Inactivation of *Streptomyces hydrogenans* 20β-Hydroxysteroid Dehydrogenase by an Enzyme-Generated Ethoxyacetylenic Ketone in the Presence of a Thiol Scavenger†

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Received June 12, 1986; Revised Manuscript Received August 11, 1986

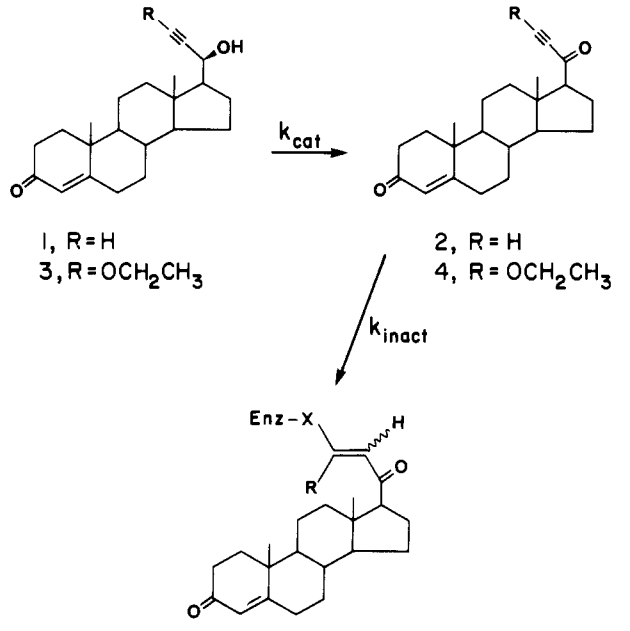
ABSTRACT: Replacement of the 21-methyl group of 20β-hydroxypregn-4-en-3-one with an ethoxyacetylene (EtO—C≡C—) group yields a compound that is an excellent substrate (pH 7.4, K_m = 2.3 μM, V_{max} = 4.6 nmol min^{-1} μg^{-1}) for the *Streptomyces hydrogenans* NAD(H)-dependent 20β-hydroxysteroid dehydrogenase (EC 1.1.1.53). The enzyme-generated ethoxyacetylenic ketone product is a potent inactivator of the enzyme. Gel filtration chromatography of enzyme inactivated with radiolabeled steroid demonstrates that covalent modification of the enzyme has occurred. Both NAD and NADH retard the rate of inactivation, suggesting that only free enzyme is susceptible to covalent modification. Consequently, enzymatically formed ethoxyacetylenic ketone does not react with the enzyme while it is part of the ternary complex. Moreover, the kinetically preferred release of this reactive ketone prior to NADH release assures that enzyme inactivation occurs only when released ketone subsequently encounters free enzyme. Kinetic analysis of inactivations carried out with chemically prepared ethoxyacetylenic ketone and enzyme at pH 7.4 and 9.2 yields bimolecular rate constants for the inactivation process of 1.15 × 10^4 L mol^{-1} s^{-1} and 6.94 × 10^4 L mol^{-1} s^{-1}, respectively. This bimolecular reaction is faster than the bimolecular reaction of the ethoxyacetylenic ketone with either glutathione, mercaptoethanol, or dithiothreitol. Thus, complete inactivation by ketone generated from 5 μM alcohol and 5 μM NAD occurs in 30 min at pH 7.4 in the presence of 1 mM glutathione.

Oxidation of vinyl, acetylenic, and allenic alcohols by hydroxysteroid dehydrogenases (HSDs) has been shown to lead to enzyme inactivation attributed to Michael addition of an enzyme nucleophile to the newly formed α,β-unsaturated carbonyl compounds (Strickler et al., 1980; Balasubramanian & Robinson, 1981; Balasubramanian et al., 1982; Tobias et al., 1982; Thomas et al., 1983; Auchus & Covey, 1986). For those studies in which the protective effects of thiols were described, it was found that complete protection was provided by these nucleophilic compounds, and it is very likely that the enzyme-generated electrophilic ketones caused inactivation after their initial release and subsequent return to the active site. Rando (1974) has discussed factors (affinity, reactivity, etc.) that generally influence whether enzyme-generated electrophiles will covalently modify the protein before they leave the active site. The factors that specifically have precluded the unambiguous demonstration of HSD inactivation prior to release of enzyme-generated reactive ketones, however, are not known in detail.

One approach that might provide insight about these factors in HSD inactivation involves the preparation of a series of enzyme-generated acetylenic ketones whose terminal acetylenic carbon atom contains either an electron donating or withdrawing group. In this series of compounds, the reactivity of the Michael acceptor is varied without greatly altering the geometry of the reactive species. Of course, the substituents will also affect both the affinity of the compounds for the enzyme (in binary and ternary complexes) and their efficiency as substrates. Using this approach, one might obtain information about the parameters that need to be accessed if one is to achieve HSD inactivation prior to release of the enzyme-generated reactive ketone.

This report describes our results with an enzyme-generated ethoxyacetylenic ketone (**4**, Scheme I) inactivator of the *Streptomyces hydrogenans* NAD(H)-dependent 20β-HSD (EC 1.1.1.53). Although inactivation did not occur without prior release of this ketone, its rapid rate of 20β-HSD inactivation allowed reaction conditions to be chosen so that this enzyme could be inactivated in the presence of millimolar concentrations of thiols. Previous examples of Michael acceptors that have been used to inactivate enzymes in the presence of high concentrations of thiols are unknown to us.

Scheme I



Experimental Procedures

Materials. Ethyl magnesium bromide and 2,3-dichloro-5,6-dicyano-1,4-benzoquinone were purchased from Aldrich Chemical Co., Milwaukee, WI. [4-^{14}C]Deoxycorticosterone

† D.F.C. is the recipient of Research Career Development Award CA-00829 from the National Institutes of Health.

