Secosteroid Mechanism-Based Inactivators and Site-Directed Mutagenesis as Probes for Steroid Hormone Recognition by 3α-Hydroxysteroid Dehydrogenase†

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ABSTRACT: Rat liver 3α-hydroxysteroid dehydrogenase (3α-HSD, EC 1.1.1.50) inactivates circulating androgens, progestins, and glucocorticoids. 3α-HSD is a member of the aldo–keto reductase superfamily, and the X-ray structure of the apoenzyme shows the presence of an $(\alpha/\beta)_8$ barrel [Hoog, S. S., Pawlowski, J. E., Alzari, P. M., Penning, T. M., & Lewis, M. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91,* 2517–2521]. As yet, a three-dimensional structure of the ternary complex E·NADPH·steroid is unavailable. To identify regions of the enzyme involved in steroid hormone recognition, we have employed mechanism-based inactivators and site-directed mutagenesis. (3*RS*)-1,10-Seco-5α-estr-1-yne-3,17β-diol (**1**) and (17*RS*)-17-hydroxy-14,15-secoandrost-4-en-15-yn-3-one (**3**) are secosteroids which contain latent Michael acceptors (α,β-unsaturated alcohols) at opposite ends of the steroid nucleus (at the C-3 and C-17 positions, respectively). It was found that compounds **1** and **3** inactivated 3α-HSD only in the presence of NAD^+. The requirement for cofactor implies that **1** and **3** are oxidized to the corresponding α,β-unsaturated ketones for inactivation to occur. Chemically prepared 17β-hydroxy-1,10-seco-5α-estr-1-yn-3-one (**2**) and 14,15-secoandrost-4-en-15-yne-3,17-dione (**4**), the presumed products of **1** and **3** oxidation, behaved as stoichiometric inactivators of 3α-HSD. In the presence and absence of NAD^+, **2** and **4** inactivated >50% of the enzyme in 10 s or less. These results provide evidence for the backward binding of partial steroid substrates and their turnover to reactive acetylenic ketones which alkylate 3α-HSD. Affinity-labeling studies with (bromoacetoxy)-steroids have previously identified Cys-170, Cys-217, and Cys-242 as sites of contact for steroid hormones. The crystal structure shows that only Cys-217 resides in the core of the barrel near the presumptive steroid binding site. To determine whether Cys-217 is the reactive nucleophile alkylated by the secosteroids, **1–4** were used to inactivate the following mutants: C170A, C242A, and C217A. The first two mutants demonstrated inactivation kinetics similar to that of native 3α-HSD. The C217A mutant, however, was highly resistant to inactivation by **1–4** and supports the role of Cys-217 in inactivation by the secosteroids. These data are rationalized by molecular modeling.

Mammalian hydroxysteroid dehydrogenases (HSDs)[1] are involved in the biosynthesis and inactivation of steroid hormones. 3α-Hydroxysteroid dehydrogenase (3α-HSD, EC 1.1.1.50) from rat liver cytosol is a representative peripheral HSD whose principal function is to inactivate circulating androgens, progestins, and glucocorticoids. Thus, 3α-HSD reduces 5α-dihydrotestosterone (a potent androgen) to 3α-androstanediol (a weak androgen) (Hoff & Schriefers, 1973). It also catalyzes the second step in glucocorticoid metabolism, since it will convert 5β-dihydrocortisone to tetrahydrocortisone (Tomkins, 1956).

Molecular cloning indicates that HSDs belong to at least two distinct protein families: the short-chain alcohol dehydrogenases and the aldo–keto reductase (AKR) superfamily (Hoog et al., 1994). The cDNA encoding rat liver 3α-HSD has been cloned and sequenced (Pawlowski et al., 1991; Cheng et al., 1991; Stolz et al., 1991), and the protein has been overexpressed in *Escherichia coli* (Cheng et al., 1991; Pawlowski & Penning, 1994). 3α-HSD is a member of the AKR family containing 58% sequence identity with human placental aldose reductase (ADR) at the amino acid level (Chung & LaMendola, 1989). This similarity raises the issue of how closely related enzymes can recognize such different substrates (aldo–keto sugars vs steroid alcohols). Previous experiments utilizing (bromoacetoxy)steroids as affinity-labeling agents identified three cysteine residues as potential points of contact for steroid hormone recognition in 3α-HSD (Penning et al., 1987, 1991). However, mutagenesis of each residue only supported the involvement of Cys-217 (Pawlowski & Penning, 1994).

Recently, the crystal structure of 3α-HSD has been solved to near atomic (3.0 Å) resolution (Hoog et al., 1994). The apoenzyme adopts an $(\alpha/\beta)_8$ barrel structure (triosephosphate isomerase barrel motif) and lacks a Rossmann fold for binding NAD(P)(H). A large hydrophobic cavity is located near the

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[1] Abbreviations: HSDs, hydroxysteroid dehydrogenases; 3α-HSD, 3α-hydroxysteroid dehydrogenase or 3α-hydroxysteroid:NAD(P)$^+$ oxidoreductase (EC 1.1.1.50); r3α-HSD, wild-type recombinant 3α-HSD; AKR, aldo–keto reductase; ADR, aldose reductase; C-3 acetylenic alcohol, or **1**, (3*RS*)-1,10-seco-5α-estr-1-yne-3,17β-diol; C-3 acetylenic ketone, or **2**, 17β-hydroxy-1,10-seco-5α-estr-1-yn-3-one; C-17 acetylenic alcohol, or **3**, (17*RS*)-17-hydroxy-14,15-secoandrost-4-en-15-yn-3-one; C-17 acetylenic ketone, or **4**, 14,15-secoandrost-4-en-15-yne-3,17-dione; androsterone, 3α-hydroxy-5α-androstan-17-one; androstanedione, 5α-androstane-3,17-dione; DHEA, dehydroepiandrosterone or 3β-hydroxyandrost-5-en-17-one.

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top of the barrel. The cavity is about 11 Å deep and projects toward the center of the barrel, and it is large enough to accommodate steroid ligands. The hydrophobic amino acids in this cavity are conserved with respect to ADR and to bovine testicular and rabbit ovarian 20α-HSD (Warren et al., 1993; Lacy et al., 1993), both of which are members of the AKR superfamily. Also, Cys-217 is located in the core of the barrel, consistent with a possible role in steroid recognition. $(\alpha/\beta)_8$ barrel structures are known to undergo large conformational changes upon binding ligand (Wierenga et al., 1992), and without a crystal structure of the ternary complex (E·NADPH·steroid), it is difficult to deduce which residues are final points of contact for steroid ligands.

In an effort to clarify the structural determinants for steroid hormone recognition, we have used novel mechanism-based inactivators of 3α-HSD and site-directed mutagenesis as probes for the steroid binding site. The general approach for the development of mechanism-based inactivators of HSDs has been to incorporate an α,β-acetylenic alcohol (a latent Michael acceptor) into the steroid side chain. Enzymatic oxidation of the alcohol to a ketone is required to convert these substrates into alkylating agents. In the case of 3α-HSD, the acetylenic alcohol would have to be introduced at the C-3 position. However, the incorporation of an acetylenic alcohol into this position is impossible due to the rigidity imposed by the steroid ring structure. This problem may be circumvented by incorporating the latent Michael acceptor into an open A ring. In this paper we show that secosteroids which contain latent Michael acceptors incorporated into either an open A or D ring are turned over and inactivate 3α-HSD. This provides evidence for backward binding and turnover of secosteroid substrates. Using a series of cysteine site-specific mutants, we demonstrate that inactivation takes place through covalent modification of Cys-217. This occurs irrespective of whether the acetylenic alcohol is on an open A or D ring and provides evidence that this residue is an important determinant for enzyme inactivation by secosteroids.

