Mechanism-Based Inactivation of 17β,20α-Hydroxysteroid Dehydrogenase by an Acetylenic Secoestradiol†

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ABSTRACT: 14,15-Secoestra-1,3,5(10)-trien-15-yne-3,17β-diol (**1**) is a mechanism-based inactivator of human placental 17β,20α-hydroxysteroid dehydrogenase (estradiol dehydrogenase, EC 1.1.1.62). Inactivation with alcohol **1** requires NAD-dependent enzymic oxidation and follows approximately pseudo-first-order kinetics with a limiting $t_{1/2}$ of 82 min and a "K_i" of 2.0 μM at pH 9.2 and 25 °C. At saturating concentrations of NAD, the initial rate of inactivation is slower than in the presence of 5 μM NAD, suggesting that cofactor binding to free enzyme impedes the inactivation process. Glutathione completely protects the enzyme from inactivation at both cofactor concentrations. Inactivation with 45 μM tritiated alcohol **1** followed by dialysis and gel filtration demonstrates a covalent interaction and affords an estimated stoichiometry of 1.4 molecules of steroid per subunit (2.8 per dimer). Chemically prepared 3-hydroxy-14,15-secoestra-1,3,5(10)-trien-15-yn-17-one (**2**) rapidly inactivates estradiol dehydrogenase with biphasic kinetics. From the latter phase, a K_i of 2.8 μM and a limiting $t_{1/2}$ of 12 min at pH 9.2 were determined. Estradiol, NADH, and NAD all retard this latter inactivation phase. We propose that enzymatically generated ketone **2** inactivates estradiol dehydrogenase after its release from and return to the active site of free enzyme.

Since estradiol (E_2)[1] is a much more potent estrogen than E_1 (Cook et al., 1962; Shutt & Cox, 1972), placental E_2-HSD, an enzyme that catalyzes the pyridine nucleotide dependent interconversion of E_1 and E_2, is ideally situated for modulating estrogenic potency in vivo. 17β-HSD activities have been described in placenta (Langer & Engel, 1958), uterus (Tseng & Gurpide, 1974), and breast tissue (Geier et al., 1975). In uterus, the 17β-HSD activity is believed to diminish estrogenic potency via oxidation of E_2 to the less potent E_1, which diffuses out of the tissue more readily than E_2 (Tseng & Gurpide, 1975). On the other hand, the role(s) of these 17β-HSD activities in placenta (Pons et al., 1977) and breast (Bonney et al., 1983), particularly in relation to reproductive endocrinology and the development of estrogen-dependent neoplasms, remains (remain) unresolved. Potent, specific inhibitors of this enzyme, consequently, could prove to be useful pharmacological tools and possibly therapeutic agents.

E_2-HSD has been purified to homogeneity from human term placenta (Jarabak, 1969; Murdock et al., 1986), and it has been used as a model to study steroid–protein interactions (Pons et al., 1977; Warren et al., 1977). Mechanism-based inactivators (Abeles, 1983; Walsh, 1982) that utilize both this enzyme's 20α activity (Tobias et al., 1982) and its 17β activity (Thomas et al., 1983) have been reported previously. The acetylenic ketone **3b** rapidly inactivates E_2-HSD (limiting $t_{1/2}$, <1 min), but the corresponding 20α alcohol **3a** is a very poor substrate (at pH 9.2, K_m = 435 μM, V_{max} = 40 nmol min^{-1} mg^{-1}) (see Scheme I). Allylic alcohol **4a**, in contrast, is an excellent substrate (at pH 9.2, K_m = 8 μM, V_{max} = 2.8 μmol min^{-1} mg^{-1}), but vinyl ketone **4b** has very poor affinity (K_i = 261 μM at pH 7.0 and a higher value at pH 9.2), and it is a slower inactivator than **3b**. In an attempt to combine the superior affinity of **4a** with the reactivity of **3b** and to extend our studies of acetylenic steroids as enzyme inactivators (Covey et al., 1981, 1986; Penning & Covey, 1982; Penning et al., 1981; Strickler et al., 1980), we synthesized alcohol **1** and ketone **2** and evaluated these compounds as inactivators of E_2-HSD.

Experimental Procedures

Materials. Biological chemicals and Reactive Blue 2–agarose were obtained from Sigma Chemical Co., St. Louis, MO. Disposable PD-10 Sephadex G-25M columns (9.1-mL bed volume) were purchased from Pharmacia, Inc., Piscataway, NJ. Silica gel was purchased from Universal Scientific Co., Atlanta, GA, and TLC plates (silica gel GF/B, 250-μm thickness) were purchased from Analtech, Newark, DE.