0006-2960/86/0425-7288$01.50/0

was from New England Nuclear, Boston, MA. Steroids, pyridine nucleotides, glutathione, and 20β-HSD [10.5 units/mg of protein (biuret)] were obtained from Sigma Chemical Co., St. Louis, MO. Silica gel for column chromatography was dry column grade and was purchased from Universal Scientific Co., Atlanta, GA. Prepacked disposable PD-10 columns containing Sephadex G-25M (bed volume, 9.1 mL) were from Pharmacia, Inc., Piscataway, NJ. Ethoxyacetylene was obtained from Farchan Labs, Willoughby, OH. Protein assays were determined with a Bio-Rad assay kit from Bio-Rad Laboratories, Richmond, CA. Bovine serum albumin was used as the standard, and results were multiplied by 2.18 to give the equivalent biuret values. Liquid scintillation counting was done in Budget Solve from Research Products International, Mount Prospect, IL, at 91% efficiency on a Beckman LS-3133T scintillation counter.

Chemical Methods. General. Melting points were determined on a Kofler micro hot stage and are uncorrected. Proton magnetic resonance spectra (NMR) were, unless stated otherwise, recorded in $CDCl_3$ on a Varian Associates Model T-60 spectrometer. Chemical shifts (δ) are given in units of parts per million (ppm) downfield from tetramethylsilane internal standard. The multiplicity is identified by s (singlet), t (triplet), q (quartet), m (multiplet), or br (broad); and coupling constants (J) are reported in hertz. Infrared spectra (IR) were recorded in KBr. Characteristic bands are listed in units of reciprocal centimeters. The letters s, m, w, and br represent strong, medium, weak, and broad bands, respectively. Ultraviolet spectra were recorded on a Beckman DU-8. Maxima are reported in nanometers and extinction coefficients as M^{-1} cm^{-1}. High-performance liquid chromatography (HPLC) was performed on a Waters Associates system using either a RCM100 C_{18} 10-μm cartridge for aqueous solvents or a RCM100 silica 10-μm cartridge for nonaqueous solvents. Flow rates were 2.0 mL/min, and components were detected by using either a variable-wavelength detector or a differential refractometer. High-resolution mass spectroscopy was done on a Kratos MS-50/DS-55 system at a dynamic resolution of ~7500. Elemental analyses were performed by Micro-Analysis, Inc., Wilmington, DE.

17β-[(1RS)-1-Hydroxy-3-ethoxy-2-propynyl]androst-4-en-3β-ol (**5a** *and* **5b**). Ethoxyacetylene magnesium bromide was generated in situ by refluxing ethyl magnesium bromide (8.2 mL of a 3.0 M solution in ether), ethoxyacetylene (4.0 mL), and ether (120 mL) in a flask equipped with a magnetic stirrer, condenser, and drying tube for 2.5 h. Then 3β-hydroxyandrost-4-ene-17β-carboxaldehyde (Caspi, 1956; Gelbart & Thomas, 1978) was added (1.46 g, 4.83 mmol, dissolved in 40 mL of ether) over a 15-min period, and the reaction mixture was refluxed (1.5 h). Aqueous 5% ammonium chloride (100 mL) was added, the layers were separated, and the aqueous layer was extracted with ether (2 × 100 mL). The combined ether layers were washed with water (100 mL), dried over anhydrous sodium sulfate, and filtered, and the solvents were removed to yield a yellow oil (0.67 g). Column chromatography of this oil on silica gel with methylene chloride/methyl *tert*-butyl ether (9:1) as eluent give unresolved diols **5a** and **5b** as a pale yellow solid (0.27 g, 15% yield). HPLC using acetone/water (65:35) as solvent separated these diols. The retention times of **5a** and **5b** were 4.1 and 2.8 min, respectively.

17β-[(1S)-1-Hydroxy-3-ethoxy-2-propynyl]androst-4-en-3β-ol (**5a**): mp 155–157 °C; NMR (pyridine-d_5) δ 0.87 (s, 3, C-18, CH_3), 0.95 (s, 3, C-19, CH_3), 1.17 (t, 3, J = 7, CH_3CH_2O), 3.87 (q, 2, J = 7, CH_3CH_2O), 4.10–4.60 (m, 2, C-3 and side-chain C-1, C*H*OH), 5.48 (br s, 1, H—C≡C); IR 3355 (s, br, OH), 2255 (s, C≡C), 1650 (w, C=C) cm^{-1}. Anal. Calcd for $C_{24}H_{36}O_3$: C, 77.38; H, 9.74. Found: C, 77.22; H, 9.66.

17β-[(1R)-1-Hydroxy-3-ethoxy-2-propynyl]androst-4-en-3β-ol (**5b**): mp 74–76 °C; NMR (pyridine-d_5) δ 0.82 (s, 3, C-18, CH_3), 1.00 (s, 3, C-19, CH_3), 1.23 (t, 3, J = 7, CH_3CH_2O), 4.02 (q, 2, J = 7, CH_3CH_2O), 4.23–4.73 (m, 2, C-3 and side-chain C-1, C*H*OH), 5.62 (br s, 1, H—C≡C); IR 3380 (s, br, OH), 2260 (s, C≡C), 1655 (w, C=C) cm^{-1}. Anal. Calcd for $C_{24}H_{36}O_3$: C, 77.38; H, 9.74. Found: C, 73.40; H, 8.86. Although these analytical results are not satisfactory, the spectroscopic data along with the chemical conversion of **5b** to **6**, which did give satisfactory analytical results, support the given structure.

17β-[(1S)-1-Hydroxy-3-ethoxy-2-propynyl]androst-4-en-3-one (**3**). Diol **5a** (80 mg, 0.22 mmol) was stirred with 450 mg of nickel peroxide (Nakagawa et al., 1962) in benzene (40 mL) at 50 °C for 20 h. The nickel peroxide was removed by vacuum filtration through a fine scintered glass funnel. Evaporation of the solvent left a pale yellow solid (73 mg). Purification by HPLC with hexane/acetone (85:15) as solvent gave enone **3** (retention time, 7.0 min), which was recovered as a white solid (48.5 mg, 60% yield): mp 149–151 °C; NMR δ 0.78 (s, 3, C-18, CH_3), 1.21 (s, 3, C-19, CH_3), 1.35 (t, 3, J = 7, CH_3CH_2O), 4.03 (q, 2, J = 7, CH_3CH_2O), 4.30 (overlapped m, 1, side-chain C-1, C*H*OH), 5.73 (br s, 1, H—C≡C); IR 3460 (s, OH), 2250 (s, C≡C), 1665 (s, C=O), 1620 (m, C=C) cm^{-1}; UV (ethanol) λ_{max} 240.6, ϵ = 16 000. Anal. Calcd for $C_{24}H_{34}O_3$: C, 77.80; H, 9.25. Found: C, 77.97, H, 9.09.

