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AN ACTIVE-SITE-DIRECTED IRREVERSIBLE INHIBITOR OF Δ^5-3-KETOSTEROID ISOMERASE

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SUMMARY

The α and β isomers of spiro-3-oxiranyl-5α-androstan-17β-ol were tested as possible inhibitors of Δ^5-3-ketosteroid isomerase of Pseudomonas testosteroni. The β-oxirane causes a first-order irreversible inactivation of the enzyme and shows saturation kinetics (K_I, 17 μM). Protection against inactivation is exhibited by 19-nortestosterone, a competitive inhibitor of the isomerase. Although the α-oxirane was found to be a good reversible inhibitor (K_i, 21 μM), prolonged incubation with it failed to produce any inactivation of the isomerase. The results obtained are consistent with the presence of a nucleophilic group situated near the 3-keto group of the substrate in the enzyme-steroid complex.

The enzyme Δ^5-3-ketosteroid isomerase (EC 5.3.3.1) from Pseudomonas testosteroni has been extensively examined in recent years (1,2). This enzyme, which catalyses the conversion of $\Delta^{5,6}$ and $\Delta^{5,10}$-3-ketosteroids to their $\Delta^{4,5}$-isomers, is of particular interest due to its remarkably high turnover number (9×10^6 min^{-1}). In order to account for this extraordinary catalytic ability, it seems reasonable to suppose that there is a group on the enzyme which activates the 3-keto group of the substrate toward enolization. We report here our finding of active-site-directed irreversible inhibition of the isomerase by spiro-3β-oxiranyl-5α-androstan-17β-ol, a compound designed to probe the enzymatic environment in the vicinity of the 3-keto group of the substrate.

MATERIALS AND METHODS

All water employed was double distilled. Dried cells of Pseudomonas testosteroni (strain ATC 11996) were purchased from Worthington Biochemical Corporation. Bovine serum albumin (BSA) used for dilutions was from Sigma

Abbreviations used: 1α, spiro-3α-oxiranyl-5α-androstan-17β-ol; 1β, spiro-3β-oxiranyl-5α-androstan-17β-ol; BSA, bovine serum albumin; NT, 19-nortestosterone; nmr, nuclear magnetic resonance; hplc, high performance liquid chromatography.

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Chemical Company, while BSA added to inactivation solutions was from Boehringer (electrophoretically pure). Dialysis tubing (Spectra por 2, molecular weight cutoff of 12-14,000 from Spectrum) was treated with 1 mM EDTA and washed with water before use. Buffers were prepared from reagent grade chemicals. Dehydroisoandrosterone, dihydrotestosterone (Aldrich) and 19-nortestosterone (Sigma) were recrystallized before use. All organic solvents (reagent grade) were from Baker.

Enzyme Purification Δ^5-3-Ketosteroid isomerase was purified from dried cell preparations of Pseudomonas testosteroni by published procedures (3,4). After elution from the affinity column, the enzyme was dialyzed against 0.01 M potassium phosphate buffer (pH 7.0) and kept frozen until used. Specific activities were estimated using the $E^{0.1\%}_{1\ cm}$ value at 280 nm of 0.336 ml/mg (5). Enzyme concentrations are expressed as the molar concentration of monomer, assuming a monomer molecular weight of 13,394 (6). The purified enzyme used in these studies had a specific activity of 35,000 units/mg. It appeared homogeneous by both polyacrylamide electrophoresis and chromatography on Sephadex-G-100 and showed the characteristic UV spectrum of the isomerase.

Steroids The α and β isomers of spiro-3-oxiranyl-5α-androstan-17βol (1α and 1β) were prepared from dihydrotestosterone as described by Cook, et al. (7), and gave mp, rotation, and nmr spectrum in agreement with those reported (7). Androst 5-ene-3,17-dione was prepared by the oxidation of dehydroisoandrosterone using the general procedure of Corey and Suggs (8). Chromatography on silica (chloroform) followed by recrystallization from ether at -20° yielded product which was homogeneous by tlc on silica (ether/chloroform; 1:9). Uv analysis of the substrate and the α,β ketone formed by acid catalyzed isomerization in 0.1 N HCl indicated that the substrate was >99% pure.

