Modification of an Enzyme Carboxylate Residue in the Inhibition of 3-Oxo-Δ^5-steroid Isomerase by (3*S*)-Spiro[5α-androstane-3,2′-oxirane]-17β-ol. Implications for the Mechanism of Action

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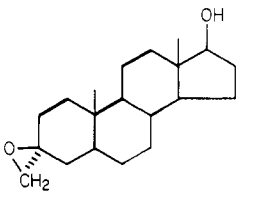
Abstract: The affinity label (3*S*)-spiro[5α-androstane-3,2′-oxirane]-17β-ol (**1β**) inhibits 3-oxo-Δ^5-steroid isomerase with a stoichiometry of one molecule of inhibitor to one enzyme monomer. The trypsin digest of the inhibited enzyme shows two distinct steroid-bound peptides, both of which are derived from residues 14–45 of the enzyme. The two modified peptides (TPS_1 and TPS_2) were isolated by HPLC, and each was shown to have an ester bond linking the steroid to the peptide fragment. Hydrolysis of each of the two peptide–steroid adducts releases a different steroid. TPS_1 gives 3α-(hydroxymethyl)-5α-androstane-3β,17β-diol (S_1) whereas TPS_2 releases 3β-(hydroxymethyl)-5α-androstane-3α,17β-diol (S_2). Tracer studies with ^{18}O-labeled **1β** show the carboxylate residue(s) involved in the ester linkages to be located on the α-face of the bound steroid. Implications of this result for the catalytic mechanism are discussed.

The 3-oxo-Δ^5-steroid isomerase (EC 5.3.3.1) from *Pseudomonas testosteroni* is a remarkably potent catalyst for the conversion of 3-oxo-$\Delta^{5(6)}$- and 3-oxo-$\Delta^{5(10)}$-steroids to their respective Δ^4-isomers.[1] This enzyme has been obtained as a homogeneous, crystalline solid[2] and the amino acid sequence is known.[3] Although a mechanism inolving a dienol intermediate was postulated[4] as far back as 1965, the identification of amino acid residues involved in catalysis and their specific functions has remained elusive.

The pH–rate profile for the isomerase[5] is indicative of two catalytically important groups with pK values of about 5.6 and >9.3 in the enzyme–substrate complex. It has been proposed that the group of pK 5.6 may be a histidine or a carboxylate which acts as the base to effect proton transfer,[1] but recent NMR studies by Benisek and Ogez[6] suggest that the group of pK 5.6 is not a histidine. Substantial evidence exists that Asp-38 is present at the active site and involved in the mechanism.[7-9] Photoionactivation of the isomerase by 3-oxo-4-estren-17β-yl acetate causes a chemical modification of Asp-38 and this residue has been shown to be "hyperreactive".[8,9] In addition, Talalay and co-workers[10] have identified Asn-57 at the active site by degradation of the enzyme inhibited with the mechanism-based inhibitor 5,10-secoestr-5-yne-3,10,17-trione.

We have recently reported that (3*S*)-spiro[5α-androstane-3,2′-oxirane]-17β-ol (**1β**), a compound designed to probe the enzymatic environment in the vicinity of the 3-oxo group of the substrate, is an active-site-directed irreversible inhibitor of the isomerase.[11] In this report we present evidence that inhibition of the enzyme by **1β** involves formation of a covalent linkage with a carboxylate residue of the enzyme. In addition, we find that this carboxylate is located on the α-face of the bound steroid, a fact that may have important implications for the enzymatic mechanism.



1β

Results

Stoichiometry of Inactivation. The time course of isomerase inactivation and the binding of inhibitor to the isomerase were simultaneously followed by use of (3*S*)-spiro[5α-androstane-3,2′-oxirane]-17α-*t*-17β-ol ([^{3}H]-**1β**). When the isomerase (6.32 μM) is incubated at 22 °C with [^{3}H]-**1β** (9.6 μM) there is a time-dependent loss of enzyme activity concurrent with an increase in protein-bound steroid (Figure 1A). Figure 1B shows that the relationship is linear and that complete loss of enzyme activity corresponds to the binding of 1.13 mol of inhibitor per mol of enzyme monomer. In a separate experiment, isomerase (19.5 μM) was incubated at pH 7 (0.01 M potassium phosphate, 4% methanol, 22 °C) with **1β** (10.0 μM). After 16 h the residual enzyme activity did not change with further incubation and was found to be 48% of the original activity. This loss of enzyme activity corresponds to 1.02 mol of enzyme monomer inactivated per mol of inhibitor. The observed stoichiometry of 1:1 under both conditions of enzyme excess and of substrate excess suggests that there is little or no enzyme-catalyzed hydrolysis of the oxirane during the incubation.

Identification of Covalently Modified Peptides. As inactivation of the isomerase proceeds there are striking changes detectable in the HPLC chromatogram of the tryptic digest of the enzyme (Figure 2). Isomerase (19.5 μM) was incubated in three parallel solutions containing 0, 10, and 25 μM **1β** (pH 7, 16 h, 22 °C)

(1) (a) Batzold, F. H.; Benson, A. M.; Covey, D. F.; Robinson, C. H.; Talalay, P. *Adv. Enzyme Regul.* **1976**, *14*, 243–267. (b) Talalay, P.; Benson, A. M. In "The Enzymes", 3rd ed.; Boyer, P. D., Ed.; Academic Press: New York, 1972; Vol. 6, pp 591–618.
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(3) Benson, A. M.; Jarabak, R.; Talalay, P. *J. Biol. Chem.* **1971**, *246*, 7514–7525.
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(9) Ogez, J. R.; Tivol, W. F.; Benisek, W. F. *J. Biol. Chem.* **1977**, *252*, 6151–6155.
(10) (a) Penning, T. M.; Covey, D. F.; Talalay, P. *J. Biol. Chem.* **1981**, *256*, 6842–6850. (b) Penning, T. M; Talalay, P. *Ibid.* **1981**, *256*, 6851–6858. (c) Penning, T. M; Heller, D. N.; Balasubramanian, T. M.; Fenselau, C. C.; Talalay, P. *Ibid.* **1982**, *257*, 12589–12593.
(11) Pollack, R. M.; Kayser, R. H.; Bevins, C. L. *Biochem. Biophys. Res. Commun.* **1979**, *91*, 783–790.