17β-[(1R)-1-Hydroxy-3-ethoxy-2-propynyl]androst-4-en-3-one (**6**). Diol **5b** (36.4 mg, 0.10 mmol) was oxidized with nickel peroxide (350 mg) in benzene (20 mL), isolated, and purified by the same procedure used for the preparation of **3**. **6** was obtained as a white crystalline solid (16.7 mg, 43% yield): mp 136–138 °C; NMR δ 0.72 (s, 3, C-18, CH_3), 1.15 (s, 3, C-19, CH_3), 1.32 (t, 3, J = 7, CH_3CH_2O), 4.00 (q, 2, J = 7, CH_3CH_2O), 4.25–3.92 (overlapped m, 1, side chain C-1, C*HOH*), 5.60 (br s, 1, H—C≡C); IR 3380 (s, OH), 2255 (s, C≡C), 1660 (s, C=O), 1610 (m, C=C) cm^{-1}; UV (ethanol) λ_{max} 241.4, ϵ = 14 800. Anal. Calcd for $C_{24}H_{34}O_3$: C, 77.80; H, 9.25. Found: C, 78.15; H, 9.13.

17β-(1-Oxo-3-ethoxy-2-propynyl)androst-4-en-3-one (**4**). **3** (40 mg, 0.11 mmol) was stirred with 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (25 mg, 0.11 mmol) in *tert*-butyl alcohol (8.0 mL) at 43 °C for 30 min. A dry ice/acetone bath was used to freeze the *tert*-butyl alcohol, and after removal of the bath, the *tert*-butyl alcohol was removed by sublimation in vacuo. The residue was column chromatographed on silica gel with methylene chloride/methyl *tert*-butyl ether (9:1) as eluent. The recovered steroid was then further purified by HPLC using hexane/acetone (90:10) as solvent. **4** (retention time, 9.0 min) was recovered as a white solid (12 mg, 30% yield): mp 96.5–98 °C; NMR δ 0.70 (s, 3, C-18, CH_3), 1.22 (s, 3, C-19, CH_3), 1.40 (t, 3, J = 7, CH_3CH_2O), 4.18 (q, 2, J = 7, CH_3CH_2O), 5.55 (br s, 1, H—C≡C); IR 2220 (s, C≡C), 1670 (s, C=O), 1650 (s, C=O), 1615 (m, C=C) cm^{-1}; UV λ_{max} 238, ϵ = 24,700; MS m/e 368.2430 ($C_{24}H_{32}O_3$ requires 368.2351).

[4-^{14}C]-**3**. [4-^{14}C]Deoxycorticosterone (24.32 μCi, 136 μg; specific activity, 58.5 mCi/mmol) was diluted with deoxycorticosterone (5 mg) to a specific activity of 1.55 mCi/mmol and converted to radiolabeled **3** (1.2 mg, 20.8% overall yield) by a small-scale repetition of the same reaction sequence.

Biochemical Methods. Enzyme Inactivations. Reactions were done at room temperature in either 50 mM sodium carbonate buffer, pH 9.2, or potassium phosphate buffer, pH 7.4. Incubations (1.0 mL) contained enzyme (42 μg), NAD (when required) in buffer (0.95 mL), and steroids **3** or **4** in ethanol (50 μL). Whenever additional steroids were included in the incubations, the total ethanol concentration was maintained at 5%. Inactivations were initiated by enzyme addition. At various times aliquots (50 μL) were removed and added to tubes that were cooled in an ice bath. Each tube contained 50 mM potassium phosphate buffer, pH 8.0 (0.65 mL), 1.4 mM NADH in distilled water (0.1 mL), and 10 mM glutathione in buffer (0.1 mL). Control experiments showed that reaction aliquots diluted into the tubes underwent no further inactivation and that the remaining active enzyme was stable. Enzyme assays were prepared by brifly hand warming each tube and transferring the entire 0.9-mL solution to a 1.0-cm path length quartz cuvette. The addition of cortisone (1.8 mM) in ethanol (0.1 mL) initiated the assay. The slope of the initial linear decrease in absorbance at 340 nm due to NADH oxidation as a function of time was used to calculate enzyme activity. Assays were performed at 25 °C in a Beckman DU-8 spectrophotometer.

K_m and V_{max} Determinations. Incubations (1.0 mL) contained steroids dissolved in ethanol (50 μL) and NAD (140 μM). Enzyme concentrations, pH, and steroid concentrations used are summarized in Table I. Reaction velocities were calculated, unless stated otherwise, from measurements of initial linear increase in absorbance at 340 nm due to NAD reduction as a function of time. Kinetic results were analyzed by $1/v$ vs. $1/[S]$ plots (Lineweaver & Burk, 1934). Regression lines for kinetic data were drawn according to a least-squares fit.

HPLC Analysis of 20β-HSD Incubations with **3** *and* **4.** Incubations (1.0 mL) containing the constituents reported in Figure 2 (see Results) were performed at room temperature, and at various times aliquots (200 μL) were directly injected into and analyzed by HPLC. The column used was an RCM-100 C_{18} 10-μm cartridge eluted at 2.0 mL/min with 80:20 methanol/water. **3** and **4** had retention times of 7.4 and 9.0 min, respectively. The compounds were detected by their UV absorbance at 238 nm and displayed on a strip chart recorder set at 0.04 absorbance units full scale.