Irreversible Inhibition The inactivation of Δ^5-3-ketosteroid isomerase was studied at 21.0 ± 0.2° in solutions containing 33 mM potassium phosphate buffer at pH 7, 0.3 μM of isomerase, and various concentrations of steroid added in methanol. The final volume was 0.5 ml and contained 4% (v/v) methanol. In some cases BSA was also present to solubilize the steroid (see Results). Aliquots (20 μl) were removed and diluted into 0.2% BSA, and these diluted solutions were assayed for enzyme activity in the standard assay system (2). Final concentrations of steroid inhibitors in the assay under these conditions were negligible ($\lesssim$1 nM) due to the dilution before assaying. Controls containing no steroid were stable for >12 hours. Linear regression plots of ln(% activity) vs time yielded pseudo-first order rate constants of inactivation (k_{obs}).

Hydrolysis rates of simple epoxides indicate that 1α and 1β should be quite stable at pH 7. Direct evidence for the stability of the β-oxirane (1β) in the buffer solution used for the irreversible inactivation experiments was obtained by hplc analysis. Thus, 1β (50 μM) was incubated at 22° in 200 ml of 0.03 M potassium phosphate buffer, (pH 7.0) with BSA (0.3%) and methanol (5% v/v). Aliquots (50 ml) were removed after 2 min, 5 hrs, and 24 hrs. and extracted with ether (2 x 20 ml). The solvent was removed under vacuum, and the residue taken up in methanol (0.3 ml). Portions (100 μl) of these concentrated methanol solutions were analyzed by hplc (on a μ Bondapak/C_{18} column, 3.9 mm x 30 cm; solvent, 75% methanol-25% water, flow rate 2.0 ml/min) using a refractive index detector. The only steroid-derived compound eluted was the β-oxirane and comparison with standard solutions showed that >90% of the β-oxirane was recovered from all three aliquots.

Dialysis Experiments The isomerase was inactivated with 10 μM 1β as described above until 10-20% of the initial enzymatic activity remained. A 20 μl aliquot was then diluted into 4 ml of 0.1% BSA and the BSA solution dialyzed against 2 liters of buffer at 4°. Three separate experiments with different buffers were performed. Thus, inactivated isomerase was dialyzed against: 1) 1 mM potassium phosphate pH 7 for 43 hrs. with three buffer changes, 2) 10 mM potassium phosphate pH 7 containing 0.1% BSA and 1% methanol for 88 hrs. with three buffer changes, and 3) 17 mM pyrophosphate pH 8.5 containing 0.5% methanol for 18 hrs. with one buffer change. In all three cases assays of the dialyzed

enzyme showed no restoration of enzymatic activity. Controls confirmed that active enzyme is stable to the dialysis conditions.

Reversible Inhibition Constants Isomerization rate constants (k_1) at 25.0° were measured in the presence of inhibitors under conditions where the increase in absorbance (248 nm) due to product formation is a first-order process. The reaction cell contained 3 ml of 33 mM potassium phosphate buffer (pH 7.0), methanol (4%, v/v), 5-androstene-3,17-dione (10 μM) as substrate, and various amounts of inhibitor (0-20 μM); the reaction was initiated by addition of enzyme diluted in 0.1% BSA. The amount of enzyme was sufficient to insure complete (>98%) isomerization in <5 min. Under these conditions the enzyme was stable and spontaneous isomerization of the substrate was negligible. Irreversible inactivation of the enzyme by 1β was also negligible in the short time required for substrate isomerization. First order rate constants (k_1) were obtained using a nonlinear least squares program.

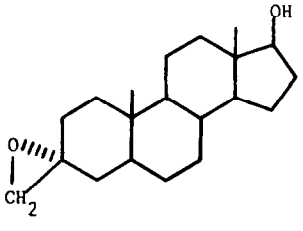
In the presence of a reversible inhibitor of the isomerase (I), the observed first-order rate constant (k_1) may be expressed as shown in equation 1

$$k_1 = \frac{k_{cat}[E]}{K_m([I]/K_i + 1)} \qquad (1)$$

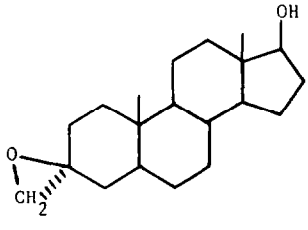
and a plot of $1/k_1$ vs. [I] can be used to calculate K_i from the slope and intercept.