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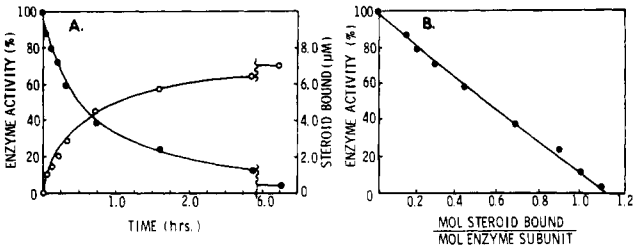


Figure 1. Stoichiometry of the inactivation of the isomerase by [^{3}H]-**1**β. (A) Isomerase (6.3 μM) in 1.2 mL of 0.01 M potassium phosphate buffer (pH 7, 5% methanol) was incubated with [^{3}H]-**1**β (9.6 μM, 3.43 μCi/μmol) at 22 °C. At indicated time intervals, two aliquots were simultaneously removed. One (20 μL) was extensively diluted in 0.1% BSA solution to determine the residual enzymatic activity (○). The second (100 μL) was extracted with two 1-mL portions of ethyl acetate (saturated with 0.01 M phosphate). The organic extracts were combined, the solvent was removed under a gentle stream of nitrogen gas, and the amount of extractable radioactive steroid was determined. The amount of enzyme-bound steroid was calculated by subtracting the amount of extractable steroid at each time from the amount of steroid initially present in the reaction medium (●). (B) The fraction of remaining activity (%) was then correlated with the moles of inhibitor bound per mole of active enzyme monomer initially present in the reaction medium.

giving 0%, 52%, and 100% inactivation, respectively. The solutions were then heated to denature the protein, treated with trypsin, and finally analyzed by HPLC. Inactivation of the isomerase by **1**β is accompanied by a loss of the peak eluting at 118 mL [TP] and the appearance of two new peaks eluting at 132 mL [TPS_1] and 133.5 mL [TPS_2]. No other significant changes are seen. In addition, the sum of the areas of the three peaks [TP, TPS_1, and TPS_2] is the same in all three tryptic digests (±10%). At 52% inactivation, 45% of this total area resides in the two new peaks and 55% of the total area is present in the TP peak. These results imply that the peptide eluting at 118 mL (TP) is modified during the inactivation by **1**β and that TPS_1 and TPS_2 are modifications of this peptide.

A sample of TP was obtained by collecting this peak from an HPLC separation of the tryptic digest of the enzyme which had not been inactivated. End-group analysis by dansylation and amino acid analysis revealed that this peptide is the tryptic fragment T_2 (residues 14–45) as reported by Benson et al.[3]

In order to determine if the two peaks that appear as inactivation products contain covalently-bound steroid, the isomerase (38.6 μM) was inactivated with [^{3}H]-**1**β (15.7 μM), denatured, digested with trypsin, and analyzed by HPLC. Assay of the radioactivity of the HPLC eluate revealed that 41% of the radioactivity was present in TPS_1 and 33% of the radioactivity was present in TPS_2. It was also found that the ratio of radioactivity recovered to peak area is the same for each of these two products. These results together with the stoichiometry experiments suggest that 1 mol of inhibitor is bound per mol of peptide in each of these two peptides and that inactivation by **1**β proceeds with the formation of two different covalently bound peptide–steroid complexes. The ratio of these peptide–steroid complexes (1.3:1.0) is constant throughout the course of inactivation and does not change with prolonged tryptic digestion nor with incubation in either neutral or acidic medium. End-group analysis confirmed that tyrosine is the amino terminal residue in both TPS_1 and TPS_2, as well as TP.

In order to establish that the protein portions of TPS_1 and TPS_2 were TP, samples of TPS_1 and TPS_2 were separately incubated at pH 12.5 and 22 °C for 45 min. Analysis by HPLC revealed that neither TPS_1 nor TPs_2 were present after base treatment. A new peak corresponding to TP appeared in the chromatogram from each solution with an area >90% of the peak area of the compounds prior to base treatment. Coinjection of a sample of TP with both the TPS_1 and TPS_2 hydrolysis products showed that all three elute as one peak. Furthermore, end-group analysis confirmed that the amino terminal residue of TP, TPS_1, and TPS_2 is the same amino acid, tyrosine. The only tryptic fragment of the isomerase with tyrosine as the amino terminal residue is the

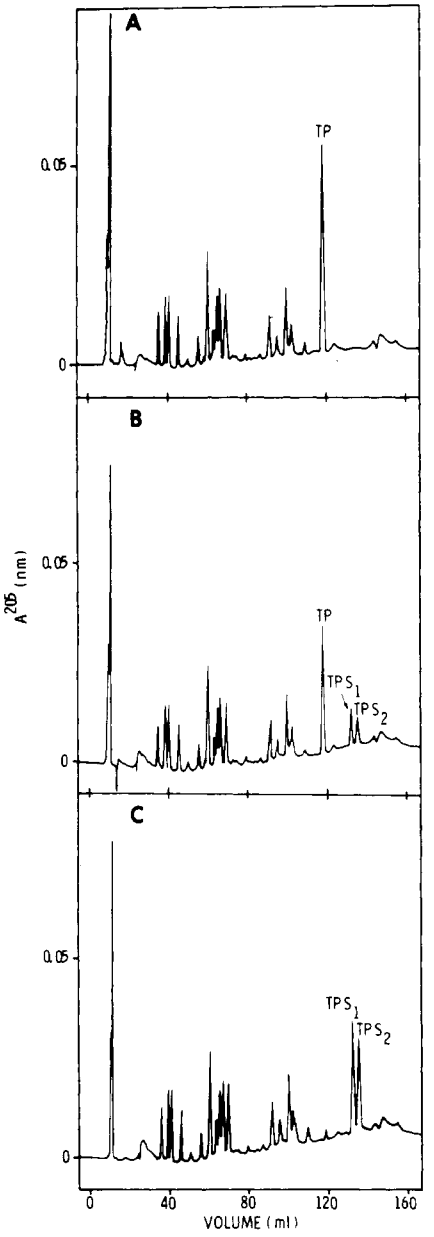


Figure 2. HPLC analysis of tryptic digests. Isomerase (19.5 μM) was incubated with **1**β (pH 7, 22 °C) for 16 h. (A) No **1**β; (B) 10 μM **1**β; (C) 25 μM **1**β. An aliquot of each solution was digested with trypsin and analyzed by HPLC using a 30-min linear gradient from 0–48% acetonitrile/water with 0.1% (v/v) phosphoric acid in the water.