EXPERIMENTAL PROCEDURES

Materials. Steroids were purchased from Steraloids (Wilton, NH). β-NAD^+ was purchased from Boehringer Mannheim (Indianapolis, IN). The synthesis of (3*RS*)-1,10-seco-5α-estr-1-yne-3,17β-diol (**1**) and 17β-hydroxy-1,10-seco-5α-estr-1-yn-3-one (**2**) has been described elsewhere (Hu & Covey, 1993). The synthesis and structural characterization of (17*RS*)-17-hydroxy-14,15-secoandrost-4-en-15-yn-3-one (**3**) and 14,15-secoandrost-4-en-15-yne-3,17-dione (**4**) will be described separately.[2] All other reagents were ACS grade or better.

Preparation of Native, Wild-Type, and Mutant 3α-HSDs. Homogeneous 3α-HSD from male Sprague-Dawley rat liver was prepared using the published procedure (Penning et al., 1984). The native enzyme had a specific activity of 2.2 μmol of androsterone oxidized min^{-1} mg^{-1} under standard assay conditions (see below). Construction of the prokaryotic expression vector containing the full-length cDNA (pKK3α-HSD) and its site-directed mutagenesis using the polymerase chain reaction, as well as the expression, purification, and characterization of wild-type recombinant 3α-HSD (r3α-HSD) and its mutants, have been previously described (Pawlowski & Penning, 1994). Aliquots (400 μL) of all enzymes were stored in 20 mM potassium phosphate buffer, pH 7.0, containing 30% (v/v) glycerol, 1 mM EDTA, and 1 mM β-mercaptoethanol at −70 °C and thawed as needed. Before use, enzyme was diluted with 10 mM potassium phosphate buffer, pH 7.0, containing 1 mM EDTA and dialyzed overnight against three changes of the dilution buffer.

Enzyme Activity. The standard spectrophotometric assay for determining 3α-HSD activity was conducted in 1-mL systems containing 75 μM androsterone, 2.3 mM NAD^+, and 100 mM potassium phosphate buffer, pH 7.0, with 4% acetonitrile as cosolvent at 25 °C. The reactions were initiated by the addition of enzyme, and the increase in absorbance at 340 nm was followed continuously for 5 min. A Gilford 260 UV–vis spectrophotometer was used for all measurements, using a molar extinction coefficient of 6270 M^{-1} cm^{-1} for NADH.

Inactivation Studies. Enzyme (80 μL) was incubated in 100-μL systems containing 100 mM Tris-HCl, pH 9.0, in the presence or absence of 2.3 mM NAD^+, with 8% dimethyl sulfoxide as a cosolvent at 25 °C. The reaction was initiated by the addition of varying amounts of inactivator. Aliquots (5 μL) were withdrawn from the incubation mixture and diluted into the standard (1 mL) assay system, and the amount of enzyme activity remaining was determined. Inactivation data obtained with the acetylenic alcohols were plotted as log % initial enzyme activity vs time. Semilog plots were fitted to the first-order equation $-dE/dt = k[I]$, where it is assumed that the disappearance of enzyme activity over time is related to the concentration of acetylenic alcohol, [I], multiplied by k, a rate constant. This rate constant is for the rate-limiting step in the inactivation event; this step could be either the turnover of the latent species by the enzyme (k_{+2}) or covalent modification (k_{+3}) (see eq 1). Data presented in the Results section indicate that turnover (k_{+2}) may be rate-limiting, in which case the first-order relationship will still hold:

$$E + I \underset{k_{-1}}{\overset{k_{+1}}{\rightleftarrows}} E{\cdot}I \xrightarrow{k_{+2}} E{\cdot}I' \xrightarrow{k_{+3}} E{\cdot}I_{inact} \quad (1)$$

(where E = enzyme, I = acetylenic alcohol, I′ = acetylenic ketone, and $E{\cdot}I_{inact}$ = inactivated enzyme).

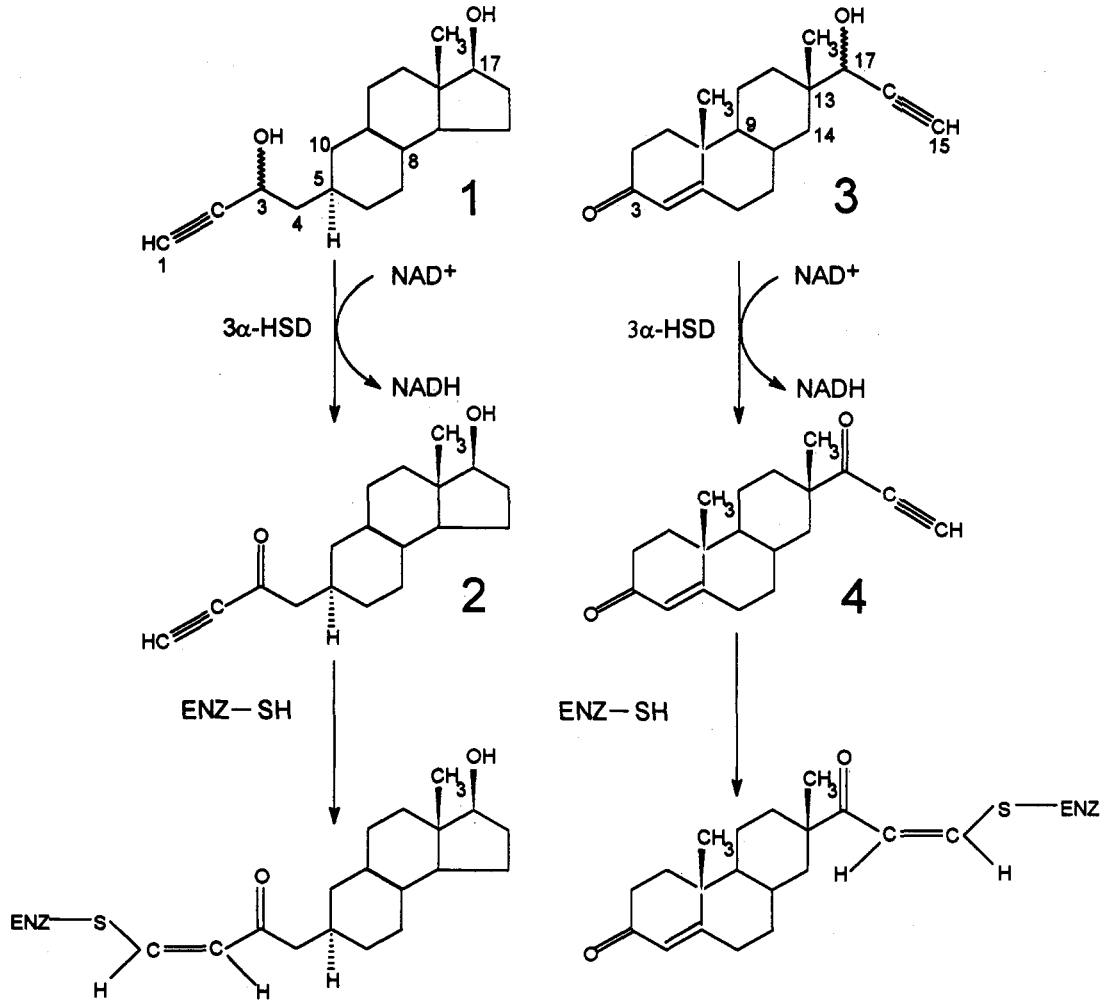
In this treatment of the Kitz–Wilson equation [$k_{app} = k_{inact} \cdot [I]/(K_i + I)$] k_{inact} approaches k_{cat} for turnover of the acetylenic alcohol, k_{app} approaches k_{+2}, and $K_i = K_I$. K_I[3] is the concentration of the acetylenic alcohol which in the presence of NAD^+ will produce a half-maximal rate of inactivation. In our experiments the rate constant k for each inactivation reaction was calculated from the appropriate semilogarithmic plot. Plots of $1/k$ vs $[I]^{-1}$ were used to generate the limiting rate constant for inactivation (k_{inact}) and K_I. Each plot was fitted to the equation for the hyperbola using the ENZFITTER program (Leatherbarrow, 1987).