RESULTS AND DISCUSSION

The β isomer of spiro-3-oxiranyl-5α-androstan-17β-ol (1β) inactivates the isomerase in a time dependent manner which obeys first-order kinetics over 3-4 half-lives (Figure 1) and leads to complete (>99%) loss of enzymatic activity.



1α



1β

Preliminary inactivation experiments suggested that 1β forms micelles at concentrations >10 μM resulting in a leveling off of k^{obs} due to saturation of the solution with steroid. Similar problems with steroid solubility have previously been reported in studying the isomerase (9,10). Satisfactory solubilization of 1β (10 - 30 μM) was obtained, however, by including 0.03% BSA (~4.5 μM) in the 4% methanol reaction buffer. Hplc analysis confirmed that the

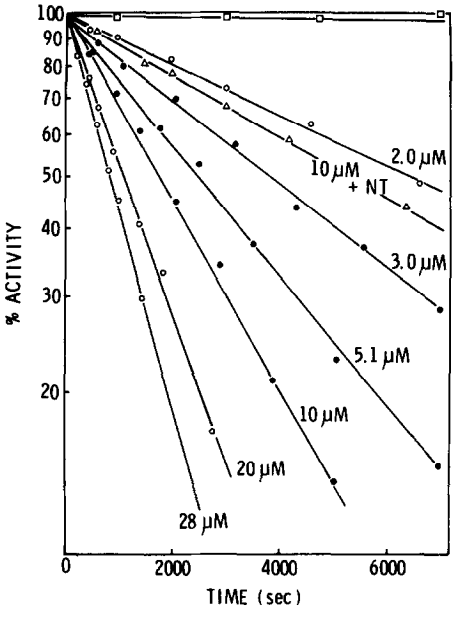


Figure 1. Pseudo-first-order inactivation of Δ^5-3-ketosteroid isomerase by 3β-oxiranyl-5α-androstan-17β-ol (1β). Reactions were run at 21° and contained in a final volume of 0.5 ml, 33 mM potassium phosphate buffer, ∿ 0.3 μM isomerase, and 2-28 μM 1β in 20 μl of methanol, with no BSA (●), and with 0.03% BSA present (o). Also shown are: control with no steroid or BSA (□), and the protective effect of 22.8 μM 19-nortestosterone (NT) during inactivation by 1β (Δ).

β-oxirane (1β) is stable to incubation with BSA. The rate constants obtained for the inactivation in both the presence and absence of 0.03% BSA are shown in Figure 2.

The errors in k^{obs} (∿10%) are such that no significant differences in k^{obs} (for $[1\beta]$ = 2-10 μM) were observed when the concentration of BSA was varied from 0 to 0.03% BSA. Interactions between steroids and serum albumin are well known (11-13), and the steroid is apparently solubilized by a weak association with BSA which disrupts micelle formation. Even with 2 μM β-oxirane, however, the effect of .03% BSA on k^{obs} is minimal indicating that the fraction of steroid bound to the BSA is effectively quite small and can safely be neglected.

The pseudo-first-order rate constants (k^{obs}) were analyzed by the method of Kitz and Wilson (14) and the results are consistent with saturation kinetics. Thus, the best weighted least squares line for the double reciprocal

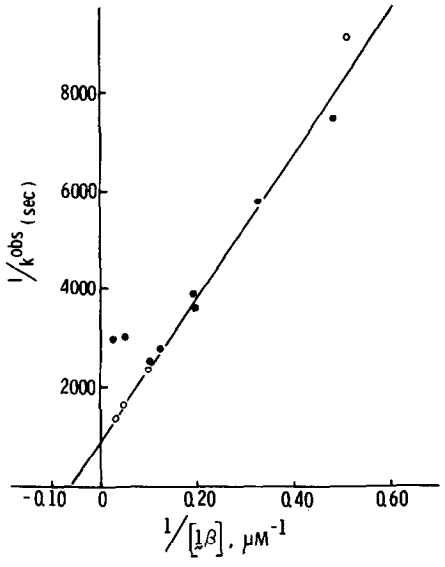


Figure 2. Irreversible inactivation of Δ^5-3-ketosteroid isomerase by 3β-oxiranyl-5α-androstan-17β-ol (1β). Double reciprocal plot of the pseudo-first-order rate constant of inactivation (k^{obs}) with respect to inhibitor concentration ([1β]). Reactions were run in the presence of 0.03% BSA (o), or with no BSA present (•) as described in Figure 1. Two points (20 and 30 μM 1β) obtained without BSA present were not included in calculating the weighted-least-squares line shown.