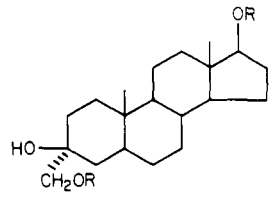
fragment labeled T_2 by Benson et al.[3]

The electrophoretic mobility of active and inactivated isomerase in polyacrylamide with 8 M urea (pH 8.5) was studied. Isomerase (19.5 μM) was incubated in three parallel solutions with 0, 10, or 25 μM **1**β for 16 h at 22 °C. At the end of this time, the solutions were assayed for residual enzymatic activity, heated to 75 °C for 10 min, and then analyzed by slab gel electrophoresis (7.5% acrylamide). Enzyme incubated in the absence of inhibitor retained activity, and this sample migrated as a single band with a relative mobility (R_f) of 0.27. Enzyme incubated with an excess of inhibitor lost >99.5% of its activity and migrated as a single band with an R_f of 0.23. When excess isomerase (19.5 μM) was incubated with **1**β (10 μM) there was a 52% loss of original enzyme activity, and this sample showed distinct protein bands of comparable intensity with R_f's of 0.23 and 0.27.

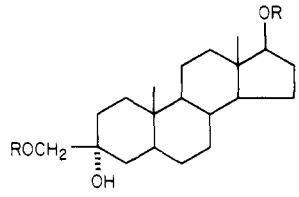
The stability of the covalent bond between the isomerase and **1**β was examined by incubating enzyme inhibited with [^{3}H]-**1**β in 7.2 M urea with several concentrations of acid and base. At various time intervals, the amount of radioactivity released was determined by extracting with ethyl acetate and assaying the

radioactivity in the ethyl acetate fraction. In dilute basic solution (0.01 M NaOH) at 25 °C, only ca. 1.5% of the radioactive steroid is extractable after 20 min. At 0.05 M hydroxide 92% is extractable after 20 min, and at 0.2 M sodium hydroxide, there is quantitative cleavage of the steroid–protein linkage within 2 min. In contrast, incubation in acid causes little cleavage of the covalent bond. After 20 h at 38 °C in 1 M hydrochloric acid, only 7.6% of the radioactivity is extracted by ethyl acetate.

Identification of the Steroids Released by Base Hydrolysis of TPS_1 and TPS_2. Isomerase inactivated with [^{3}H]-**1**β was digested with trypsin, and samples of TPS_1 and TPS_2 were isolated by HPLC. Each of these compounds was treated with base and neutralized, and the radioactive steroid was extracted with methylene chloride. The steroids were identified as S_1 from TPS_1



S_1, R = H
S_1(Ac), R = Ac



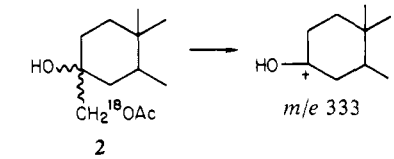
S_2, R = H
S_2(Ac), R = Ac

and S_2 from TPS_2 by the following series of experiments. The radioactive steroid from TPS_1 was diluted with a mixture of unlabeled S_1 and S_2. The mixture was then separated by HPLC and the radioactivity eluting with S_1 and S_2 determined to be 92% and 5%, respectively. A similar experiment with TPS_2 showed only 1% of the total radioactivity eluting with S_1, while 84% was associated with S_2.

The steroid products were also characterized as the diacetates S_1(Ac) and S_2(Ac) by hydrolyzing radioactive TPS_1 and TPS_2 and acetylating the methylene chloride extracts. Each acetylated compound was diluted with a mixture of nonradioactive S_1(Ac) and S_2(Ac) and analyzed as above. The steroid isolated in this manner from TPS_1 showed 86% of its radioactivity eluting with S_1(Ac) and <1% with S_2(Ac). Similarly, the steroid from TPS_2 showed 2% of its radioactivity associated with S_1(Ac) and 86% with S_2(Ac).

Location of ^{18}O in the Hydrolysis Products S_1 and S_2 from Enzyme Inhibited with [^{18}O]Oxirane. The 3β-oxirane **1**β was synthesized enriched with 50% ^{18}O in the oxirane oxygen. Samples of TPS_1 and TPS_2 were isolated from enzyme inhibited with [^{18}O]-**1**β and hydrolyzed in base, and the steroids as the diacetates were analyzed by mass spectrometry to determine the location of the ^{18}O.

Analysis of the acetates was facilitated by making use of the fact that the base peak for both S_1(Ac) and S_2(Ac) corresponds to M − CH_2OAc (*m/e* 333). Thus, a comparison of the peak intensities at *m/e* 333 and 335 directly gives the amount of ^{18}O in the tertiary alcohol oxygen. An authentic sample of **2** prepared from **1**β and 99% $H_2{}^{18}O/{}^{18}OH^-$ gave less than 1% enhancement the *m/e* 335 peak. Similarly, a sample of **3** prepared from