General Procedures. Enzyme activity was assayed by the method of Ryan and Engel (1958). NADH production was quantitated by measuring A_{340} every 15 s for 2.25 min at 25 °C in a Perkin-Elmer DU-8 spectrophotometer. Steroids were weighed on a Cahn 4400 or a Cahn C30 microbalance. Radioactivity was quantitated by mixing the sample with 5 mL of Budget Solve (Research Products International, Mt. Prospect, IL) and counting for 5 min in a Beckman LS-3800 scintillation counter. Efficiency was determined by adding a known amount of [^{3}H]toluene to the samples of interest. Protein was estimated by a modification of the method of Smith et al. (1985) using crystalline bovine serum albumin as standard. The sample was added to an equal volume of working reagent [80 mM K_2HPO_4/20 mM Na_3PO_4 containing 0.1% $CuSO_4 \cdot 5H_2O$, 0.2% sodium tartrate, and 1% bicinchoninic acid (Pierce Chemical Co., Rockford, IL)]. After

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[1] Abbreviations: E_2, estradiol; E_1, estrone; E_2-HSD, estradiol dehydrogenase (EC 1.1.1.62); 17β-HSD, 17β-hydroxysteroid dehydrogenase; $Na(H)CO_3$, sodium carbonate–bicarbonate; KP_i, potassium mono- and dihydrogen phosphate; NMR, nuclear magnetic resonance spectroscopy; THF, tetrahydrofuran; TLC, thin-layer chromatography; δ, chemical shift in parts per million downfield from tetramethylsilane; SD, standard deviation.

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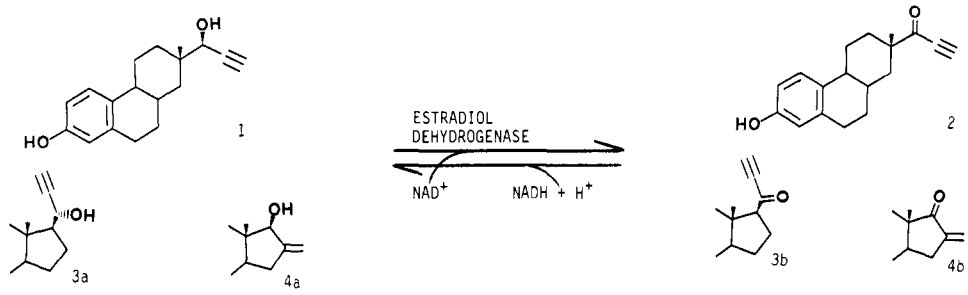
Scheme I

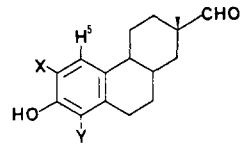


the samples were mixed and incubated at 60 °C for 30 min, they were cooled, and absorbance at 562 nm was measured. Compounds on TLC plates were visualized by charring with 10% sulfuric acid in methanol. NMR spectra were recorded at 300 MHz (^{1}H) on a Varian XL-300 spectrometer.

Enzyme Purification. Using the method of Murdock et al. (1986), 5–10 mg of homogeneous protein per placenta was obtained with a specific activity of 3 ± 1 units/mg according to our protein assay.

Syntheses. The syntheses of (2*S*,4a*S*,10a*S*)-1,2,3,4,4a,9,10,10a-octahydro-7-hydroxy-2-methyl-2-phenanthrenecarboxaldehyde (**5**), alcohol **1**, and ketone **2** are described elsewhere (Auchus et al., 1986).

Monobromocarboxaldehydes **6a** *and* **6b**. A solution of aldehyde **5** (25 mg, 0.1 mmol) in glacial acetic acid (2 mL) was stirred at room temperature and treated dropwise with 5% w/w Br_2 in AcOH until a yellow color persisted for 10 s (approximately 450 μL, 0.14 mmol of Br_2). After being stirred at room temperature for 15 min, the reaction mixture was diluted into 10 mL of H_2O plus 1 mL of saturated NaCl and extracted 3 times with 3 mL of EtOAc. The pooled organic layers were dried over Na_2SO_4, filtered, and concentrated in vacuo. The residue was purified on a 5 × 60 mm column of silica gel with 10% EtOAc/hexanes and crystallized from CH_2Cl_2, affording 25 mg (78% yield) of a white solid. A mixture (~1:1) of 6- and 8-monobromides was observed by ^{1}H NMR ($CDCl_3$): **6a**, δ 7.34 (s, 1, H^5) and 6.76 (s, 1, H^8); **6b**, δ 7.19 and 6.89 (dd, J = 8.4 Hz, 1 each, H^5, H^6).