plot for 1β was drawn in Figure 2 using values of k^{obs} obtained both with .03% BSA (for 2-30 μM β-oxirane) and without BSA (for 2-10 μM β-oxirane). The slope and intercept were used to calculate values of the apparent dissociation constant for the initial reversibly formed complex between the enzyme and 1β (K_I = 17 μM) and the first-order rate constant for conversion of the complex to inactive enzyme (k_3 = 1.13 x 10^{-3} sec^{-1}). A binding constant for 1β for competitive inhibition was also determined from short-term studies of its inhibition of the enzymatic isomerization of the substrate under conditions where irreversible inactivation was negligible. The binding constant determined for 1β by this method (19 μM) is in good agreement with the value (17 μM) obtained from the irreversible inactivation experiments.

Evidence that the inhibition is active-site-directed can also be seen in the fact that 19-nortestosterone (NT), a known competitive inhibitor of the enzyme (15), significantly supresses the rate of inactivation (Figure 1). A plot of $1/k^{obs}$ vs [NT] at a constant concentration (10 μM) of 1β,

according to equation 2, was linear and gave a value of 6.5 μM for the binding constant of NT (K_{NT}). This value is in reasonable agreement with the bind-

$$\frac{1}{k^{obs}} = \frac{K_i[NT]}{k_3 K_{NT}[1\beta]} + \frac{[1\beta] + K_i}{k_3[1\beta]} \qquad (2)$$

ing constant (9.1 μM) determined for NT from its inhibition of the enzymatic isomerization of the substrate under similar conditions (4% methanol, 25°). This agreement is further evidence that the substrate, 19-nortestosterone, and 1β are competing for the same site on the enzyme.

The inhibitory properties of the corresponding α isomer (1α) were also examined. Although 1α was found to be a good reversible inhibitor (K_i = 21 μM), it failed to show any irreversible inactivation of the isomerase under conditions identical to those used for 1β, even after prolonged reaction times (>10 hrs) over a broad concentration range of 1α (5-100 μM with or without 0.03% BSA present).

The inability of the α isomer to inactivate the isomerase is significant. Both isomers of 1 possess essentially the same reactive group (epoxide) and differ only in the configuration at the spiro linkage. Thus, the specificity exhibited by the enzyme for reaction with the oxiranes, coupled with the protective effects shown by 19-nortestosterone, strongly suggest that 1β is reacting at the active-site of the isomerase.

Several dialysis experiments indicate that the inactivation of the isomerase by 1β is irreversible. Prolonged dialysis of partially inactivated enzyme against phosphate buffer (pH 7, with or without 1% MeOH) did not restore the lost activity. Dialysis vs pH 8.5 buffer also failed to produce any reactivation of the inactivated enzyme.

The irreversible nature of the inactivation by 1β indicates that a covalent bond is probably formed with an active-site nucleophile. The lack of reactivity of 1α also has interesting implications concerning a mechanism for the inactivation by 1β. If inactivation by 1β proceeds by nucleophilic attack at the oxirane ring carbon, the active site nucleophile will presumably be

positioned on the α side of the steroid. The α oxirane has the oxirane ring carbon on the β side and, therefore, the reactive site carbon in 1α may be too far removed for the nucleophile to attack efficiently. In this regard, it is interesting to note that, although the oxiranes are reported to undergo nucleophilic attack [by piperidine (7)], the conditions required are rather drastic (reflux for 48 hours in the presence of phenol). This suggests that the oxirane is being activated in some way during reaction at the active site, quite possibly by the same amino acid residue that activates the 3-keto group of the substrate.

Several irreversible inhibitors (16,17) and photoaffinity labels (18) for the isomerase have been previously reported. These compounds probably react with an active-site group which is positioned close to the $\Delta^{5,6}$ double bond of the substrate. Since 1β appears to react with a residue responsible for activation of the 3-keto group of the substrate, the use of 1β may present a unique opportunity to further explore the catalytic mechanism of the isomerase.

ACKNOWLEDGMENT

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