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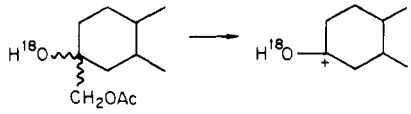
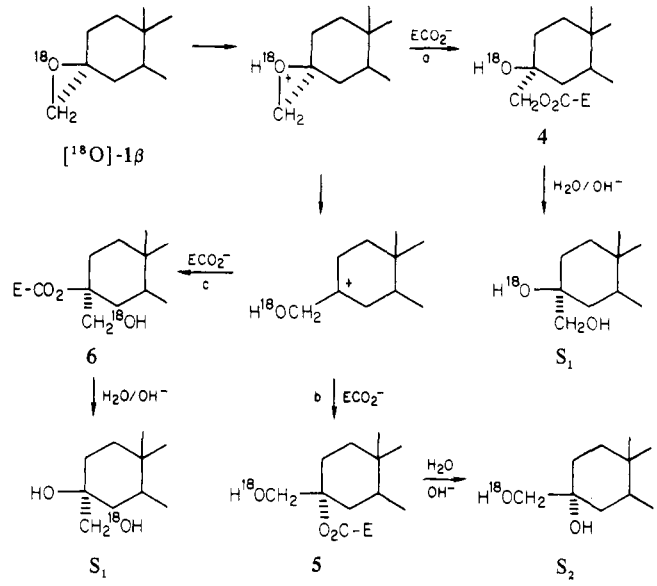


$H_2{}^{16}O/{}^{16}OH^-$ hydrolysis of [^{18}O]-**1**β had greater than 97% of the expected increase at *m/e* 335.

Mass spectral analysis of S_1(Ac) obtained from TPS_1 showed that 91% of the expected ^{18}O was present at the tertiary alcohol

Scheme I



position (**3**). In contrast, S_2(Ac) from hydrolysis of TPS_2 gave no enhancement of *m/e* 335 relative to a sample not enriched in ^{18}O showing that the ^{18}O originally in the oxirane is ultimately found in the primary acetate.

Discussion

Nature of the Covalent Bond in the Inhibited Enzyme. We previously reported[11] that 3β-oxiranyl steroids are active-site-directed irreversible inhibitors of the 3-oxo-Δ^5-steroid isomerase, and evidence was presented that a covalent bond is formed between the inhibitor and the enzyme. We now find that the stoichiometry of inhibition is 1 mol of inhibitor per enzyme subunit. In addition, tryptic digestion of isomerase inhibited by **1**β gives *two* peptides with steroid covalently bound in a ratio of 1.3:1.0. In the case of both of these peptides, several lines of evidence suggest that the covalent bond between the steroid and the peptide is an ester linkage resulting from attack of an enzyme carboxylate residue on the oxirane. (1) The bond is fairly stable to acid but quite labile in base. (2) Hydrolysis of both TPS_1 and TPS_2 at pH 12.5 (45 min, 22 °C) gives quantitative (>90%) regeneration of TP (as analyzed by HPLC), consistent with cleavage of an ester to regenerate a carboxyl group in TP. (3) the inactivated enzyme has a reduced electrophoretic mobility toward the anode, suggesting the conversion of a negatively charged carboxylate group in the native enzyme to a neutral ester function in the inactivated enzyme.

It is significant that both of the steroid-bound peptides (TPS_1 and TPS_2) generate the same peptide (TP) upon base hydrolysis yet give different steroid products. This result suggests that there may be one carboxylate of the enzyme which can attack the oxirane in two ways. In this regard, we have shown that 17β-oxiranes also are active-site-directed irreversible inhibitors of the isomerase[12] and give a tryptic map virtually identical with the one in the present study. In the case of the 17β-oxiranes, we identified the reactive amino acid as Asp-38 for both TPS_1 and TPS_2.[7] We have not as yet been able to identify the carboxylic acid group(s) involved in the inhibition by 3β-oxiranes, but the similarity of the tryptic map to that for the 17β-oxiranes suggests Asp-38 as a viable possibility.

Identification of the Steroids Produced by Hydrolysis of TPS_1 and TPS_2. Although 3β- and 17β-oxiranes reportedly undergo nucleophilic attack, the conditions required are rather drastic (reflux for 48 h with piperidine in the presence of phenol).[13] This suggests that the oxiranes are being activated in some way during

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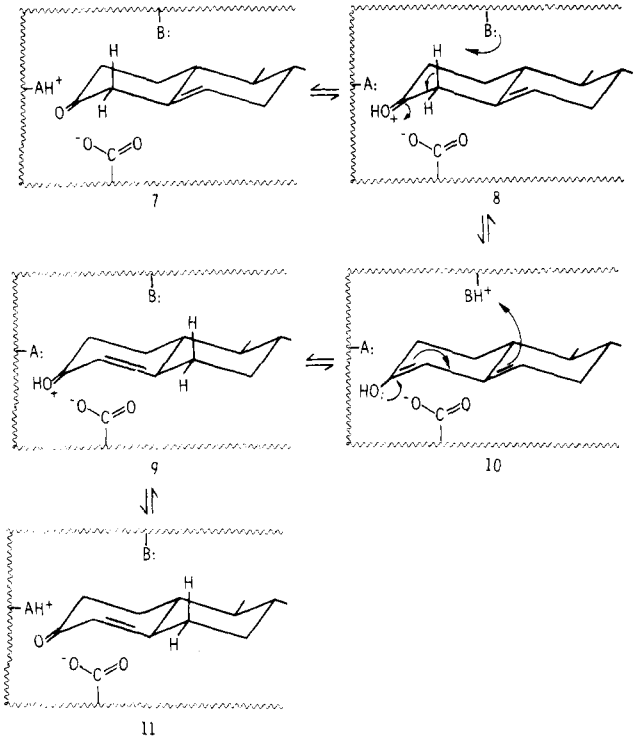
Scheme II



reaction at the active site, quite possibly by an amino acid that protonates the 3-oxo group of the substrate. Upon protonation, epoxides are known to be quite susceptible to nucleophilic attack.[14]

Several different structures might result from the reaction of **1β** with a carboxylate residue. The most reasonable candidates are **4**–**6** (Scheme I). Structure **4** would be formed by S_N2 displacement from the α-side of the steroid at the methylene carbon (path a) whereas **5** and **6** could come from attack at the tertiary carbon either from the α-side of the steroid (**5**, path b) or the β-side (**6**, path c) in an S_N1 fashion. Hydrolysis of **5** would produce S_2, and hydrolysis of both **4** and **6** would produce S_1. The identification of S_2 from TPS_2 suggests **5** as the structure for TPS_2, and the isolation of S_1 from TPS_1 implicates either **4** or **6** as TPS_1.