Turnover Studies. Evaluation of acetylenic alcohols **1** and **3** as substrates for 3α-HSD was performed in a 1-mL system containing 10 μM wild-type recombinant 3α-HSD, 2.3 mM NAD^+, and 50 mM glycine, pH 9.0, in the presence or absence of 300 μM β-mercaptoethanol as a scavenging reagent, with 8% dimethyl sulfoxide as a cosolvent. Turnover of acetylenic ketones **2** and **4** was measured in a 1-mL system containing 0.03 μM C217A mutant, 180 μM NADH, and 100 mM potasssium phosphate buffer, pH 7.0, with 8% dimethyl sulfoxide as cosolvent. The C217A mutant was the preferred enzyme for these studies since it is resistant to inactivation by the acetylenic ketones (see Results).

[2] Y. Hu, P. F. Sherwin, and D. F. Covey, unpublished results.

[3] "K_I" in this case is defined as the concentration of **1** or **3** that, in the presence of NAD^+, produces a half-maximal rate of inactivation. This constant is not a true K_i (Kitz & Wilson, 1962) since it contains a rate constant for turnover.

Scheme 1: Mechanism-Based Inactivation of 3α-HSD by Acetylenic Alcohols **1** and **3**



Molecular Modeling. Molecular modeling was performed on a Silicon Graphics Iris Indigo Elan 4000 computer using the Sybyl molecular modeling software, version 6.0, from Tripos Associates, Inc., St. Louis, MO.

RESULTS

Inactivation of 3α-HSD by C-3 Acetylenic Alcohol **1**. It was found that (3*RS*)-1,10-seco-5α-estr-1-yne-3,17β-diol (**1**; Scheme 1) caused inactivation of 3α-HSD in a time- and concentration-dependent manner (Figure 1). Inactivation followed pseudo-first-order kinetics over approximately 3 half-lives, i.e., until inactivation was 90% complete. Analysis of the data by the method of Kitz and Wilson (1962) gave a limiting $t_{1/2}$ of 42 s and showed that inactivation was half-maximal at K_I = 680 μM. Inactivation had an obligatory requirement for NAD^+. The requirement for cofactor suggests that oxidation of **1** to the corresponding acetylenic ketone [17β-hydroxy-1,10-seco-5α-estr-1-yn-3-one (**2**); Scheme 1] precedes inactivation and that it is the enzyme-generated ketone which covalently modifies the enzyme by Michael addition. Both glutathione and β-mercaptoethanol (2 mM) protected 3α-HSD against inactivation by **1**, implying that the acetylenic ketone can be scavenged by these thiols. Since glutathione is a tripeptide and presumably too large to gain access to the active site, the enzyme-generated acetylenic ketone must leave the catalytic site and then return to inactivate the enzyme. Dialysis of the inactivated enzyme failed to restore enzyme activity, supporting the contention that covalent modification had occurred.

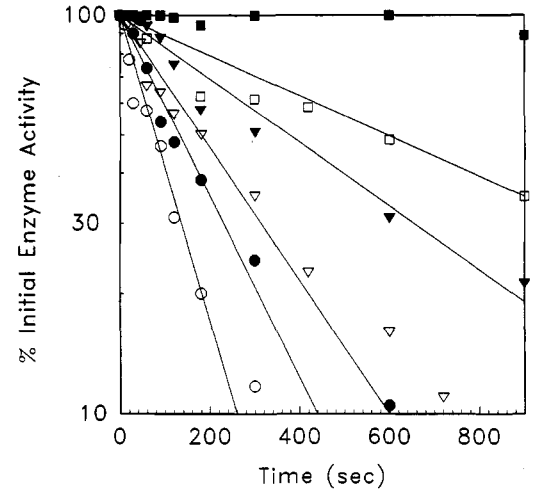


FIGURE 1: Inactivation of 3α-HSD by acetylenic alcohol **1**. Enzyme (9.0 μM) was incubated with 2.3 mM NAD^+ in the presence of one of the following: 65 (□), 100 (▼), 200 (▽), 400 (●), or 800 μM **1** (○) or 800 μM **1** and no NAD^+ (■).

Inactivation of 3α-HSD by C-3 Acetylenic Ketone **2**. The enzyme-generated acetylenic ketone (**2**) was synthesized and caused inactivation of 3α-HSD in a concentration-dependent manner (Figure 2A). At each concentration, inactivation was so rapid ($t_{1/2}$ < 10 s) that the kinetics of inactivation could not be followed. The extent of inactivation observed with **2** showed that the enzyme activity could be titrated so that approximately 80% inactivation was observed with equimolar amounts of inactivator (Figure 2B). Inactivation by **2** did not

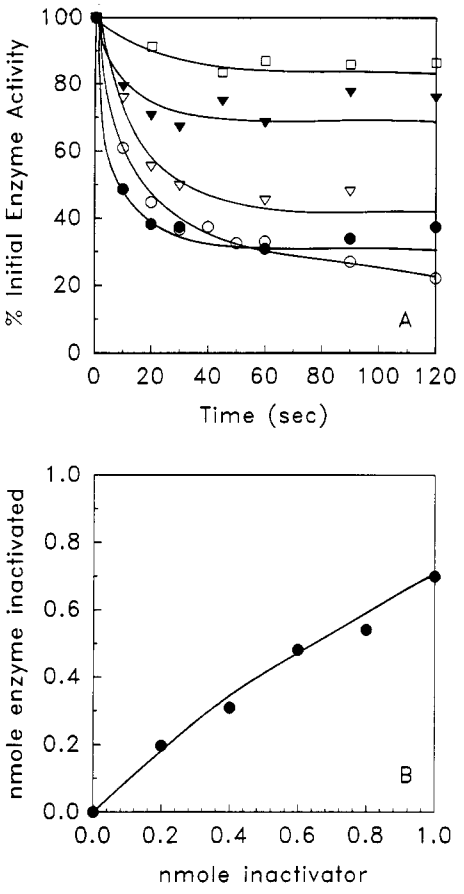


FIGURE 2: Inactivation of 3α-HSD by acetylenic ketone **2**. Panel A: Enzyme (10.2 μM) was incubated with one of the following: 2 (□), 4 (▼), 6 (▽), 8 (●), or 10 μM **2** (○). The total amount of enzyme inactivated with each concentration of inactivator was determined from the endpoint of each line. Panel B: A plot of nanomoles of enzyme inactivated vs nanomoles of inactivator.

require NAD^+. When saturating quantities of NAD^+ were added, inactivation by **2** still occurred at a robust rate ($t_{1/2}$ < 60 s; Figure 3). Complete inactivation of 10 μM enzyme was observed using 40 μM **2** in the presence of saturating amounts of NAD^+, indicating that **2** must be able to covalently modify free enzyme as well as the E·NAD^+ complex. The experiments with **1** and **2** were replicated with r3α-HSD, and no significant difference was observed in the kinetic constants.