Irreversibility of 20β-HSD Inactivation. Duplicate incubations (0.5 mL) were made containing enzyme (42 μg), 1 mM glutathione, 10 μM NAD, and 20 μM (3.2×10^4 cpm) radiolabeled steroid **3** in 50 mM buffer, pH 7.4, containing 5% ethanol. After 1 h at room temperature, when no enzyme activity was detectable, the duplicate incubations were separately applied to Sephadex G-25M columns and eluted with 50 mM buffer, pH 7.4 (20 × 1-mL portions). An aliquot (50 μL) of each fraction (1.0 mL) from one incubation was assayed for enzyme activity, and another aliquot (400 μL) from the same fraction was assayed for protein content. The equivalent fraction from the duplicate incubation was mixed with scintillation cocktail (10 mL) and counted for 5 min on the combined ^3H,^{14}C channel of a Beckman LS-3133T scintillation counter.

Results

Inactivation of 20β-HSD by Enzyme-Generated Ethoxyacetylenic Ketone. Incubation of 20β-HSD at pH 7.4 (Figure 1, upper panel) with alcohol **3** in the absence of NAD results in no loss of enzyme activity relative to a no inhibitor control. Incubation of enzyme, NAD, and alcohol **6**, the epimeric 20α-ethoxyactylenic alcohol which is not a 20β-HSD substrate,

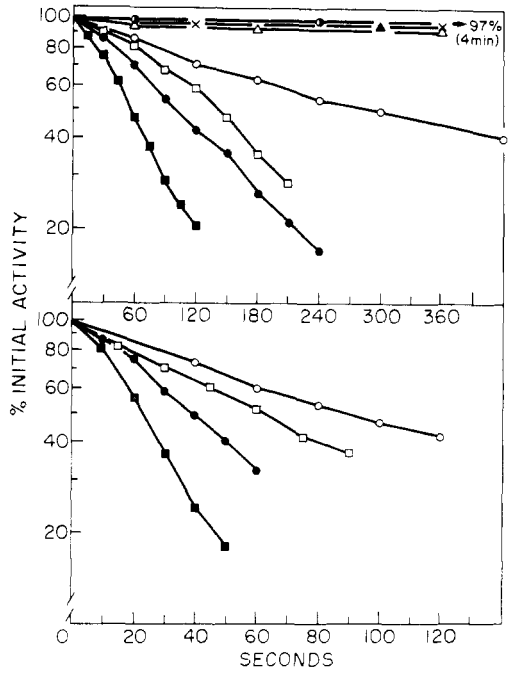


FIGURE 1: Inactivation of 20β-HSD by ethoxyacetylenic alcohol **3** at pH 7.4 (upper panel) and 9.2 (lower panel). Upper panel: Enzyme and the following: 140 μM NAD (open triangles); 5 μM **3** (×); 140 μM NAD and 5 μM **6** (half-filled circles); 140 μM NAD, 1 mM glutathione, and 5 μM **3** (filled triangle); 140 μM NAD and **3** at 0.5 (open circles), 1 (open squares), 2 (filled circles), or 5 μM (filled squares). Lower panel: Enzyme, 140 μM NAD, and **3** at 0 5 (open circles), 1 (open squares), 2 (filled circles), or 5 μM (filled squares). See Biochemical Methods for additional details. Results are the average of duplicate experiments, which agreed within ±3% at all time points.

also results in no loss of enzyme activity. These controls demonstrate that the ethoxyacetylenic alcohols are not capable of directly inactivating the enzyme. Incubation of 20β-HSD at either pH 7.4 or pH 9.2 (lower panel) with alcohol **3** and NAD results in a rapid time-dependent loss of 20β-HSD activity. The rate of inactivation is proportional to the concentration of **3** and, after an initial lag period (observable at the highest concentration evaluated), follows pseudo-first-order kinetics (determined, but not shown, by regression analysis of the data presented). The lag period is consistent with enzyme-generated **4** leaving the active site and later returning to inactivate the enzyme. The fact that the inclusion of 1 mM glutathione in the incubation buffer (Figure 1, upper panel) completely prevents enzyme inactivation also supports this conclusion. As will be demonstrated later, protection by glutathione is reduced (or lost) when experimental conditions are chosen that increase the free enzyme concentration by decreasing the amount of enzyme present in binary complexes with pyridine nucleotides.

HPLC Analysis of Incubations. HPLC analysis of various incubation mixtures (Figure 2) demonstrates that oxidation of alcohol **3** by NAD does not occur in the absence of 20β-HSD (panel A), that ketone **4** formation occurs rapidly when 20β-HSD is present (panel B), and that 1 mM glutathione scavenges, but not instantaneously, the accumulated enzyme-generated ketone **4** (panels C and D). The results shown in panels E–H (Figure 2) were obtained from experiments done to investigate the reduction of ketone **4** by NADH and 20β-HSD. Incubation of 5 μM ketone **4**, 135 μM NAD, and 5 μM NADH for 15 min results in no detectable reduction of ketone **4** (panel E). Reduction of ketone **4** can be demonstrated, however, when 140 μM NADH is used in the enzyme incubations (panels F and G). As expected, no reduction

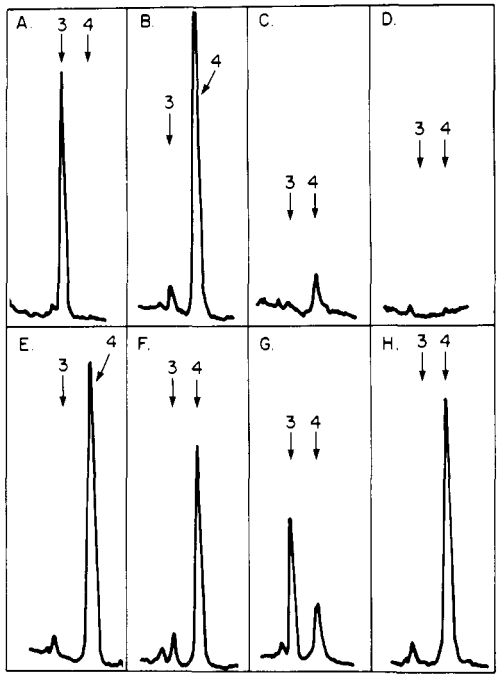


FIGURE 2: HPLC profiles of 20β-HSD incubations at pH 7.4 with **3** and **4**. Incubations in panels B–H contained enzyme (42 μg) and the additions reported in each panel. The positions of **3** and **4** are marked by arrows. The unmarked peak that eluted ahead of **3** is found in the buffer/ethanol blank. Panels: A, **3** (5 μM) and NAD (140 μM), no enzyme after 1 min; B, **3** (5 μM) and NAD (140 μM) after 1 min; C, **3** (5 μM), NAD (140 μM), and 1 mM glutathione after 1 min; D, as in panel C after 15 min; E, **4** (5 μM), NAD (135 μM), and NADH (5 μM) after 15 min; F, **4** (5 μM) and NADH (140 μM) after 1 min; G, as in panel F after 15 min; H, **4** (5 μM) and NAD (140 μM) after 1 min. See Biochemical Methods for additional details.