Discrimination between **4** and **6** was accomplished by the use of [^{18}O]-**1β**. Since greater than 90% of the ^{18}O label in S_1 was found in the tertiary hydroxyl group, TPS_1 can be represented by structure **4**. The assignment of structure **5** to TPS_2 and **4** to TPS_1 implies that the active-site carboxyl group(s) involved in attack on the oxirane ring is located on the α-face of the bound steroid molecule. Since the oxirane linkage is probably protonated prior to attack by carboxylate, our results are consistent with the presence of an acidic amino acid residue near the β-oxirane oxygen and, by inference, near the 3-keto oxygen of the natural substrates.

Implications for the Catalytic Mechanism of 3-Oxo-Δ^5-steroid Isomerase. It is clear from the above analysis that there is a carboxylate anion within residue 14–45 which is located in the vicinity of C-3 of the bound steroid. Although we do not as yet know the identity of this group, the fact that Asp-38 has been shown to be present near the A ring[8] and that it has been shown to be "hyperreactive"[9] indicates that Asp-38 may well be the group involved. Other suggestive evidence is our finding that Asp-38 is the amino acid modified by 17β-oxiranes.[7] It is, however, impossible on the basis of current evidence to rule out another acidic residue as the site of attachment since the relevant peptide contains other carboxylate residues.

It is interesting to speculate on the role(s) that this carboxylate may play in the enzymatic mechanism for isomerization of Δ^5-3-ketones. Malhotra and Ringold[4] have proposed that the enzymatic reaction proceeds through a dienol intermediate. Their proposal, which takes into account the fact that proton transfer is predominantly **4β** to **6β**,[4,15] is shown in Scheme II. The carboxylate which is alkylated by **1β** is positioned on the α-surface of the steroid A ring. Since this carboxylate is on the α-face of the steroid and proton transfer is **4β** to **6β**, *this group cannot be the base involved in the proton transfer.*

It is, of course, possible that this carboxyl is functioning as the acid AH in Scheme II, but we believe this to be improbable. The rate of the catalytic reaction depends[5] upon the deprotonation of a group with pK 5.6 and the protonation of a group with pK >9.3 in the enzyme–substrate complex. If the group with pK >9.3 is assigned to the acid AH, then it is unlikely to be a carboxylic acid.

How then could this group function to increase the enzymatic rate? We propose that this carboxylate functions via electrostatic catalysis to stabilize the protonated ketone intermediates **8** and **9**. As these structures are doubtless the highest energy ones along the reaction pathway, their stabilization should lead to a significant rate enhancement. This type of electrostatic catalysis is similar to what has long been postulated for Asp-52 in the mechanism of action of lysozyme.[16]

Another function of this carboxylate might be to stabilize AH$^+$ in the free enzyme by hydrogen bonding. Support for this mechanism may be found in the fact that only the 3β-oxiranes and not their 3α-isomers are irreversible inhibitors of the isomerase. Since nucleophilic attack on epoxides by relatively weak nucleophiles is enhanced by acid catalysis,[14] alkylation by the 3β-oxiranes suggests that there is an acid group of the enzyme on the β-face of the steroid in the vicinity of the C-3 oxygen and a carboxylate on the α-face near C-3. Formation of the E–S complex with the inhibitor (or substrate) would result in breaking the hydrogen bond between the carboxylate on the α-face and the acid AH$^+$ on the β-face. The acidity of AH$^+$ in the E–S complex would then be increased relative to free enzyme, facilitating protonation of the carboxyl.

Another possible mechanism for catalysis is formation of a covalent acylal intermediate followed by E2 elimination to form the dienol. This pathway is less plausible since virtually all acid-catalyzed enolizations occur via loss of a proton from the protonated aldehyde or ketone. However, P. Y. Bruice[17] has recently presented evidence for catalysis of enolization of oxaloacetic acid by tertiary amines which proceeds through an addition–elimination mechanism.

The existence of a carboxyl group on the α-face could also explain the fact[15] that some epimerization at C-4 is observed during catalysis of isomerization. The 4α-proton could be abstracted by the carboxylate, followed by protonation of C-4 on the β-face by protonated BH$^+$ (of Scheme II) or some other acid.

Experimental Section

Acetonitrile and water used in the HPLC analysis were HPLC grade (Baker); all other solvents and reagents were reagent grade (Baker). Sodium borohydride-*t* was purchased from Amersham Searle. Water enriched in ^{18}O (98.3 atom %) was obtained from Prochem. Dried cells of *Pseudomonas testosteroni* were purchased from Sigma. Trypsin pretreated with L-1-*p*-toluenesulfonamido-2-phenylethyl chloromethyl ketone was obtained from Worthington. Polyamide TLC sheets were obtained from Schleicher and Schuell. (3*S*)-Spiro[5α-androstane-3,2′-oxirane]-17β-ol (**1β**) was prepared as previously described.[11] General procedures concerning isomerase purification and inhibition as well as the trypsin digestion, HPLC analyses, and amino acid analysis have been described.[7,11,12] The purified enzyme used in this work had a specific activity of 47 000 units/mg.

(3*S*)-Spiro[5α-androstane-3,2′-oxirane]-17-one. (3*S*)-Spiro[5α-androstane-3,2′-oxirane]-17β-ol (**1β**) was oxidized with 2 equiv of pyridinium chlorochromate by using the general procedure of Corey and Suggs.[18] Chromatography on silica (Woelm, dry pack, hexane/CH_2Cl_2,

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4:1) followed by recrystallization from hexane yielded the desired ketone as long needles: mp 163.5–165 °C; NMR ($CDCl_3$) δ 0.90 and 0.88 (6 H, 18-CH_3 and 19-CH_3), 2.56 (2 H, AB pattern, J = 5 Hz, oxirane CH_2); IR ($CHCl_3$) 1730 cm^{-1} (C=O). Anal. ($C_{20}H_{30}O_2$) C, H.