Examination of C-3 Acetylenic Alcohol **1** *as a Substrate for 3α-HSD.* It is apparent that **2** is a much more efficient inactivator of 3α-HSD than **1**, suggesting that the rate-limiting step is oxidation of **1** rather than covalent modification by **2**. Because neither compound provides a distinctive absorbance spectrum, attempts were made to measure turnover of **1** by monitoring conversion of NAD^+ to NADH at 340 nm. Since it was anticipated that large quantities of enzyme would be required for these experiments, r3α-HSD was used. Also, β-mercaptoethanol was added to prevent inactivation by the enzyme-generated ketone **2**. Unfortunately, adduct formation in Tris-HCl produced a change in absorbance at 340 nm, making it impossible to measure NADH production using this buffer. Turnover was measured using 100 μM **1** in 50 mM glycine, pH 9.0, to circumvent this problem. Although oxidation of **1** does occur, this compound is a poor substrate for 3α-HSD. The specific activity for oxidation of **1** under these conditions was determined to be 4.27 nmol oxidized/min per milligram of enzyme, which is 0.2% of the specific activity observed using androsterone as a substrate under standard assay conditions. At this rate of turnover the amount of **2** produced would be sufficient to account for the total

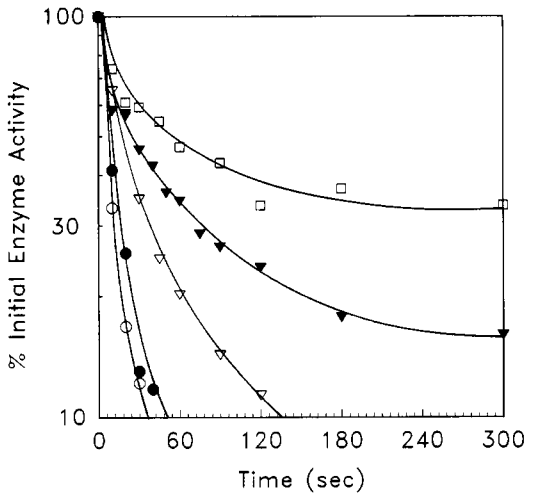


FIGURE 3: Inactivation of the E·NAD^+ complex by acetylenic ketone **2**. Enzyme (11.2 μM) was incubated with 2.3 mM NAD^+ in the presence of one of the following: 10 (□), 15 (▼), 20 (▽), 30 (●), or 40 μM **2** (○). The concentration of cofactor was chosen to approximate $10K_d$.

amount of enzyme inactivated by **1**. Turnover was complete after 30 min, with 2.5 nmol of NADH produced per nanomole of r3α-HSD. In the absence of β-mercaptoethanol, when both substrate oxidation and enzyme inactivation are occurring unimpeded, an estimate of the partition ratio can be calculated (i.e., the number of moles of inactivator turned over prior to the inactivation of 1 mol of enzyme; Walsh, 1984). Under these conditions, turnover was again complete after 30 min, and the specific activity for the oxidation of **1** was 2.44 nmol oxidized/min per milligram of enzyme. Addition of androsterone to the assay mixture at the end of the experiment permitted the determination of the amount of enzyme activity remaining. Assuming that inactivation follows psuedo-first-order kinetics, the $t_{1/2}$ was determined to be 10 min. Therefore inactivation was taking place at a rate of 0.378 nmol of enzyme/min, while compound **2** was being produced at a rate of 0.683 nmol/min, giving a partition ratio of 1.8. The lower turnover observed in the absence of β-mercaptoethanol is a reflection of the enzyme inactivation that is occurring under these conditions.

Inactivation of 3α-HSD by C-17 Acetylenic Alcohol **3**. Human placental ADR is an enzyme with 58% amino acid sequence identity to 3α-HSD. Although ADR is primarily an aldo–keto reductase, which uses aldose sugars as its principal substrates, it is also capable of catalyzing the reduction of several isocorticosteroids (minor metabolites of corticosterone) that contain an aldehyde functionality at C-21 (Wermuth & Monder, 1983). 3α-HSD has no 20α/β-HSD activity, yet the high degree of homology between ADR and 3α-HSD suggested that the latter enzyme may be able to bind steroids backward. Furthermore, bovine testicular and rabbit ovarian 20α-HSD (Warren et al., 1993; Lacy et al., 1993) possess high sequence identity with the ADRs. These observations led us to examine (17*RS*)-17-hydroxy-14,15-secoandrost-4-en-15-yn-3-one (**3**) as a potential mechanism-based inactivator of 3α-HSD. Compound **3** caused time-dependent inactivation of 3α-HSD which followed approximately psuedo-first-order kinetics over 3 half-lives. Transformation of the data gave a limiting $t_{1/2}$ of 8.3 min with half-maximal inactivation occurring at a K_I = 70 μM (Figure 4). Again, the rate constants for 3α-HSD and r3α-HSD were identical. The inactivation of 3α-HSD by **3** had an absolute requirement for NAD^+, β-mercaptoethanol protected against inactivation, and enzyme activity was not restored by extensive dialysis. Thus, the arguments presented for **1** hold true for **3**: the acetylenic

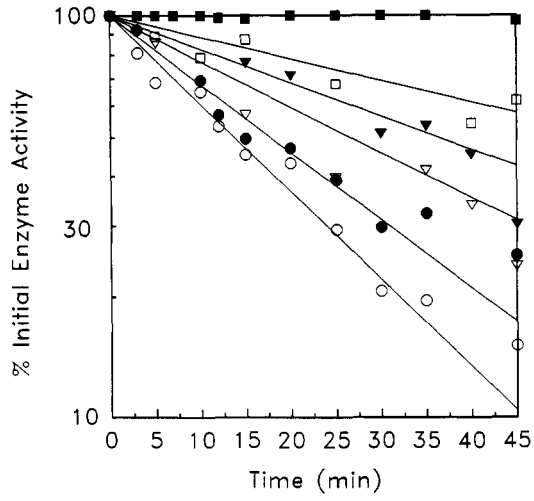


FIGURE 4: Inactivation of 3α-HSD by acetylenic alcohol **3**. Enzyme (11.1 μM) was incubated with 2.3 mM NAD^+ in the presence of one of the following: 15 (□), 20 (▼), 30 (▽), 50 (●), or 100 μM **3** (○) or 100 μM **3** and no NAD^+ (■).

alcohol **3** is oxidized to form the corresponding ketone, which then leaves and returns to the enzyme before covalent modification and inactivation of 3α-HSD occurs. Since inactivation is dependent upon turnover of the C-17 acetylenic alcohol, 3α-HSD must be able to perform catalysis at both the C-3 and C-17 positions of these secosteroids.