6a, X=Br Y=H
6b, X=H Y=Br

[6-^{3}H]-5, X=^{3}H Y=H

[8-^{3}H]-5, X=H Y=^{3}H

Tritiated Carboxaldehyde **5** (*6- and 8-*^{3}*H*). This reaction was performed by New England Nuclear, Research Products Division, Boston, MA, as a custom synthesis. **6a** and **6b**, 25 mg, were dissolved in 2 mL of ethanol and 0.03 mL of triethylamine. To this were added 25 mg of 5% Pd/C and an atmosphere of tritium gas. The reaction was stirred at room temperature for 1 h, and an uptake of 1.5 mL was observed. A total of 2.3 Ci of nonvolatile material was obtained.

Unlabeled compound **5** (12 mg) was added to 100 mCi of tritiated **5**, and the mixture was purified on a 5 × 50 mm column of silica gel with 10–15% EtOAc/hexanes. The fractions containing radiolabeled **5** were pooled and concentrated under N_2 to a white solid.

Tritiated Alcohol **1** (*2-*^{3}*H and 4-*^{3}*H, Steroid Numbering*). A solution of approximately 0.2 M ethynylmagnesium bromide in THF (predried over KOH and distilled from $LiAlH_4$) was prepared (Auchus et al., 1986) under N_2. Part of this reagent (1 mL, approximately 0.2 mmol) was added dropwise to tritiated aldehyde **5** in 0.5 mL of dry THF under N_2 with agitation. A thick white precipitate formed, and after 15 min at room temperature, the reaction mixture was diluted with 6 mL each of EtOAc and 0.1 N HCl, vortexed, and separated. The organic layer was washed with 5 mL of saturated NaCl, dried over Na_2SO_4, filtered, and concentrated under N_2. The residue was purified on a 5 × 50 mm column of silica gel with 20% EtOAc/hexanes and recrystallized with Et_2O/hexanes. The solid was dissolved in 3 mL of absolute ethanol and stored at −20 °C. The specific activity was determined to be 1.53 ± 0.06 Ci/mmol, and the product was homogeneous by TLC (R_f = 0.34, 25% EtOAc/hexanes).

Inactivation by Enzyme-Generated Ketone **2**. Alcohol **1** in 5 μL of absolute ethanol was incubated at 25 °C with 72 μg of E_2-HSD in 100 mM $Na(H)CO_3$ buffer (pH 9.2, 20% glycerol) with or without NAD and/or glutathione in a total volume of 1.0 mL. At intervals, 50-μL aliquots were withdrawn and added to 0.95 mL of assay cocktail [100 mM $Na(H)CO_3$ buffer, pH 9.2, 60 μM E_2, 3.3% (v/v) propylene glycol, 200 μM NAD, and 0.8% (w/v) human albumin]; activity was determined as described.

Inactivation by Chemically Prepared Ketone **2**. Ketone **2** in 5 μL of absolute ethanol was incubated at 25 °C with 72 μg of E_2-HSD in 100 mM $Na(H)CO_3$ buffer (pH 9.2, 2% glycerol) with or without E_2, NADH, or NAD in a total volume of 1.0 mL. At intervals, 50-μL aliquots were withdrawn and added to 0.9 mL of quench buffer [100 mM $Na(H)CO_3$ buffer, pH 9.2, 60 μM E_2, 3.3% propylene glycol, 0.8% (w/v) human albumin, and 5 mM glutathione]. Assays were initiated by adding 50 μL of 4 mM NAD (final concentration, 200 μM).

Evaluation of Alcohol **1** *as a Substrate.* Alcohol **1** in 5 μL of absolute ethanol was incubated with 286 μg of E_2-HSD and 200 μM NAD in 1.0 mL of 100 mM $Na(H)CO_3$ buffer [pH 9.2, 20% glycerol, 0.5 % (w/v) human albumin] at 25 °C for 15 min. Absorbance at 340 nm was measured at 1-min intervals. Five concentrations of alcohol **1** (20–200 μM) were assayed.

Enzymic Reduction of Ketone **2**. Ketone **2** (40 μM) in 5 μL of absolute ethanol was incubated at 25 °C with 100 μg of E_2-HSD and 400 μM NADH in a total of 1 mL of 100 mM $Na(H)CO_3$ buffer (pH 9.2, 2% glycerol). Absorbance at 340 nm was measured at 15-s intervals for 2–6 min.