occurs when ketone **4** and enzyme are incubated with NAD (panel H). Thus, although the reduction of ketone **4** is possible, this reduction is not significant when alcohol **3** is oxidized in the presence of 140 μM NAD (i.e., the NAD concentration used for the experiments presented in Figure 1).

Substrate Characteristics of Alcohol **3**. Since conditions were found that allowed the oxidation of alcohol **3** to occur without loss of enzyme activity, the apparent K_m and V_{max} of the compound were determined (Table I). Comparison of these constants with those determined earlier for alcohol **1** shows that at pH 9.2 alcohol **3** binds ~70-fold more tightly and is maximally oxidized ~10-fold more slowly. The ethoxy group of alcohol **3** may afford some favorable binding interaction with the enzyme since 17β-[(1*S*)-1-hydroxy-2-butynyl]androst-4-en-3-one, a compound in which the ethoxy group is replaced by a methyl group, is not bound tightly by the enzyme. The kinetic constants for 20β-hydroxy-4-pregnen-3-one are included in Table I to demonstrate that alcohol **3** is utilized (compare K_m/V_{max} for each compond) nearly as efficiently as this excellent substrate.

Effects of Other Steroids and NAD Concentration on the Inactivation Resultant from Alcohol **3** *Oxidation.* In other experiments done with 140 μM NAD (not shown), 20β-hydroxy-4-pregnen-3-one (10 μM), a substrate for the enzyme, competes against alcohol **3** (2 μM) for enzyme catalysis and retards the rate of inactivation (percent inactivation at 120 s decreases from 58% to 13%). Estradiol (10 μM), a compound that is not a substrate, although it is a weak inhibitor (Gibb & Jeffrey, 1972), has only minimal effects (percent inactivation at 120 s decreases to 50%) on the activation rate. These results further substantiate that alcohol **3** is converted to the actual inactivator at the enzyme active site.

Table I: Kinetic Constants

compd[a]	pH	K_m (μM)	V_{max} (nmol min^{-1} μg^{-1})
20β-hydroxy-4-pregnen-3-one (10–100 μM)[b]	9.2	6.0	68.3
alcohol **1**	9.2	145[c]	63[c]
alcohol **3** (3–10 μM)	9.2[d]	2.1	6.4
	7.4[d]	2.3	4.6
17β-[(1*S*)-1-hydroxy-2-butynyl]-androst-4-en-3-one (5–30 μM)[e]	9.2	69	0.19

[a] Values in parentheses indicate the range of four different concentrations of steroid used for initial rate determinations. [b] Assays contained 0.1 μg of enzyme. [c] Values are from Strickler et al. (1980). [d] Buffers contained 1 mM glutathione and 2.5 μg of enzyme. Initial rates were measured at 350 nm (ε = 5300) to avoid interference due to an absorbance (λ_{max} = 300 nm) from the glutathione adduct formed with steroid **4**. [e] Assays contained 25 μg of enzyme. Insolubility of the steroid in the buffer precluded its use at higher concentrations. This previously unreported compound had analytical and spectroscopic data consistent with the reported structure.

The effect of NAD concentration on inactivation was also investigated (data not shown). If the rate of inactivation is determined solely by how rapidly ketone **4** is produced, then at a fixed concentration of alcohol **3** (2 μM), this rate should increase to a maximal value as the NAD concentration is increased. This is not what is observed. Although increasing NAD from 40 to 90 μM increases the reaction velocity and the resultant inactivation rate (59% and 65% inactivation at 120 s, respectively), further increases in NAD to 140 μM and 1.4 mM slow the inactivation rate (56% and 26% inactivation at 120 s, respectively). Since this enzyme shows a great preference for the formation of binary complexes with pyridine nucleotides rather than with steroids (Betz & Warren, 1968; Betz & Taylor, 1970), these results indicate (1) that inactivation does not occur in abortive E·NAD·**4** complexes (if they are formed) because the rate of inactivation would have increased at high NAD concentrations and (2) that free enzyme is readily inactivated by ketone **4** since decreasing the free enzyme concentration through formation of E·NAD complexes decreases the inactivation rate.

The question of the relative amount of inactivation that occurs in the evolutive E·NADH·**4** ternary complex was addressed by observing the efficiency of glutathione as a scavenger at high and low NAD concentrations. As shown earlier (Figure 1), the addition of 1 mM glutathione to the buffer completely protects the enzyme against ketone **4** generated from 5 μM alcohol **3** in the presence of 140 μM NAD. This is not the case when the scavenger addition experiment is repeated with 5 μM NAD (Figure 3). Although the rate of inactivation is slowed by the glutathione, only partial protection is observed at pH 9.2, and complete inactivation still occurs at pH 7.4.[1] Since the concentration of E·NADH·**4** competing against glutathione is lower at the lower NAD concentration, glutathione cannot be a less effective scavenger if enzyme inactivation in the E·NADH·**4** ternary complex is quantitatively important. The result is consistent, however, with increased amounts of free enzyme (caused by lowering the amount of enzyme present in E·NAD binary complexes) effectively competing against glutathione for ketone **4** and indicates that inactivation of free enzyme by ketone **4** is the quantitatively important inactivation event.

