **6**β binds very tightly (K_I = 5.4 μM) but also inhibits slowly ($k_3 = 1.7 \times 10^{-4}$ s^{-1}). In essence, the inhibitor molecule, when bound in the nonproductive mode, "protects" the active site from covalent modification.

All other 3β- and 17β-oxiranes tested show substantially higher values for K_I and k_3 than either **5**β or **6**β but comparable values for k_3/K_I (Table I). Since the value of k_3/K_I reflects only the energy difference between the free reactants (enzyme plus inhibitor) in solution and the transition state, it is unaffected by the existence of backward binding. A comparison of k_3/K_I values then allows the relative energies of the transition states for inactivation by the 3β- and 17β-oxiranes (relative to the ground states) to be determined. The similarity in the k_3/K_I ratios for the oxiranes of both the 3β and 17β series indicates that the transition state for the inactivation is of comparable energy for all of the oxiranes. This result suggests that the dissociation constants for A mode binding of the 3β-oxiranes and D mode binding of the 17β-oxiranes are comparable. Similarly, the fact that the second-order rate constants for inactivation (k_3/K_I) for the 17β-oxiranes vary only slightly with the nature of the A and B rings suggests that when a steroid is bound in the D mode the interactions with the A and B rings are unimportant.

Chart II



Multiple binding modes must be considered in interpreting studies of the relationship between structure and affinity of steroids for the active site of the isomerase. For example, the effect of substitution at the 3- and 17-positions of estradiol on the K_i values in 10% methanol (Weintraub et al., 1977) can be satisfactorily accounted for by the backward binding hypothesis. Both the 17-acetate (**12**) and the 3-methoxy derivative (**13**) of estradiol (see Chart II) are competitive inhibitors of the isomerase, but the 3,17-dimethoxy compound (**14**) shows no evidence of binding. In terms of the multiple binding model, substitution at the 17-position would give a steroid that still can bind in mode A if the D ring (in mode A) is in an environment that is accessible to solvent and can accommodate a bulky substituent. Substitution at the 3-hydroxyl, on the other hand, may allow only D mode binding if there are severe steric constraints at the normal A ring binding site. Substitution at both the 3- and 17-positions then would prevent binding in either mode.

It is reasonable to suspect that the existence of wrong-way binding might be common to many enzymes of steroid metabolism and, possibly, to steroid receptors as well. For example, the enzyme 3β(17β)-hydroxysteroid dehydrogenase from *Pseudomonas testosteroni* is known to show activity toward both 3β- and 17β-alcohols (Talalay & Marcus, 1956; Delin et al., 1964). This fact can most easily be explained if there are two modes of binding of the substrate, similar to what we have proposed for the reaction of 3β- and 17β-oxiranes with the isomerase. The possibility of backward binding has also recently been dicussed for other steroid-transforming enzymes. Both Sweet and Samant (1980) and Strickler et al. (1980) have suggested that the dual activity of 3α(20β)-hydroxysteroid dehydrogenase may lie in its ability to bind steroids in two ways. A similar proposal has been put forth to explain how purified steroid alcohol sulfotransferase has the ability to react with hydroxyl groups at both the 3- and 17-positions of the steroid nucleus (Adams & McDonald, 1981). The results presented here for the isomerase are clearly consistent with these ideas and may be indicative of a general phenomenon associated with steroid binding proteins. It is interesting to note that backward binding for the 3α(20β)-hydroxysteroid dehydrogenase and the hydroxysteroid sulfotransferase involves "flipping" the plane of the steroid nucleus so that the β-surface

[2] The agreement of the extinction coefficients for ionized **5**β (log ϵ = 4.56) and **5**β reversibly bound to the isomerase (log ϵ = 4.76) supports the contention that reversible binding of **5**β takes place almost exclusively in mode A.

is down. In our model the β-surface remains up.

In summary, we have accumulated compelling evidence that two modes exist for the binding of oxiranyl steroid affinity labels to 3-oxo-Δ^5-steroid isomerase and have partially characterized a transient enzyme–steroid complex. It is reasonable to assume that multiple binding modes may characterize other high-affinity steroid binding proteins. Particular consideration should be given to this phenomenon in the interpretation of affinity labeling experiments.

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Registry No. **5β**, 75347-04-5; **6β**, 16669-01-5; **8β**, 51057-16-0; 3-oxo-Δ^5-steroid isomerase, 9031-36-1.

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