Time Course and Stoichiometry of NADH Production. Alcohol **1** in 5 μL of absolute ethanol (100 μM) was incubated with 72 μg of E_2-HSD (2.1 nmol of subunits) and 200 μM NAD in 1.0 mL of 100 mM $Na(H)CO_3$ (pH 9.2, 20% glycerol) at 25 °C for 6 h in the DU-8 spectrophotometer. Absorbance at 340 nm was measured at 5-min intervals and converted to nanomoles of NADH by using $\epsilon = 6.23 \times 10^3$ M^{-1} cm^{-1} (<10% initial activity remained after 6 h).

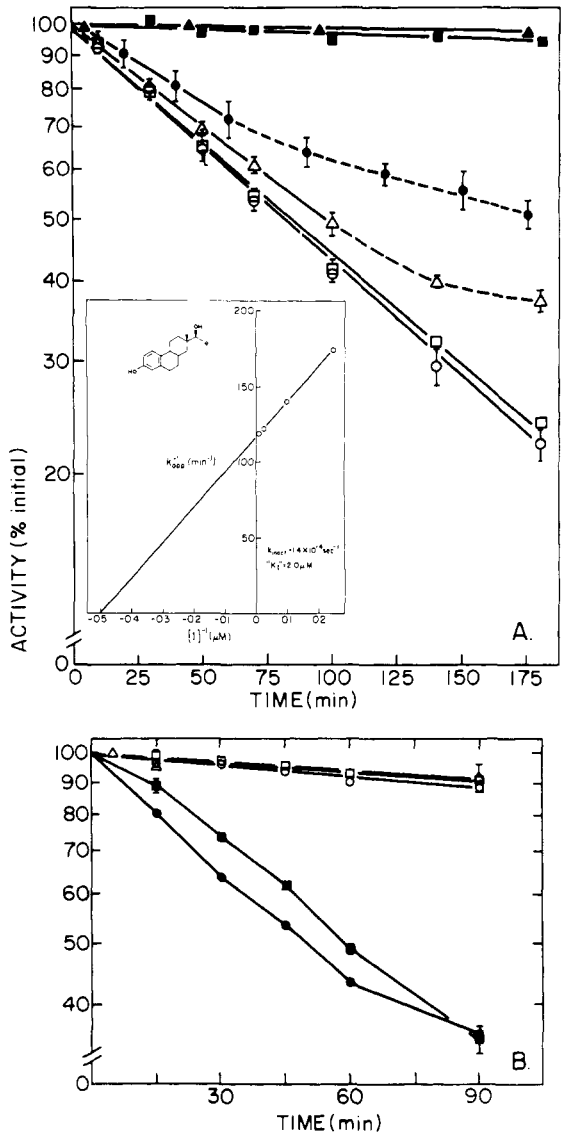


FIGURE 1: Inactivation of E_2-HSD by enzyme-generated ketone **2**. Panel A: E_2-HSD was incubated with 200 μM NAD and the following: (▲) no steroid; (●) 4, (△) 10, (□) 40, or (○) 100 μM alcohol **1**; or (■) 40 μM alcohol **1** plus 5 mM glutathione. Data points indicate the mean ± SD of three experiments (except ■, $n = 2$). Lines drawn are least-squares fits ($r \geq 0.998$). Insert: Double-reciprocal plot to determine "K_i" and limiting $t_{1/2}$ ($=0.693/k_{inact}$) values. Panel B: E_2-HSD was incubated with 40 μM alcohol **1** and the following: (●) 5 μM NAD, no glutathione; (○) 5 μM NAD plus 1 mM glutathione; (■) 200 μM NAD, no glutathione; or (□) 200 μM NAD plus 1 mM glutathione. (△) E_2-HSD was incubated with 5 μM NAD but *no* steroid. Data points indicate mean ± range of duplicate determinations.

Demonstration of Covalent Interaction and Stoichiometry. Tritiated alcohol **1** in 6 μL of absolute ethanol (final concentration, 45 μM) was incubated with 100 μM NAD and 78 μg of E_2-HSD in a total of 1.2 mL of 100 mM $Na(H)CO_3$ (pH 9.2, 20% glycerol) at 25 °C for 12 h and assayed; 6–9% of the initial activity remained. A control incubation (alcohol **1** omitted) lost 17–20% activity.

A second control (alcohol **1** omitted) was chilled to 4 °C and mixed with 45 μM tritiated alcohol **1**. After 1 min at 4 °C, 1.0 mL was removed and dialyzed against 250 mL of 20 mM KP_i, pH 7.0, for 4 h at 4 °C, with a buffer change at 1.5 h. A 1-mL aliquot of the 12-h incubation was removed and similarly dialyzed.