Inactivation of the enzyme at pH 7.4 in the presence of 1 mM glutathione as reported in Figure 3 is clearly biphasic. The fast and slow phases are due to inactivation under non-

[1] Inactivations done in the presence of 1 mM dithiothreitol or 10 mM mercaptoethanol gave the same results.

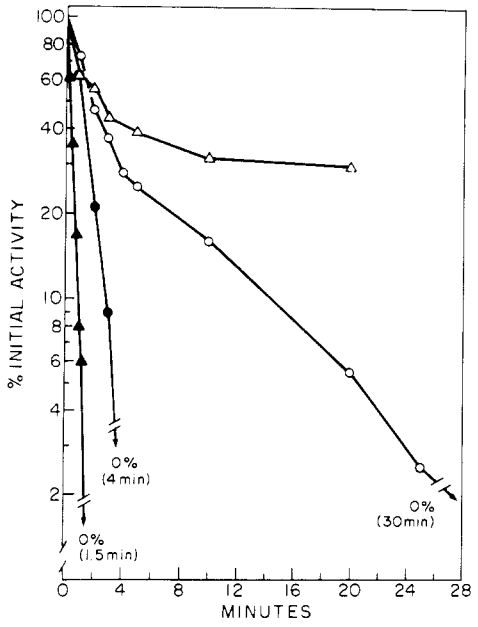


FIGURE 3: Effect of 1 mM glutathione on the inactivation of 20β-HSD by alcohol **3**. Enzyme, 5 μM alcohol **3**, and 5 μM NAD in pH 9.2 buffer with (open triangles) and without (filled triangles) 1 mM glutathione or in pH 7.4 buffer with (open circles) or without (filled circles) 1 mM glutathione.

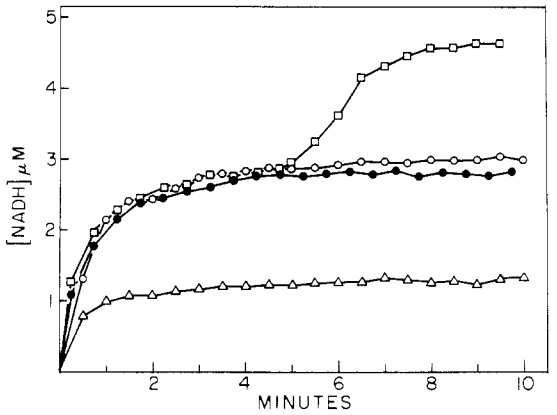


FIGURE 4: Time course of NADH production during 20β-HSD inactivation at pH 7.4 in the presence of 1 mM glutathione. Enzyme, 5 μM alcohol **3**, and 5 μM NAD (filled circles); same as initial conditions but with an addition of enzyme at 5 min (open circles); same as initial conditions but with an addition of 5 μM each of alcohol **3** and NAD at 5 min (open squares); enzyme and 2.5 μM each of alcohol **3**, NAD, and NADH (open triangles).

equilibrium and equilibrium conditions, respectively. The dissociation constants of NAD and NADH for their binary complexes with 20β-HSD as determined from fluorescence quenching experiments at pH 8.0 are 104 μM and 16 μM, respectively (Szymanski & Furfine, 1977). This difference in dissociation constants for the pyridine nucleotides, the low concentration of NAD used in the experiment, and the failure of glutathione to react instantaneously with ketone **4** are the factors that allow inactivation under equilibrium conditions to be achieved after approximately 4 min. That equilibrium has indeed been achieved after this time is demonstrated by the experiments shown in Figure 4.

When NADH production is measured during the course of the inactivation (Figure 4) it is found to reach ~2.8 μM after 4 min and to remain essentially unchanged after this time. Since 28% of the initial enzyme activity remains after 4 min (Figure 3) and another burst of NADH production can be obtained when additional NAD and alcohol **3** are added at

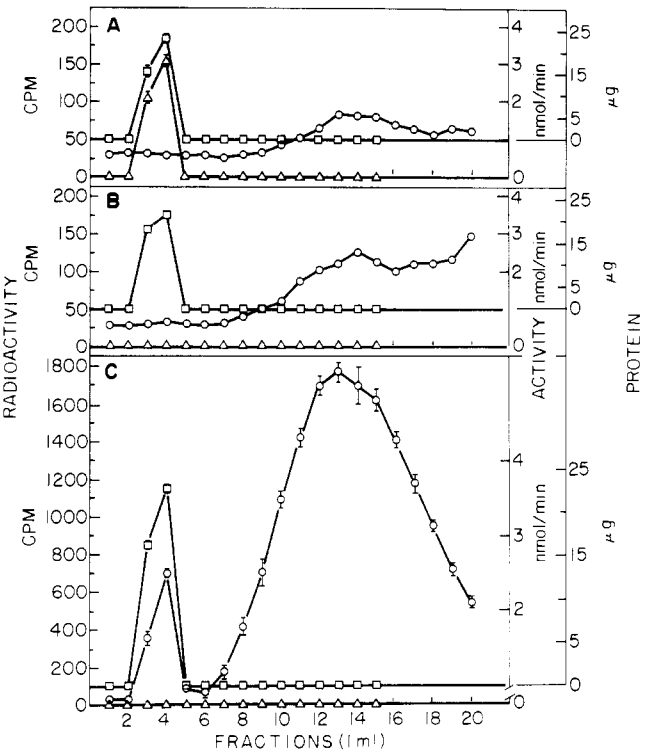


FIGURE 5: Profile of Sephadex G-25M column fractions from inactivations of 20β-HSD by radiolabeled alcohol **3**. Panel A: One control incubation contained enzyme (42 μg) and NAD (10 μM) in buffer, pH 7.4, and column fractions were analyzed for enzyme activity (triangles) and protein (squares). A second control incubation contained radiolabeled alcohol **3** (20 μM, ~32 000 cpm) in buffer, pH 7.4, and column fractions were analyzed for radioactivity (circles). Panel B: Enzyme (42 μg) was incubated with NAD (10 μM) and unlabeled **3** (10 μM) in buffer, pH 7.4, for 30 min. Radiolabeled **3** (20 μM, ~32 000 cpm) and additional NAD (10 μM) were then added, and after an additional 30 min column fractions were collected and analyzed. Panel C: Enzyme (42 μg) was incubated with NAD (10 μM) and radiolabeled **3** (20 μM, ~32 000 cpm) in buffer, pH 7.4, for 30 min, and then column fractions were collected and analyzed. See Biochemical Methods for additional details. Results shown in panels A and C are the averaged results of duplicate experiments.