(3*S*)-Spiro[5α-androstane-3,2′-oxirane]-17α-*t*-17β-ol ([^{3}H]-1β). (3*S*)-Spiro[5α-androstane-3,2′-oxirane]-17-one (99 mg) was reduced with sodium borohydride-*t* (14 mg, 509 mCi/mmol) diluted ~1:1 with unlabeled sodium borohydride for 4.5 h in isopropanol at room temperature. The crude product (87 mg) was chromatographed on silica (methylene chloride/hexane, 4:1). The labeled compound had a specific activity of 82.3 Ci/mol. TLC analysis (silica, methylene chloride/ethyl acetate) indicated that the steroid was both chemically and radiochemically homogeneous. An identical procedure using nonradioactive sodium borohydride gave a product with physical and spectral properties identical with authentic **1β**.

3-Oxo-5α-androstan-17β-ol-*3*-^{18}*O*. A solution of 100 mg of 3-oxo-5α-androstan-17β-ol in 3.0 mL of dioxane containing 0.01 M HCl and 270 μL of $H_2{}^{18}O$ was prepared under nitrogen and heated at 75 °C for 24 h in a sealed tube. The reaction mixture was cooled and worked up at 4 °C to prevent excessive back exchange. The solution as poured into 20 mL of ice-cold 0.01 M phosphate buffer (pH 7) and immediately extracted with methylene chloride (3 × 6 mL). The organic layers were combined and dried over magnesium sulfate and the solvent removed on a rotary evaporator. Yield, 100 mg (100%). Mass spectrum, m/z 292 (M^+), 277 (M − CH_3), 259 (M − CH_3 − H_2O). Incorporation of ^{18}O, 90%.

(3*S*)-Spiro[5α-androstane-3,2′-oxirane-^{18}*O*]-17β-ol ([^{18}O]-1β). 3-Oxo-5α-androstan-17β-ol-*3*-^{18}*O* was converted to [^{18}O]-**1β** as described previously.[11] Mass spectrum, m/z 306 (M^+, ^{18}O), 304 (M^+, ^{16}O). Incorporation of ^{18}O, 50%.

3α-(Hydroxymethyl)-5α-androstane-3β,17β-diol (S_1). (3*S*)-Spiro-[5α-androstane-3,2′-oxirane]-17β-ol (250 mg) dissolved in dioxane (4 mL) was added to a stirred solution (70 °C) of dioxane/1 M NaOH (1:1, 180 mL). The solution was purged with nitrogen, sealed, cooled to 55 °C, and stirred at this temperature for 6 days. The solution was cooled to room temperature and 13.6 gm $KHPO_4{\cdot}H_2O$ was added. After the solid had dissolved, the dioxane was removed by rotary evaporation at room temperature. The suspended white solid material that formed was isolated by suction filtration and washed with water (~50 mL). The steroid was then dissolved in EtOAc/methanol (1:1) and the solvent evaporated to yield a white solid (240 mg, 90%). Recrystallization from methanol/ether/hexane (1:2:1) gave white crystals: mp 210–213 °C; NMR (Me_2SO-d_6) δ 0.63 (3 H, s, 18-CH_3), 0.78 (3 H, s, 19-CH_3), 3.30 (2 H, d, CH_2OH), 3.41 (1 H, m, 17α-H), 3.98 (1 H, s, 3α-OH), 4.23 (1 H, t, CH_2OH), 4.39 (1 H, d, 17β-OH). Anal. ($C_{20}H_{34}O_3$) C, H.

3β-(Hydroxymethyl)-5α-androstane-3α,17β-diol (S_2) was prepared similarly from (3*R*)-spiro[5α-androstane-3,2′-oxirane]-17β-ol[11] in 95% yield. Recrystallization from ether gave white crystals: mp 135–138 °C; NMR (Me_2SO-d_6) δ 0.62 (3 H, S, 18-CH_3), 0.70 3 H, S, 19-CH_3), 3.08 (2 H, d, CH_2OH), 3.42 (1 H, m, 17α-H), 3.72 (1 H, s, 3β-OH), 4.39 and 4.42 (2 H, m, overlapping, 17β-OH and CH_2OH). Repeated purifications by recrystallization and HPLC did not yield a sample that gave a satisfactory C analysis. The diacetate S_2(Ac), however, did give an acceptable C, H analysis.

3α-(Hydroxymethyl)-5α-androstane-3β-^{18}*O*-3β,17β-diol was prepared by the same method as the unlabeled compound from 10 mg of (3*S*)-spiro[5α-androstane-3,2′-oxirane-^{18}*O*]-17β-ol. Purification was effected by HPLC on a C_{18} μBondapack column with elution by 35% acetonitrile. Only one peak was apparent. Mass spectrum, m/z 293 [(^{18}O)M − CH_2OH)], 291 [(^{16}O)M − CH_2OH)], isotopic purity 50%.

3α-(Hydroxymethyl-^{18}*O*)-5α-androstrane-3β,17β-diol was prepared by the same method as the unlabeled compound from 10 mg of (3*S*)-spiro[5α-androstane-3,2′-oxirane]-17β-ol and $NaOH/H_2{}^{18}O$ (ca. 1 M). Mass spectrum, m/z 291 [(^{18}O)M − $CH_2{}^{18}OH$)].

3α-(Acetoxymethyl)-17β-acetoxy-5α-androstan-3β-ol S_1(Ac). Treatment of 3α-(hydroxymethyl-5α-androstane-3β,17β-diol (99 mg) with 2 mL of pyridine (redistilled from sodium hydroxide) and 0.5 mL of acetic anhydride (redistilled from sodium acetate) for 22 h at room temperature gave the diacetate, which was isolated by diluting the reaction mixture with neutral potassium phosphate buffer (50 mL, 0.013 M), collecting the precipitate, and washing with water (110 mg, 88%). Recrystallization from ether gave small rosettes: mp 153–154.5 °C; NMR ($CDCl_3$) δ 0.78 (3 H, s, 18-CH_3), 0.86 (3 H, s, 19-CH_3), 2.03 (3 H, s, 17-$OCOCH_3$), 2.11 (3 H, s, CH_2OCOCH_3), 4.16 (2 H, s, CH_2OCOCH_3), 4.3–4.8 (1 H, m, 17α-H). Anal. ($C_{24}H_{38}O_5$) C, H.