Samples (1.0 mL) from each dialysis bag were applied to separate PD-10 columns equilibrated with 20 mM KP_i, pH 7.0, and eluted with the same buffer. Fractions (1 mL) were collected, counted (100 μL), and assayed for protein (400 μL). Fractions containing protein were assayed in duplicate.

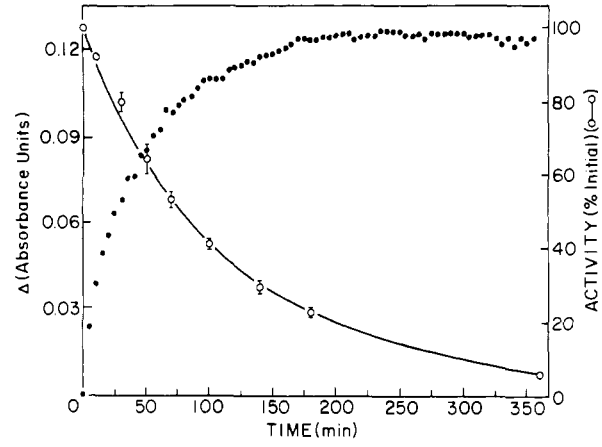


FIGURE 2: NADH production by E_2-HSD with alcohol **1**. E_2-HSD was incubated with 200 μM NAD and 100 μM alcohol **1**. Results of a representative experiment are shown. Turnover (total NADH production) was calculated from the absorbance change (●) between $t = 0$ and equilibrium ($t \geq 4$ h). Activity data (○) were taken from the experiments shown in Figure 1A.

RESULTS

Inactivation of E_2-HSD by Enzyme-Generated Ketone **2**. Incubation of alcohol **1** with E_2-HSD and 200 μM NAD (pH 9.2, 25 °C) results in time-dependent loss of activity (Figure 1A). Inactivation follows approximately pseudo-first-order kinetics for a length of time proportional to the initial concentration of alcohol **1**. The deviation from linearity probably reflects the system's reaching equilibrium (vide infra). By use of the linear portion of the curves and a plot of $1/k_{app}$ vs. $[S]^{-1}$ (Kitz & Wilson, 1962), a limiting $t_{1/2}$ of 82 min was calculated (Figure 1A, insert). The rate of inactivation is half-maximal at "K_i" = 2.0 μM.[2] Glutathione (5 mM) completely protects the enzyme from inactivation resultant from enzymic oxidation of 40 μM alcohol **1** (Figure 1A). If NAD is not included, 89.0 ± 1.7% control activity remains after 120 min in the presence of 100 μM alcohol **1**.

The inactivation experiments in Figure 1A were performed at saturating NAD concentrations to minimize effects due to changing NAD concentrations. To ascertain if the concentration of cofactor could influence inactivation kinetics, we conducted inactivation experiments at lower NAD concentrations. At early times, inactivation is more rapid and apparently more linear at 5 μM than at 200 μM NAD (Figure 1B).[3] These data indicate that excess cofactor actually slows the rate of inactivation. Glutathione (5 mM) completely protects the enzyme from inactivation at both concentrations of NAD (Figure 1B).

Alcohol **1** *as a Substrate.* Since inactivation occurs over minutes to hours, we were able to evaluate alcohol **1** as a substrate under inactivation conditions. Alcohol **1** ($K_m = 79$ μM, $V_{max} = 8.1$ nmol min^{-1} mg^{-1}, $r = 0.999$ for $1/v$ vs. $1/[S]$ plot) is a poorer substrate than estradiol ($K_m = 6.2$ μM, $V_{max} = 3.4$ μmol min^{-1} mg^{-1}), being oxidized about 400 times more slowly under these conditions.

[2] "K_i" in this case is defined as the concentration of alcohol **1** that, in the presence of NAD, produces a half-maximal rate of inactivation. This constant is *not* a true K_i (Kitz & Wilson, 1962) since it is not necessarily equal to the concentration of the inactivating species.

[3] The discrepancy between $t_{1/2}$ for inactivation with 200 μM NAD and 40 μM alcohol **1** in panels A and B of Figure 1 is attributed to different preparations of enzyme and steroid used for the two experiments.