Inactivation of 3α-HSD by C-17 Acetylenic Ketone **4**. The presumed product of the oxidation of **3**, 14,15-secoandrost-4-en-15-yne-3,17-dione (**4**; Scheme 1), inactivated 3α-HSD in a concentration-dependent manner with a $t_{1/2}$ of <10 s (Figure 5A). Like compound **2**, compound **4** inactivated 3α-HSD in a nearly stoichiometric manner (Figure 5B). Therefore enzyme inactivation takes place regardless of whether the acetylenic ketone is at C-3 or C-17 of the secosteroid. Inactivation by **4** did not require NAD^+, and saturating quantities of NAD^+ did not retard the rate or degree of inactivation (data not shown). Like compound **2**, compound **4** inactivated the E·NAD^+ complex at a robust rate.

Examination of C-17 Acetylenic Alcohol **3** *as a Substrate for 3α-HSD*. To determine whether production of compound **4** is rate-limiting for inactivation, oxidation of **3** was measured by monitoring production of NADH at 340 nm using r3α-HSD in 50 mM glycine, pH 9.0. Compound **3** appears to be an even poorer substrate than **1**. The specific activity for the oxidation of 100 μM **3** in the presence of β-mercaptoethanol is 2.06 nmol oxidized/min per milligram of enzyme. After 30 min turnover was complete, with 1.2 nmol of NADH produced per nanomole of enzyme. In the absence of β-mercaptoethanol, the specific activity for the oxidation of **3** was 0.82 nmol oxidized/min per milligram of enzyme. Turnover was complete after 60 min, and at the end of this time course the amount of enzyme activity remaining was determined. Assuming inactivation follows psuedo-first-order kinetics, a $t_{1/2}$ of 10 min was estimated. Therefore inactivation was taking place at a rate of 0.378 nmol of enzyme/min, while compound **4** was being produced at a rate of 0.229 nmol/min, giving a partition ratio of 0.6.

It should be emphasized that the partition ratios for compounds **1** and **3** provide our best estimates of the relationship that exists between the number of moles of inactivator turned over before 1 mol of enzyme is inactivated. However, a true partition between turnover and inactivation in a single binding event is not implied since glutathione and β-mercaptoethanol can offer complete protection against inactivation by the acetylenic alcohols. Rather, the acetylenic

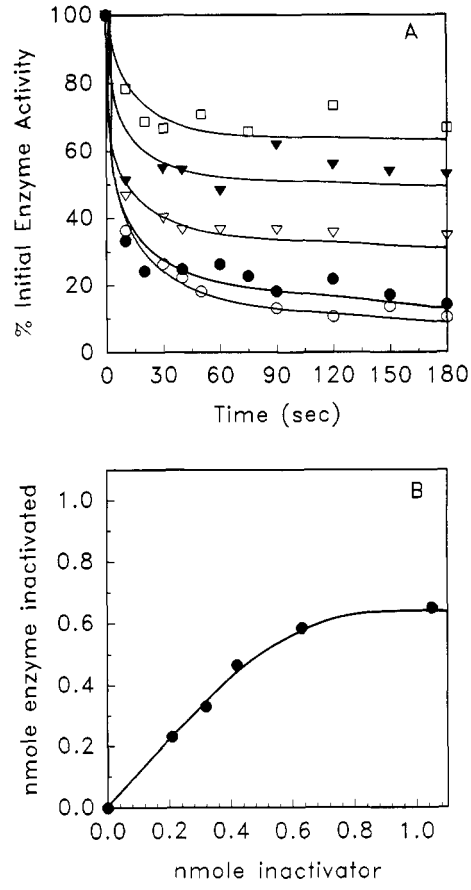


FIGURE 5: Inactivation of 3α-HSD by acetylenic ketone **4**. Panel A: Enzyme (8.5 μM) was incubated in the presence of one of the following: 2 (□), 4 (▼), 6 (▽), 8 (●), or 10 μM **4** (○). The total amount of enzyme inactivated with each concentration of inactivator was determined from the endpoint of each line. Panel B: A plot of nanomoles of enzyme inactivated vs nanomoles of inactivator.

ketones inactivate by a release and return mechanism, and their low partition ratio may reflect the fact that they are stoichiometric inactivators and are very tight binders. Furthermore, our partition ratios are subject to error since they are based on small amounts of NADH formation and extrapolated half-lives. However, our turnover experiments do help explain the different limiting $t_{1/2}$ lives for the enzyme during its inactivation by **1** and **3**. Oxidation of **1** is over twice as rapid as oxidation of **3**, which agrees with the more rapid enzyme inactivation observed with **1**. These data imply that the rate-limiting step for enzyme inactivation is turnover rather than covalent modification, and this is reflected in our analysis of the kinetic data.

Identification of Cys-217 as the Site for Covalent Modification. Since it is known that the acetylenic alcohols **1** and **3** are mechanism-based inactivators of 3α-HSD, the enzyme-generated acetylenic ketones could be used to covalently tag the steroid binding site of 3α-HSD. Previous experiments in this laboratory using (bromoacetoxy)steroids as affinity-labeling agents have identified Cys-170, Cys-217, and Cys-242 as sites for covalent modification. Elucidation of the crystal structure for 3α-HSD shows that Cys-170 and Cys-242 cannot be involved in steroid binding since they are located on the periphery of the structure. In all likelihood they are labeled because, of the nine cysteines present in the primary structure, they are the most accessible and have a local hydrophobic environment (Hoog et al., 1994). By contrast, Cys-217 is located in the core of the barrel near the presumptive steroid binding site. Further, mutation of Cys-217 to Ala-217 increases the K_m for androstanedione 4-fold, imply-

Table 1: Inactivation of Native and Recombinant 3α-HSD by the Acetylenic Alcohols and Acetylenic Ketones

kinetic constants	native 3α-HSD	r3α-HSD	C170A	C242A	C217A
alcohol **1**					
K_I (μM)	680 ± 210	440 ± 140	520 ± 90	530 ± 200	ND[a]
k_{inact} (s^{-1})	(1.64 ● 0.29) × 10^{-2}	(1.40 ● 0.21) × 10^{-2}	(1.33 ± 0.12) × 10^{-2}	(1.15 ± 0.23) × 10^{-2}	
k_{inact}/K_I (s^{-1} μM^{-1})	2.40 × 10^5	3.22 × 10^5	2.58 × 10^5	2.18 × 10^5	
alcohol **3**					
K_I (μM)	73 ± 15	25 ± 4.5	53 ± 6.6	32 ± 10	ND
k_{inact} (s^{-1})	(1.40 ± 0.16) × 10^{-3}	(0.56 ± 0.035) × 10^{-3}	(1.58 ± 0.098) × 10^{-3}	(0.86 ± 0.11) × 10^{-3}	
k_{inact}/K_I (s^{-1} μM^{-1})	1.93 × 10^5	2.22 × 10^5	2.96 × 10^5	2.70 × 10^5	
ketone **2**					
$t_{1/2}$ (s)	<10	<10	<10	<10	ND
ketone **4**					
$t_{1/2}$ (s)	<10	<10	<10	<10	ND