5 min (Figure 4), the decrease in NADH production must be due to the attainment of an equilibrium between alcohol **3** oxidation and ketone **4** reduction.[2] Other results that would be anticipated if the system were at equilibrium after ~4 min were also found. Thus, a second addition of enzyme after 5 min failed to initiate additional NADH production, and an inactivation carried out with 2.5 μM each of alcohol **3**, NAD, and NADH produced a different maximal concentration of additional NADH.

The failure to achieve total inactivation at pH 9.2 in the presence of glutathione (Figure 3) is probably due to an increase in the reactivity of glutathione (ionization of the sulfhydryl group) at this pH. A detailed analysis of the inactivation at this pH was not attempted.

Evidence for Covalent Modification. Radiolabeled alcohol **3** (20 μM), NAD (10 μM), glutathione (1 mM), and enzyme were incubated for 30 min to achieve complete inactivation, and then noncovalently bound radiolabel was removed from the protein by Sephadex G-25M chromatography. The results along with appropriate controls are shown in Figure 5. The controls show that unbound alcohol **3** does not elute in the

[2] Inactivations done in the presence of preformed glutathione adducts (prepared by reacting ketone **4** and glutathione) showed that these adducts have no effect on the equilibrium between **3** and **4**.

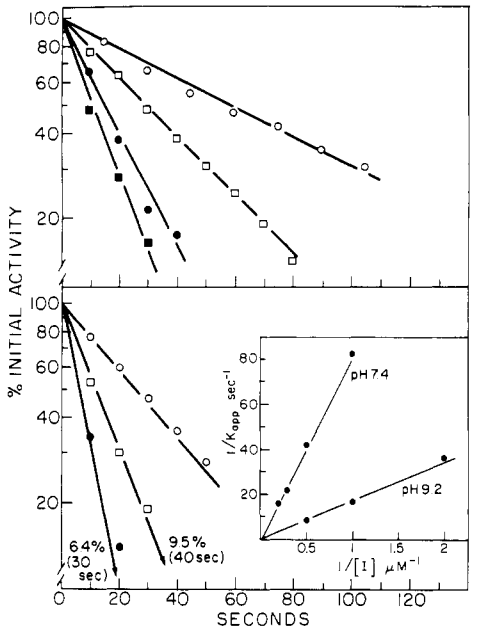


FIGURE 6: Inactivation of 20β-HSD by ethoxyacetylenic ketone **4** at pH 7.4 (upper panel) and 9.2 (lower panel). Upper panel: Ethoxyacetylenic ketone **4** at 1 (open circles), 2 (open squares), 3.5 (filled circles), or 5 μM (filled squares). Lower panel: Ethoxyacetylenic ketone **4** at 500 nM (open circles), 1 μM (open squares), or 2 μM (filled circles). Insert: Double-reciprocal plot of apparent rate constant for inactivation vs. concentration of **4** at pH 7.4 and 9.2. All lines were drawn by regression analysis. See Biochemical Methods for additional details. Results are the average of duplicate experiments, which agreed within ±3% at all time points.

same fractions as the enzyme (panel A) and that inactivation of the enzyme with unlabled alcohol **3** followed by incubation with radiolabeled alcohol **3** results in no incorporation of the labeled compound into the protein (panel B). Inactivation with radiolabeled alcohol **3** (panel C) results in the incorporation of 0.36 nmol (1100 cpm) of enzyme-generated ketone **4** into 39.6 μg of protein. The large amount of radioactive material eluting off the column after the enzyme–steroid adduct is presumed to be the glutathione–steroid adduct. This material has not been characterized.

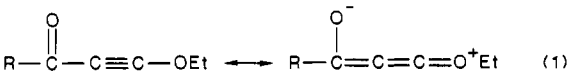
Inactivation of 20β-HSD by Ketone **4**. Since ketone **4** was prepared by chemical methods, its properties as an active-site-directed inhibitor were also examined. Incubation of this reactive ketone with the enzyme at either pH 7.4 or pH 9.2 results in an extremely rapid loss of enzyme activity that follows pseudo-first-order kinetics (Figure 6). Double-reciprocal plots of the apparent rate constant for inactivation vs. inhibitor concentration (Kitz & Wilson, 1962) provide no evidence for enzyme–ketone complex formation prior to enzyme inactivation. The bimolecular rate constants are 1.15×10^4 L mol^{-1} s^{-1} at pH 7.4 and 6.94×10^4 L mol^{-1} s^{-1} at pH 9.2.

Finally, experiments were done with ketone **4** to determine how pyridine nucleotides and other steroids affected its inactivation of the enzyme (data not shown). At pH 7.4, the percentage of 20β-HSD inactivated at 60 s by 2 μM ketone **4** decreased from 72% to 59% and 20% when 100 μM NAD and 100 μM NADH, respectively, were added to separate incubations. Similar results were obtained at pH 9.2, with the percentage of 20β-HSD inactivated at 20 s decreasing from 86% to 65% and 50%, respectively. This was expected because the earlier results obtained with enzyme-generated ketone **4** indicated that the ketone could not inactivate the enzyme in ternary complexes formed from preexisting binary E·NAD(H) complexes.

Neither 100 μM 20β-hydroxy-4-pregnen-3-one (an excellent alcohol substrate) nor 100 μM cortisone (an excellent ketone substrate) had effects on the rate of inactivation caused by 2 μM ethoxyacetylenic ketone at either pH 7.4 or pH 9.2. These results indicate that either the competing steroids are bound at a site different from that covalently modified by the ethoxyacetylenic ketone or that none of the steroids form kinetically significant binary complexes with the enzyme. Our preference is the latter explanation. As long as the concentrations of the binary complexes of the nonreactive steroids and enzyme are small, the free enzyme concentration remains essentially unchanged and no protection will be observed.