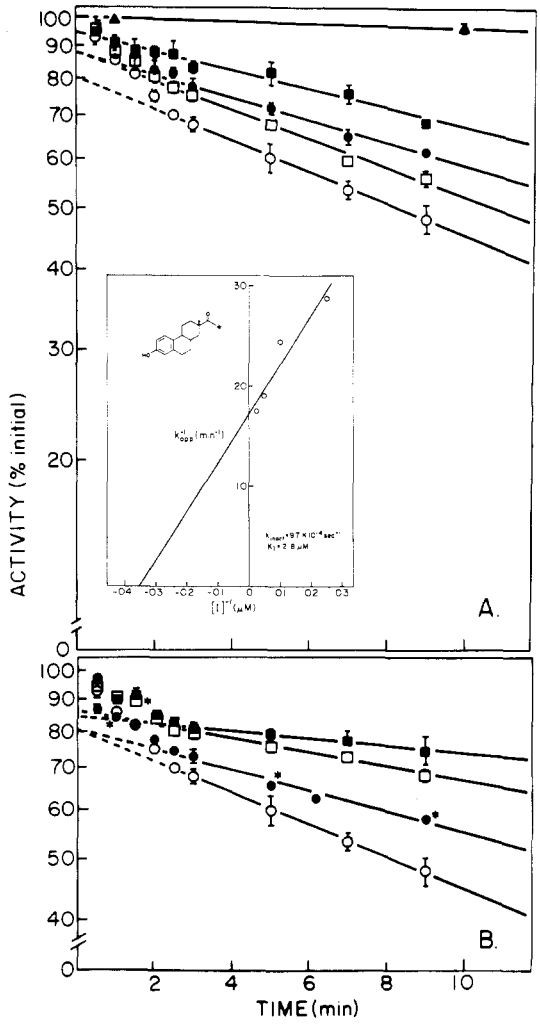


FIGURE 3: Inactivation of E_2-HSD by ketone **2**. Panel A: E_2-HSD was incubated with (▲) no steroid or (■) 4, (●) 10, (□) 20, (○) or 40 μM ketone **2**. Data points indicate mean ± range of duplicate determinations. Lines drawn are least-squares fits ($r \geq 0.965$). Inset: Double-reciprocal plot of the data. Panel B: E_2-HSD was incubated with 40 μM ketone **2** plus the following: (○) no other additions; (●) 200 μM NAD; (■) 400 μM NADH; or (□) 60 μM E_2. Concentrations of cofactors and substrate were chosen to approximate $10K_m$. Data points indicate mean ± range of duplicate determinations (except *, $n = 1$). Lines drawn are least-squares fits ($r \geq 0.981$).

Time Course and Stoichiometry of NADH Production. At 100 μM alcohol **1**, a concentration slightly above K_m but well above "K_i", the generation of 8–9 molecules of NADH per subunit of enzyme accompanies complete inactivation (Figure 2). At lower concentrations, the rate of turnover decreases more rapidly than the rate of inactivation; consequently, less NADH is produced per inactivation event.

Figure 2 also illustrates that detectable NADH production has ended by 3 h, whereas inactivation continues for several hours thereafter.[4] We interpret these data to indicate that the system has reached equilibrium after 3 h.

Inactivation of E_2-HSD by Ketone **2**.[5] Chemically prepared ketone **2**, an affinity label, inactivates E_2-HSD with biphasic kinetics. A brief, more rapid initial phase precedes pseudo-first-order loss of activity (Figure 3A). We do not understand

[4] Typically, 6–10% of initial activity remains after 7–8 h when 100 μM alcohol **1** is used.

[5] Glycerol concentrations for experiments with ketone **2** were minimized because glycerol reacts with acetylenic ketones under these conditions [$t_{1/2}$ = 22 min (Tobias et al., 1982)]. The absence of glycerol stabilization (Langer & Engel, 1958) produced minimal loss of activity during the short time course of these experiments.