[a] ND = not determined.

ing that it may contribute to steroid binding. If these secosteroids are to be used as probes for the steroid binding site, it is important to demonstrate that the release and return of the enzyme-generated acetylenic ketones do not result in the labeling of the exterior cysteines. To determine whether the secosteroid mechanism-based inactivators **1** and **3** and the corresponding ketones **2** and **4** would modify the same or different residues, all four compounds were screened against the C170A, C217A, and C242A mutants. In the case of both alcohol **1** and **3**, wild-type recombinant 3α-HSD and the C170A and C242A mutants demonstrated inactivation kinetics similar to that of native rat liver 3α-HSD (Table 1). However, the C217A mutant proved to be highly resistant to inactivation by **1** and **3** for over 2 h (Figure 6A). Ketones **2** and **4** were also assessed for their ability to inactivate the mutants. Once again, wild-type recombinant 3α-HSD and the C170A and C242A mutants displayed inactivation kinetics similar to that of native rat liver 3α-HSD, but the C217A mutant retained activity for 2 h (Figure 6B). The results of these experiments indicate that Cys-217 is the major site of covalent modification by the enzyme-generated acetylenic ketones **2** and **4** and that this modification in all likelihood occurs by Michael addition of the thiol group of this cysteine residue to the α,β-unsaturated ketone.

Examination of C-3 Acetylenic Ketone 2 and C-17 Acetylenic Ketone 4 as Substrates for the C217A Mutant. Knowing that the C217A mutant was resistant to inactivation provided a source of enzyme to determine whether productive ternary complexes of E·NADH·acetylenic ketone exist. Compounds **2** and **4** were found to be exceptionally good substrates for the C217A mutant, yielding a K_m = 41.9 ± 3.7 μM and a V_{max} = 2.69 ± 0.09 μmol/min per milligram of enzyme for **2** and a K_m = 11.3 ± 0.6 μM and a V_{max} = 0.454 ± 0.007 μmol/min per milligram of enzyme for **4** at pH 7.0. By comparison, the reduction of androstanedione by this mutant at pH 7.0 yields a K_m = 18.7 ± 2.0 μM and a V_{max} = 0.859 ± 0.041 μmol/min per milligram of enzyme (Pawlowski & Penning, 1994). The ability of the C217A mutant to turn over both of the acetylenic ketones provides further support for the binding of these compounds at the active site and the noninvolvement of Cys-217 in catalysis.

DISCUSSION

The observation that both the acetylenic alcohols **1** and **3** and their corresponding ketones inactivate 3α-HSD provides several new insights into the factors determining substrate recognition. Compounds **1** and **3** contain acetylenic alcohols at the equivalent of the C-3 and C-17 positions of the steroid nucleus, respectively, yet both compounds function as mechanism-based inactivators, suggesting that **3** must bind in an opposite orientation to that observed for **1**. The requirement

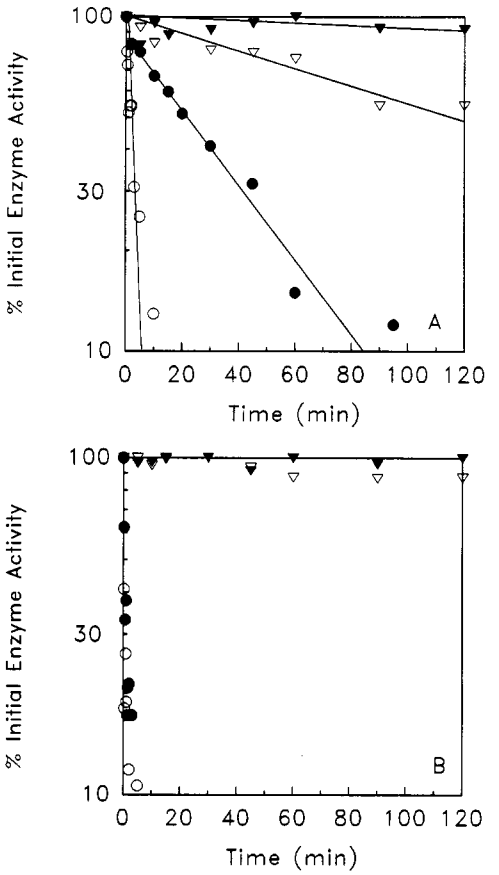


FIGURE 6: Inactivation of the C217A mutant by acetylenic alcohols **1** and **3** (panel A) and acetylenic ketones **2** and **4** (panel B). Panel A: r3α-HSD (10.5 μM) was incubated with 2.3 mM NAD^+ and 400 μM **1** (○) or 2.3 mM NAD^+ and 100 μM **3** (●). The C217A mutant (10.3 μM) was incubated with 2.3 mM NAD^+ and 400 μM **1** (▽) or 2.3 mM NAD^+ and 100 μM **3** (▼). Panel B: r3α-HSD (10.0 μM) was incubated with 10 μM **2** (○) or 10 μM **4** (●). The C217A mutant (10.3 μM) was incubated with 10 μM **2** (▽) or 10 μM **4** (▼).

for NAD^+ for each compound to inactivate 3α-HSD indicates that both alcohols are substrates for the enzyme and that oxidation of the acetylenic alcohols precedes inactivation. Therefore backward binding and catalysis of the secosteroid alcohols must occur at the active site. The use of site-directed mutants provides strong evidence that, irrespective of the position of the acetylenic ketone in the secosteroid, Cys-217 is the site of covalent attachment.

A feature of the acetylenic ketones is their ability to inactivate the E·NAD^+ complex almost as rapidly as free enzyme. 3α-HSD follows an ordered kinetic mechanism in which pyridine nucleotide binds first and leaves last (Askonas et al., 1991). Inactivation of the E·NAD^+ complex by the acetylenic ketones implies that these compounds bind to the

binary complex, gain access to the steroid binding site, and covalently modify this site. The ability of the acetylenic ketones to bind to free enzyme is consistent with the formation of abortive binary complexes that have been previously observed (Askonas et al., 1991). Previous studies on the inactivation of HSDs by enzyme-generated steroidal acetylenic ketones have shown that, irrespective of the kinetic mechanism, cofactor protects against inactivation (Auchus & Covey, 1986; Covey et al., 1986). This led to the hypothesis that to achieve true suicide inactivation of an HSD, significant concentrations of a binary E·steroid complex must form (Auchus & Covey, 1986). The low partition ratios observed for acetylenic alcohols **1** and **3** and the ability of acetylenic ketones **2** and **4** to inactivate the E·NAD^+ complex represent a limitation to this hypothesis. Inactivation, in this case, can occur in the abortive E·NAD^+·secosteroid ternary complex.