Discussion

Ethoxyacetylenic alcohol **3** binds ~70-fold more tightly than acetylenic alcohol **1** (Strickler et al., 1980) in its ternary complex with NAD and 20β-HSD. The improved affinity of alcohol **3** may result from a favorable interaction of the ethoxy group oxygen atom with the enzyme in the ternary complex. The ethoxy group also markedly shifts the equilibrium between alcohol **3** and ketone **4** more toward ketone **4** at physiological pH. The ethoxy group is electron donating due to the unpaired electrons on oxygen (eq 1), and the electron-rich system would



(1)

be a poorer hydride acceptor than the unsubstituted acetylene. This makes ketone reduction less favorable and might explain the observed shift in the equilibrium.

The conclusion that 20β-HSD inactivation results from covalent modification of the enzyme was supported by the gel filtration experiments in which 0.36 nmol of radiolabeled steroid was incorporated into 39.6 μg of protein. 20β-HSD has been shown to be an ~110000 molecular weight tetramer composed of four identical subunits (Blomquist, 1973), and the assumption that all of the protein eluted was originally active 20β-HSD would lead to a calculated stoichiometry of inactivation of 1 steroid bound per tetramer. However, since the specific activity of the commercially available enzyme used in this study was 10.5 units/mg and previously purchased material has had specific activities as high as 29 units/mg, the stoichiometry could be as high as 3 steroids bound per tetramer. Hence, the stoichiometry of inactivation remains to be unambigously established. Identification of the modified active-site amino acid also remains a target for future studies.

The ethoxyacetylenic ketone **4** used in this study differs from the acetylenic ketone **2** used earlier (Strickler et al., 1980) in two respects: (1) it inactivates the free 20β-HSD so rapidly that it can be used as an inactivator in the presence of thiols under certain reaction conditions, and (2) it does not form detectable binary complexes with the enzyme prior to the inactivation reaction. Part of the reason for the first observed difference is due to the fact that the acetylenic ketone reacted with thiols more rapidly than the ethoxyacetylenic ketone. Since the chemical kinetics of these reactions were not studied, it is not possible at this time to quantitate the difference in reaction rates. Nevertheless, this conclusion is supported by HPLC experiments (not shown) done with enzyme-generated acetylenic ketone **2**. The experiments were similar to those reported for the ethoxyacetylenic compounds in Figure 2. Regardless of NAD concentration and time of analysis, it was never possible to detect enzyme-generated acetylenic ketone **2** in the presence of 1 mM glutathione. This shows that ketone **2** is more reactive than ketone **4** toward glutathione.

More important, however, than this difference in reactivity of the two ketones toward glutathione is their difference in

reactivity toward 20β-HSD. The bimolecular rate constant for 20β-HSD inactivation at pH 9.2 by ketone **4** is 6.94×10^4 L mol^{-1} s^{-1} while that of the earlier evaluated ketone **2** (Strickler et al., 1980) was 444 L mol^{-1} s^{-1} (obtained from k_{inact}/K_I). Hence, the ethoxyacetylenic ketone inactivates 20β-HSD 156-fold faster at this pH. The difference in inactivation rates at pH 7.4 would be even greater. If ketone **4** reacted with 20β-HSD as slowly as ketone **2**, then it would probably not have been possible to inactivate the enzyme with ketone **4** in the presence of glutathione or other thiols.

Given the similarity in structure between ketones **2** and **4**, why are binary complexes with the enzyme detectable prior to inactivation only with ketone **2**? One obvious answer is that despite the structural similarity of the compounds covalent modification occurs at different sites in the protein. Another possibility is that modification of the same site occurs for both compounds but that binary complex formation is rate-limiting (i.e., $k_{inact} > k_{on}$) only for ketone **4**. This could be the situation if a slow (relative to k_{inact} for ketone **4**, but not for ketone **2**) conformational change was required before binary complex formation occurred. Sequence data on a peptide fragment containing each covalently bound steroid would establish whether or not identical sites are modified by ketones **2** and **4** and will distinguish between these possible explanations of the experimental data.

As discussed under Results, 20β-HSD inactivation by enzyme-generated ketone **4** in the ternary E·NADH·**4** complex is not a quantitatively important event. It would seem that the nucleophilic group that reacts with the ethoxyacetylenic ketone cannot get near enough to the β-acetylenic carbon for Michael addition to occur in this ternary complex. This does not necessarily mean that the nucleophilic residue is actually shielded from the reactive ketone by the cofactor. It is equally possible that the strict alignment of the catalytic amino acids in the ternary complex positions the only available nucleophilic groups proximate to the carbonyl group at a distance that precludes Michael addition to the more remote β-acetylenic carbon. In either situation the rates at which the reduced cofactor and reactive ketone leave the ternary complex to form their respective binary complexes will dictate whether inactivation can occur without release of the reactive ketone from the active site. If initial steroid release is a highly favored kinetic process, as it is for 20β-HSD (Betz & Warren, 1968; Betz & Taylor, 1970), then inactivation will essentially occur only when free enzyme is subsequently encountered by released reactive ketone. This explains why even the large increase in k_{inact} observed for ketone **4** relative to that observed for ketone **2** failed to permit 20β-HSD inactivation to occur prior to release of ketone **4**.

In summary, this study of 20β-HSD inactivation by an enzyme-generated ethoxyacetylenic ketone has provided insight into why inactivation without prior release and return of the inactivator has not yet been achieved for this enzyme. It suggests that HSDs whose ternary complexes dissociate to give kinetically significant concentrations of binary enzyme–steroid complexes will be easier targets for suicide inactivation. The following paper in this issue (Auchus & Covey, 1986) attempts to address this problem by using a HSD with a random kinetic mechanism. Finally, this study has provided an example of an enzyme-generated inactivator that can be used in the presence of thiols even though it inactivates by a release and return mechanism.

Acknowledgments

We thank Dr. Ian Jardine of the Mayo Clinic, Rochester, MN, for performing the high-resolution mass spectrometric determination and Richard Auchus for helpful discussions concerning the preparation of the manuscript.

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