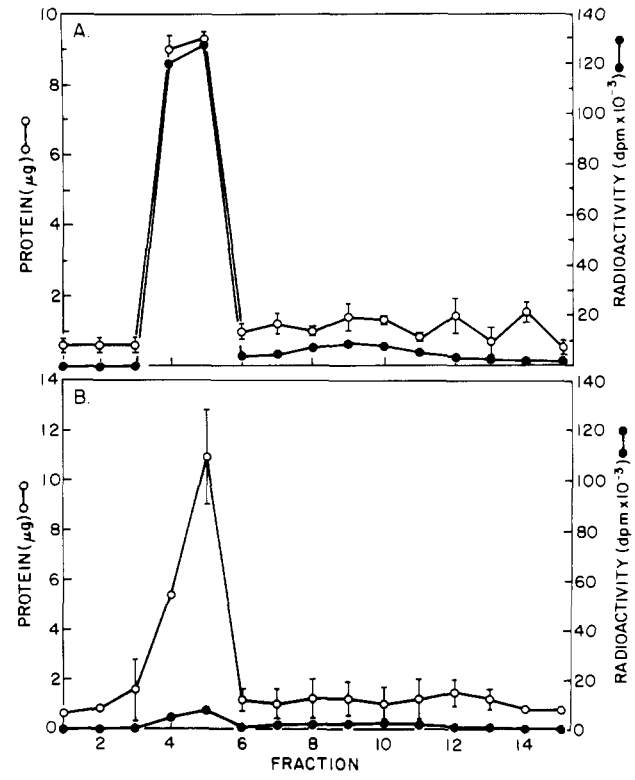


FIGURE 4: Demonstration of covalent attachment of E_2-HSD to enzyme-generated tritiated ketone **2**. Sephadex G-25 chromatography of E_2-HSD incubated with 100 μM NAD and 45 μM tritiated alcohol **1** for (panel A) 12 h, 25 °C, or (panel B) 1 min, 4 °C. Column fractions (1 mL) were assayed for protein (○) and radioactivity (●).

what the early phase represents, but it might derive from a process that is not active-site-directed. From the latter phase, a limiting $t_{1/2}$ of 12 min and a K_i of 2.8 μM were calculated (Figure 3A, insert). At approximately 10 times their respective K_m values, E_2, NADH, and to a lesser extent NAD retard the rate of the second inactivation phase (Figure 3B). These data suggest that this second inactivation phase is an active-site-directed process and that inactivation occurs slowly if at all in ternary E·**2**·NADH complexes. To ascertain if reduction of ketone **2** occurs in the ternary complex with NADH, the rate of reduction of 40 μM ketone **2** was measured under these conditions and found to be 0.35 ± 0.10 μmol min^{-1} mg^{-1}.

Evidence for Covalent Inactivation and Stoichiometry. To determine if inactivation involves covalent attachment of steroid to E_2-HSD, the enzyme was incubated with tritiated alcohol **1** plus NAD. After >90% inactivation, dialysis, and gel filtration, the majority of the radioactivity appears in the fractions containing protein (Figure 4A). Only 5% of this radioactivity cochromatographs with protein if the incubation is conducted at 4 °C for 1 min (Figure 4B). These data provide support for a covalent interaction that requires minutes to hours. From the radioactivity and protein measurements, we calculated an inactivation stoichiometry of 1.4 equiv of steroid per subunit (2.8 equiv per 68 000-dalton protein).

Discussion

By constructing a propargyl 17β-estradiol equivalent, we hoped to utilize the high reactivity of acetylenic ketones and to retain the structural characteristics common to good substrates (17β-OH) for E_2-HSD. Our first compound, alcohol **1**, is a substrate that is turned over slowly by E_2-HSD at pH 9.2. At pH 7.4, turnover appears more rapid, but so little oxidation occurs before equilibrium is reached that kinetic parameters could not be determined[6] (data not shown). The

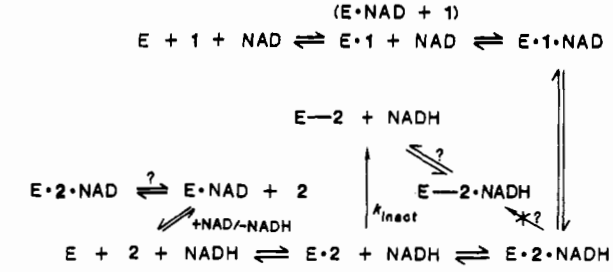
Michaelis constant of alcohol **1** is less than 10 times higher than that of E_2, indicating that the active site of E_2-HSD can accommodate this 14,15-secoestradiol structure with good affinity. As a mechanism-based inactivator of E_2-HSD, however, alcohol **1** is effective at concentrations much lower than its K_m ("K_i" = 2 μM). To understand why the "K_i" for alcohol **1** is much lower than its K_m, we evaluated the presumed inactivating species, ketone **2**, in the absence of turnover. The K_i of ketone **2** as an affinity label is almost as low as the "K_i" of alcohol **1** as a mechanism-based inactivator. As anticipated, the acetylenic ketone is a rapid, active-site-directed inactivator (Figure 3), albeit not as rapid as others we have studied (Covey et al., 1986; Tobias et al., 1982). Since inactivation by chemically prepared ketone **2** is much faster than enzymic oxidation of alcohol **1**, turnover of alcohol **1** and not the reactivity or affinity of ketone **2** limits the rate of inactivation.