3α-HSD is not the first steroid-transforming enzyme shown to bind steroid ligands backward. For example, 3(17)β-hydroxysteroid dehydrogenase from *Pseudomonas testosteroni* catalyzes the reversible oxidation of dehydroepiandrosterone (DHEA, 3β-hydroxyandrost-5-en-17-one) and testosterone (17β-hydroxyandrost-4-en-3-one) (Talalay & Dobson, 1953), and these steroids contain the reactive alcohol at either the C-3 or the C-17 position, respectively. Further, it was shown by using a single steroid substrate, e.g., DHEA, that sequential reduction of the 17-oxo group and oxidation of the 3-hydroxy group occurred without dissociation of cofactor (Minard et al., 1985). These findings indicate that both activities occur at a single active site. Two modes of steroid binding have also been demonstrated in 3α,20β-hydroxysteroid dehydrogenase from *Streptomyces hydrogenans* since (bromoacetoxy)steroid affinity-labeling agents simultaneously abolished both 3α and 20β activity (Sweet & Samant, 1980). A similar observation was made using 17β-[(1*S*)-1-hydroxy-2-propynyl]androst-4-en-3-one and its enzyme-generated affinity alkylator, 17β-(1-oxo-2-propynyl)androst-4-en-3-one (Strickler et al., 1980). Another example is Δ^5-3-ketosteroid isomerase from *Pseudomonas testosteroni*. In this case alkylating agents were used in which the reactive oxiranyl groups were located at opposite ends of the steroid nucleus. Both spiro-17β-oxiranyl steroids and spiro-3β-oxiranyl-5α-androstan-17β-ol were irreversible inactivators of the isomerase (Bevins et al., 1980) and covalently modified the same amino acid residue, Asp-38 (Kayser et al., 1983; Bevins et al., 1984).

Although our data could be explained by the existence of multiple steroid binding sites on 3α-HSD, there is compelling evidence that only one site exists. Equilibrium binding of the competitive inhibitor testosterone to 3α-HSD in the presence of NADH has shown that approximately 1 mol of steroid binds per mole of enzyme (Ricigliano & Penning, 1990). However, the most persuasive argument comes from examination of the X-ray crystal structure of the apoenzyme (Hoog et al., 1994). The amino acid residues implicated in catalysis are located at the bottom of a hydrophobic pocket that is 11 Å deep and is sufficiently large to accommodate only a single steroid molecule. These findings support the concept that 3α-HSD can bind and catalyze the oxidation of acetylenic alcohols **1** and **3** by accommodating these ligands in two orientations at the same site.

To reconcile how both **1** and **3** can act as substrates for 3α-HSD, we have taken advantage of molecular modeling. Rotation of **3** by 180° allows for surprisingly good superimposition on the tricyclic nucleus of **1**. Thus, both inactivators appear to occupy a common three-dimensional space. More specific structural constraints are suggested by the known stereochemistry of the reaction catalyzed by the enzyme. 3α-HSD catalyzes hydride transfer from the 4-*pro-R* position of the cofactor to the C-3 position of the steroid (Askonas et al., 1991). Also, 3α-HSD will stereoselectively oxidize only the 3α-hydroxy or axial alcohol. Therefore, one might surmise that during catalysis the enzyme-bound conformations of the flexible secosteroids **1** and **3** will closely mimic those of 3α-hydroxysteroid substrates. Should this be the case, then only one of the two diastereomers present in each secosteroid can place the hydroxy group and the hydrogen transferred to the cofactor in the appropriate positions when the tricyclic ring systems are laid over a steroid substrate. Work is presently underway to determine whether this is the actual case. A more detailed discussion of modeling results on secosteroids **1** and **3** awaits the outcome of this study.

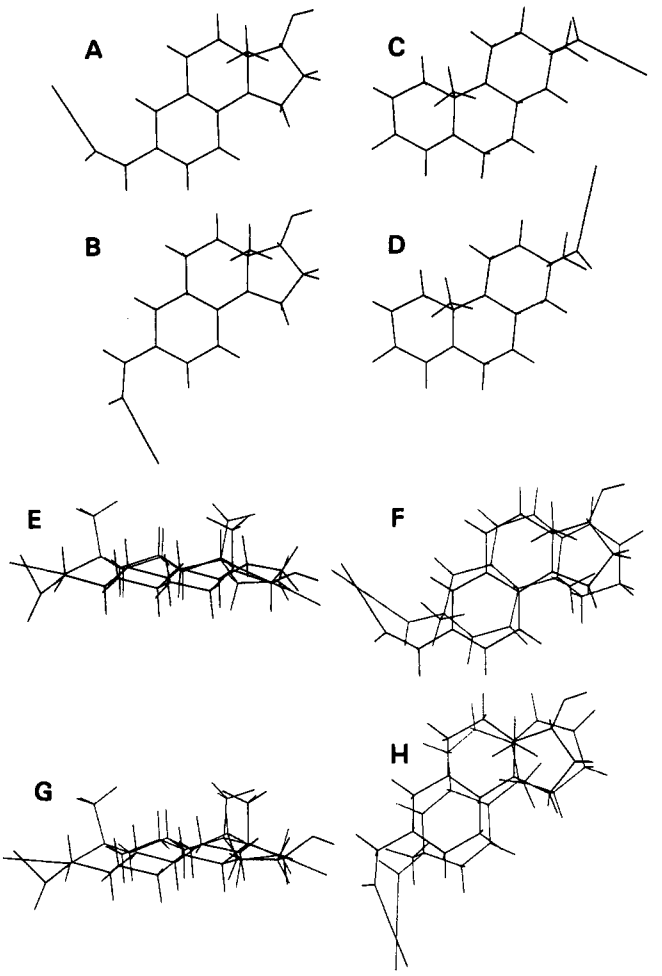


FIGURE 7: Energy-minimized structures of acetylenic ketones **2** and **4**. Panels A–D: Rotamer 1 of compound **2** (A), rotamer 2 of compound **2** (B), rotamer 1 of compound **4** (C), and rotamer 2 of compound **4** (D). Panels E–H: In all superimpositions the β-acetylenic carbons, which are the sites of modification by Cys-217, were overlaid. (E) A side view of the superimposition of C over A; C was rotated 180°, and the C-15, C-9, and C-3 positions of C were overlaid upon the C-1, C-8, and C-17 positions of A, respectively. (F) Top view of E. (G) Side view of the superimposition of D over B; D was rotated 180°, and the C-15, C-9, and C-3 positions of D were overlaid upon the C-1, C-8, and C-17 positions of B. (H) Top view of G.

Molecular modeling of acetylenic ketones **2** and **4** was also performed, and the results obtained when energy-minimized conformations of these molecules were superimposed are shown in Figure 7. Regardless of which diastereomer of secosteroid **1** or **3** is oxidized by the enzyme, a single enantiomeric product (**2** or **4**) is obtained in each case. For secosteroid **4**, bond rotation can occur about the C-13–C-17 bond, and energy minimization using the Powell minimization option (without charges) in the Sybyl program identifies two minimum-energy

conformations (rotamers), separated by 0.07 kcal/mol, for this molecule. In rotamer 1, the acetylenic group lies close to the axis of the C-16–C-17 bond of a steroid (Figure 7C). In rotamer 2, the positions of the acetylenic and carbonyl groups are changed by a 127° bond rotation (Figure 7D). Two similar conformations can be found for secosteroid **2** wherein rotamer 2 is favored over rotamer 1 by 0.73 kcal/mol (Figure 7A,B). When paired rotamers 1 and 2 from each compound are aligned in space as described in the caption of Figure 7, it is clear that excellent superimpositions can be obtained for either set of paired rotamers. In conducting these superimpositions, emphasis was placed on overlaying the β-carbons of the acetylenic ketones since this is the site of nucleophilic attack by Cys-217. We suspect that rotamer 1 of each compound is responsible for inactivation of 3α-HSD because these rotamers most closely approximate the structure of a steroid.