3β-(Acetoxymethyl)-17β-acetoxy-5α-androstan-3α-ol S_2(Ac) was prepared in a similar fashion from 3β-(hydroxymethyl)-5α-androstane-3α,17β-diol. The precipitate was dissolved in 10 mL of methylene chloride and applied to silica gel (2 gm) capped with sodium sulfate (0.5 gm). The absorbant was washed with methylene chloride (2 × 5 mL) and ether (2 × 5 mL). The filtrates were combined and the solvent evaporated to yield the diacetate as a white solid (100 mg, 84%): mp (recrystallization from ether/hexane, 1:1) 139–140.5 °C; NMR ($CDCl_3$) δ 0.77 (6 H, br s, 18-CH_3 and 19-CH_3), 2.02 (3 H, s, 17-$OCOCH_3$), 2.09 (3 H, s, CH_2OCOCH_3), 2.11 (1 H, s, OH), 3.92 (2 H, s, CH_2OCOCH_3), 4.4–4.8 (1 H, m, 17α-H). Anal. ($C_{24}H_{38}O_5$) C, H.

Acetylation of the ^{18}O-enriched triols S_1 and S_2 to produce **2** and **3** was carried out using 10 mg of reactant and the above procedure for S_2(Ac): mass spectrum (**2**), m/z 333 [(^{18}O)M − $CH_2{}^{18}OCOCH_3$)]: mass spectrum (**3**), m/z 335 [(^{18}O)M − $OCOCH_3$)], 333 [(^{16}O)M − $OCOCH_3$)]; isotopic enrichment 50%.

Stoichiometry of Inactivation. The incorporation of [^{3}H]-**1β** into protein during the course of inactivation was examined by a procedure similar to that of Penning et al.[19] as described in the legend to Figure 1. A control experiment demonstrated that enzyme activity does not change with time under the experimental conditions if the inhibitor is omitted from the reaction medium. Another control experiment showed that the inhibitor is quantitatively extractable when incubated under the experimental conditions in the absence of isomerase. A third control demonstrated that in a reaction in which an excess enzyme is incubated with inhibitor at 22 °C for 24 h ≤4% of the total radioactivity present is extractable with ethyl acetate. Acid- or base-hydrolyzed steroidal oxiranes are also quantitatively extracted by ethyl acetate.

Tryptic Digestion of Modified and Unmodified Isomerase. Aliquots of both active and inactivated enzyme were denatured by warming to 75 °C for 10 min and then treated with trypsin (4%, w/w of protein) at pH 7.8 and 37 °C. The digestion was stopped after 4–5 h by the addition of sufficient 1 M HCl to reduce the pH to ca 2.

HPLC Analysis of Tryptic Digests and Isolation of Modified Peptides. The tryptic digests were analyzed by HPLC on a C_{18} reverse-phase column using gradient elution from 0–48% acetonitrile/water with 0.1% phosphoric acid (v/v) in the water (detection at 205 nm). The peptides were isolated by HPLC using gradient elution from 20–60% acetonitrile/water with 0.04% phosphoric acid (v/v) in the water. The collected peptides were neutralized and the acetonitrile was removed under a stream of nitrogen.

HPLC Elution of Radioactive Peptides. Isomerase (28.6 μM) was incubated at 22 °C for 16 h in a medium (100 μL) containing 0.01 M potassium phosphate (pH 7), [^{3}H]-**1β** (17.4 μM), and methanol (5%). Isomerase in 0.01 M potassium phosphate was added (giving a final enzyme concentration of 38.3 μM and a final steroid concentration of 15.7 μM). Denaturation and digestion with trypsin for 16 h was carried out as above. An aliquot of this solution (80 μL) containing an estimated 1.27×10^5 dpm of radioactivity was then subjected to reverse-phase HPLC on μBondapak C_{18} (7.8 × 300 mm, 2 mL/min flow). A 6-min isocratic elution at 30% acetonitrile was followed by a 20-min linear gradient to 54% acetonitrile. The aqueous phase included 0.04% phosphoric acid. The eluate was collected in 1-mL fractions. A portion was removed for counting using 4 mL of Scint A cocktail (Research Plus). Approximately three-quarters of the radioactivity injected was accounted for in the eluate fractions (1.06×10^5 dpm total collected).

Electrophoresis. The general procedures of Davis[20] and Studier[21] were followed.

Release of Radioactivity by Acid or Base. Isomerase (12 μM) in 235 mL of 0.01 M potassium phosphate buffer (pH 7) was incubated at 22 °C for 24 h with [^{3}H]-**1β** (5.9 μM, 83 μCi/mol) added in 14 μL of methanol. A small portion (2.5 μL, 870 ± 20 cpm) was added to each of several 8 M urea solutions (20 μL) of varying pH's. The solutions were incubated at either 25 °C (basic solutions) or 38 °C (acidic solutions). At various times the solutions were adjusted to neutral pH and extracted with ethyl acetate (250 μL, saturated with water). The percent hydrolysis was determined by dividing the ethyl acetate extractable dpm's by the total dpm's present. A control experiment in 7.2 M urea at neutral pH demonstrated that ≤4% of the total counts present were extracted by ethyl acetate. Other control experiments demonstrated that the oxirane and its hydrolysis products were ≥93% extractable from each urea solution.