Identification of Cys-217 as the site of covalent modification by **2** and **4** supports the prediction that a rotamer common to both compounds must be the alkylating agent. This residue is located on the C-terminal half of the $(\alpha/\beta)_8$ barrel. Although Cys-217 is not part of the hydrophobic pocket, it is near the pyridine nucleotide binding site which is situated at the bottom of this pocket. Cys-217 is accessible only from the core of the $(\alpha/\beta)_8$ barrel. Compounds **2** and **4** must bind in the hydrophobic pocket to covalently modify this residue. In the case of **1** and **3**, the location of the catalytic residues and the pyridine nucleotide cofactor dictate that, upon binding the acetylenic alcohols, the B face of the secosteroid must be juxtaposed to the C-terminal half of the $(\alpha/\beta)_8$ barrel. Assuming that the same situation exists for the acetylenic ketones, it is predicted that the acetylenic group of rotamer 1 of each of these inactivators would react with Cys-217. The only way rotamer 2 of acetylenic ketones would have access to Cys-217 is if the secosteroids bind "upside-down" and present the wrong face for hydride transfer. Attempts to model the acetylenic ketones into the crystal structure have met with difficulty because Cys-217 is located in a disordered portion of the structure, which by analogy to other $(\alpha/\beta)_8$ barrels undergoes a conformational change upon ligand binding (Wierenga et al., 1992). Our lack of knowledge concerning the nature of this conformational change emphasizes the shortcomings of docking steroidal ligands into the crystal structures of apoenzymes.

The idea that steroids can bind in several orientations at the same site in 3α-HSD is supported by the use of alkylating agents. When acetylenic ketones **2** and **4** are added to the list of inactivators which modify Cys-217 [17β-(bromoacetoxy)-5α-dihydrotestosterone and 11α-(bromoacetoxy)progesterone; Penning et al. (1991)], alkylating agents with reactive groups on the A, C, or D ring of the steroid nucleus have all been shown to label the same residue. It appears that steroid ligands "sample" the hydrophobic pocket in different orientations. Cys-217 may be the most nucleophilic residue at or near the steroid ligand binding site, and once inactivators come into contact with it, Cys-217 becomes covalently modified. If Cys-217 is involved in steroid recognition, it would be anticipated that the C217A mutant would lose affinity for steroid ligands in a dramatic fashion. Instead, the changes in steroid affinity are less dramatic, with the K_m for androstanedione increased 4-fold in the C217A mutant. These data reflect the possibility that there may be multiple points of contact between enzyme and ligand. For example, the crystal structure of aldose reductase complexed with zopolrestat predicts 110 contacts between the inhibitor and the enzyme (Wilson et al., 1993). The change of a single cysteine residue to alanine may be too subtle to disrupt ligand binding to a greater degree.

REFERENCES

Askonas, L. J., Ricigliano, J. W., & Penning, T. M. (1991) *Biochem. J. 278*, 835–841.

Auchus, R. J., & Covey, D. F. (1986) *Biochemistry 25*, 7295–7300.

Bevins, C. L., Kayser, R. H., Pollack, R. M., Ekiko, D. B., & Sadoff, S. (1980) *Biochem. Biophys. Res. Commun. 95*, 1131–1137.

Bevins, C. L., Bantia, S., Pollack, R. M., Bounds, P. L., & Kayser, R. H. (1984) *J. Am. Chem. Soc. 106*, 4957–4962.

Cheng, K.-C., White, P. C., & Qin, K.-N. (1991) *Mol. Endocrinol. 5*, 823–828.

Chung, S., & LaMendola, J. (1989) *J. Biol. Chem. 264*, 14775–14777.

Covey, D. F., McMullan, P. C., Weaver, A. J., & Chien, W. W. (1986) *Biochemistry 25*, 7288–7294.

Hoff, H. G., & Schriefers, H. (1973) *Hoppe-Seyler's Z. Physiol. Chem. 354*, 507–513.

Hoog, S. S., Pawlowski, J. E., Alzari, P. M., Penning, T. M., & Lewis, M. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 2517–2521.

Hu, Y. F., & Covey, D. F. (1993) *J. Chem. Soc., Perkin Trans. 1, No. 4*, 417–422.

Kayser, R. H., Bounds, P. L., Bevins, C. L., & Pollack, R. M. (1983) *J. Biol. Chem. 258*, 909–915.

Kitz, R., & Wilson, I. B. (1962) *J. Biol. Chem. 237*, 3254–3259.

Lacy, W. R., Washenick, K. J., Cook, R .G., & Dunbar, B. S. (1993) *Mol. Endocrinol. 7*, 58–66.

Leatherbarrow, R. J. (1987) *ENZFITTER: A Non-linear Regression Data Analysis Program for the IBM PC (and true compatibles)*, Biosoft, Cambridge, U.K.

Minard, P., Legoy, M.-D., & Thomas, D. (1985) *FEBS Lett. 188*, 85–90.

Pawlowski, J. E., & Penning, T. M. (1994) *J. Biol. Chem. 269*, 13502–13510.

Pawlowski, J. E., Huizinga, M., & Penning, T. M. (1991) *J. Biol. Chem. 266*, 8820–8825.

Penning, T. M., Mukharji, I., Barrows, S., & Talalay, P. (1984) *Biochem. J. 222*, 601–611.

Penning, T. M., Carlson, K. E., & Sharp, R. B. (1987) *Biochem. J. 245*, 269–276.

Penning, T. M., Abrams, W. R., & Pawlowski, J. E. (1991) *J. Biol. Chem. 266*, 8826–8834.

Ricigliano, J. W., & Penning, T. M. (1990) *Biochem. J. 269*, 749–755.

Stolz, A., Rahimi-Kiani, M., Ameis, D., Chan, E., Ronk, M., & Shively, J. E. (1991) *J. Biol. Chem. 266*, 15235–15257.

Strickler, R. C., Covey, D. F., & Tobias, B. (1980) *Biochemistry 19*, 4950–4954.

Sweet, F., & Samant, B. R. (1980) *Biochemistry 19*, 978–986.

Talalay, P., & Dobson, M. M. (1953) *J. Biol. Chem. 205*, 823–837.

Tomkins, G. M. (1956) *J. Biol. Chem. 218*, 437–447.

Walsh, C. T. (1984) *Annu. Rev. Biochem. 53*, 493–535.

Warren, J. C., Murdock, G. L., Ma, Y., Goodman, S. R., & Zimmer, W. E. (1993) *Biochemistry 32*, 1401–1406.

Wermuth, B., & Monder, C. (1983) *Eur. J. Biochem. 131*, 423–426.

Wierenga, R. K., Noble, M. E. M., & Davenport, R. C. (1992) *J. Mol. Biol. 224*, 1115–1126.

Wilson, D. K., Tarle, I., Petrash, J. M., & Quiocho, F. A. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 9847–9851.