Identification of S_1 and S_2. Isomerase (29.9 μM) was incubated at 22 °C for 14 h in 0.01 M potassium phosphate (pH 7, 4% methanol) with [^{3}H]-**1β** (14.3 μM), denatured, and digested by trypsin. Tryptic peptides containing radioactive steroid (TPS_1 and TPS_2) were isolated (at 53.0 and 65.6 mL, respectively) by HPLC on μBondapak C_{18} (7.8 × 300 mm) with isocratic elution (acetonitrile/0.04% H_3PO_4, 48:52) and ultraviolet

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detection at 205 nm. The pH of the collected fraction of TPS_1 was adjusted to ca. 13 with 1 M NaOH and the solution incubated at 37 °C for 35 min. One mL of this solution was neutralized and evaporated to dryness under a stream of nitrogen. Water (1 mL) was added and four 0.5-mL portions of methylene chloride were used to extract the aqueous layer. Greater than 99% of the radioactivity present was extracted. The organic extracts were combined and mixed with 200 μL of methanol containing 3α-(hydroxymethyl)-5α-androstane-3β,17β-diol (ca. 0.7 mg) and 3β-(hydroxymethyl)-5α-androstane-3α,17β-diol (ca. 0.4 mg). The organic solvents were removed under a stream of nitrogen and the contents of the tube redissolved in 200 μL of methanol. One portion of this solution was analyzed by HPLC, and a second portion was assayed for radioactivity. By isocratic elution (35% acetonitrile/water, 2 mL/min, refractive index detection) peaks corresponding to 3α-(hydroxymethyl)-5α-androstane-3β,17β-diol (39.5 mL) and 3β-(hydroxymethyl)-5α-androstane-3α,17β-diol (45 mL) were collected. Each sample was evaporated to dryness under a stream of nitrogen and assayed for radioactivity. The same procedure was followed for analysis of TPS_2. Base-line separation of the two steroids peaks was achieved in each experiment.

Identification of the Acetylated Derivatives of S_1 and S_2. TPS_1, collected as described above, was treated with 0.1 M NaOH (1 mL) at 37 °C for 1 h. The solution was neutrallized with 1 M HCl and extracted with methylene chloride (4 × 0.5 ml). (Less than 1% of the total radioactivity was left in the aqueous phase.) The methylene chloride was removed under nitrogen and the radioactive material treated with pyridine (2 mL redistilled from sodium hydroxide) and acetic anhydride (0.25 mL, redistilled from sodium acetate) at 22 °C for 24 h. The sample was diluted with a mixture of 3β-(acetoxymethyl)-17β-acetoxy-5α-androstan-3α-ol (1.76 mg) and 3α-(acetoxymethyl)-17β-acetoxy-5α-androstan-3β-ol (2.64 mg) in methylene chloride (200 μL) and diluted with 20 mL of ether. The ether solution was washed with neutral potassium phosphate buffer (0.323 M, 5 × 5 mL) and saturated NaCl (1 × 5 mL) and dried ($MgSO_4$), and the solvent was evaporated. A faint odor of pyridine in the residue was removed by drying under vacuum for an hour. The residue was redissolved in methylene chloride (200 μL) and a portion was assayed for radioactivity. Another portion was analyzed by HPLC (μPorasil column 3.8 × 30 mm)), using methylene chloride/hexane/acetonitrile (52:42:6, R.I. detection, 4 mL/min). The peaks corresponding to 3α-(acetoxymethyl)-17β-acetoxy-5α-androstan-3β-ol (33 mL) and to 3β-(acetoxymethyl)-17β-acetoxy-5α-androstan-3α-ol (42 mL) were collected, evaporated to dryness under nitrogen, and assayed for radioactivity. A similar procedure was followed for analysis of TPS_2. Base-line separation of the two steroid peaks was achieved in each experiment.

Location of ^{18}O in S_1 and S_2. A 3.2-mL aliquot of an aqueous solution containing 0.01 M phosphate (pH 7), 22.5 μM isomerase, and 19.3 μM [^{18}O]-**1β** was incubated at 22 °C overnight until all of the enzyme activity was gone. The inactivated enzyme was denatured and digested with trypsin, and samples of TPS_1 and TPS_2 were collected by HPLC. The solvent was evaporated and each sample was incubated at 37 °C for 3 h with 3 mL of 0.1 M NaOH. The organic material was extracted with ethyl acetate (3 × 1.5 mL) and the solvent evaporated in a stream of nitrogen. Pyridine (1 ml) and acetic anhydride (200 μL) were added, and the solution was let stand overnight. Aqueous phosphate buffer (2 mL, 0.33 M, pH 7) was added and the solution was extracted with methylene chloride (2 × 1 mL) and washed with water. The solvent was evaporated and the residue analyzed by mass spectrometry.

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Registry No. 1β, 2066-43-5; [^3H]-**1β**, 90991-88-1; S_1, 90991-84-7; S_1(AC), 90991-86-9; (3β-^{18}O)-S_1, 90991-91-6; (hydroxymethyl-^{18}O)-S_1, 90991-92-7; S_2, 90991-85-8; S_2(AC), 90991-87-0; EC 5.3.3.1, 9031-36-1; 3-oxo-5α-androstan-17β-ol-3-^{18}O, 90991-89-2; (3*S*)-spiro[5α-androstane-3,2′-oxirane]-17-one, 90991-90-5.

Electronic Effects of Substituents in 6-Methyl-6-phenylfulvenes: ^{13}C NMR and Theoretical Studies[1]

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Abstract: The ability of substituents to alter the electron distribution in the fulvene π-system has been studied by using substituent-induced ^{13}C chemical shifts in a series of para-substituted 6-methyl-6-phenylfulvenes as an experimental probe, in conjunction with semiempirical molecular orbital calculations. Electron-releasing substituents lead to an overall shielding increase in the five-membered ring, but a downfield shift in the exocyclic carbon. This observation, and our CNDO calculations, suggests that (a) there is relatively little resonance interaction between the substituted phenyl group and the strongly twisted fulvene moiety and (b) the dominant mode of electron redistribution is π-polarization. Furthermore, it appears that DSP correlations may in some instances give misleading results by suggesting the operation of factors which are, in fact, absent (here, resonance effects).

While the understanding of substituent electronic effects on alternant π-systems is well advanced both experimentally[3a-c] and theoretically,[4] relatively less attention has been given to nonalternant systems, where the presence of odd-membered rings leads to markedly nonuniform π-electron distributions and high chemical reactivities.[5] Such basic questions as the role of incipiently aromatic rings in facilitating transfer of electronic perturbation, and even whether the carbons in a nonalternant π-system will be partitioned into subsets analogous to the conjugated/nonconjugated sets in alternant π-systems, and what the basis for the partitioning will be, remain largely unanswered.

In this paper we report the results of a study of the ^{13}C NMR spectra of a series of para-substituted 6-methyl-6-phenylfulvenes,[6] along with parallel semiempirical molecular orbital calculations,

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