To determine if enzyme inactivation involves a covalent bond to the steroid, E_2-HSD was inactivated by incubation with NAD and tritiated alcohol **1** for several hours at 25 °C. After dialysis and gel filtration chromatography, radioactivity cochromatographed with protein (Figure 4A). Little radioactivity eluted with protein, however, after a brief incubation at 4 °C (Figure 4B). These data provide strong support for a covalent adduct that requires enzymic catalysis. Using the protein assay of Smith et al. (1985), we calculated an inactivation stoichiometry of 1.4 molecules of steroid per subunit of enzyme. The nonintegral stoichiometry could result either from additional non-active-site modifications or, more likely, from the inherent limitations in currently available methods of quantitating small amounts of protein (that is, equal weights of the bovine serum albumin standard and E_2-HSD might not assay identically). Nonetheless, we conclude that approximately 1 molecule of steroid remains bound to each subunit of enzyme.

The complete protection from inactivation that glutathione affords at 5 and 200 μM NAD (Figure 1) suggests that the inactivating species is free and accessible to reactive nucleophiles before enzyme inactivation occurs (Abeles, 1983; Walsh, 1982). We cannot exclude the possibility that the reactive moiety of ketone **2** is exposed to solvent while bound in the active site, but we consider this possibility unlikely. Protection by thiols, when studied, has been observed for other mechanism-based inactivators of this enzyme (Thomas et al., 1983; Tobias et al., 1982). Due to the scatter in the data at early time points (Figure 1A), simple inspection could not determine if a lag phase precedes pseudo-first-order kinetics. Although one would expect a lag phase if the "release-and-return" mechanism is significant (Abeles, 1983; Walsh, 1982), linear regression analysis of the data affords lines that extrapolate very nearly through the origin at $t = 0$. We suspect that our failure to demonstrate a pronounced lag phase may be a consequence of the slow turnover rate for alcohol **1** and the high affinity of ketone **2**.

Chemically prepared ketone **2** inactivates E_2-HSD with biphasic kinetics. The second inactivation phase can be suppressed or abolished by the addition of E_2, NADH, or NAD (Figure 3B). Substrate protection demonstrates that this inactivation process is active-site-directed. NADH protection indicates that either ketone **2** cannot inactivate the enzyme in ternary E·**2**·NADH complexes or that ketone **2** is reduced faster than inactivation can occur (or both). Indeed, ketone **2** is reduced under these conditions (at 40 μM, $V_0 = 0.35 \pm 0.10$ μmol min^{-1} mg^{-1}, reaching equilibrium in <10 min), but this rate of turnover does not seem sufficient to account for the dramatic protection by NADH.

Scheme II



NAD protection suggests that (1) the enzyme cannot form abortive E·**2**·NAD complexes and protection derives from sequestering free enzyme as binary E·NAD complexes and/or (2) inactivation cannot occur in the ternary E·**2**·NAD complexes. The former is the simplest explanation; furthermore, E_2-HSD does not form abortive, binary complexes with E_1 and NAD or E_2 and NADH (Betz, 1971). We cannot, however, rule out a contribution from the latter possibility. This protection by NAD could explain why, at 5 μM NAD, inactivation by enzyme-generated ketone **2** is more rapid than at 200 μM NAD. At low NAD concentrations, ketone **2** has more *free* enzyme available to bind and to inactivate. We have observed this behavior in our previous study of the bacterial 20β-hydroxysteroid dehydrogenase (Covey et al., 1986). In addition, NAD protection could explain why inactivation kinetics at 5 μM NAD are initially linear, without any suggestion of a lag phase. At lower NAD concentrations, turnover of alcohol **1** is even slower, and ketone **2** inactivates even more efficiently than at 200 μM NAD.

The data are entirely consistent with Scheme II. Enzymic oxidation of alcohol **1** generates ketone **2** and NADH. After release of cofactor and steroid, free enzyme can bind either of the two steroids or cofactors. Since ketone **2** has such high affinity for the enzyme, inactivation commences, even when it is present at very low concentrations. At high (100 μM) concentrations of alcohol **1**, saturating concentrations of ketone **2** are generated in ~30 min; consequently, inactivation continues to follow pseudo-first-order kinetics for two half-lives despite slower turnover as the system reaches equilibrium.

In the preceding paper in this issue, we report inactivation of the 3α,20β-hydroxysteroid dehydrogenase from *Streptomyces hydrogenans* by an ethoxyacetylenic alcohol (Covey et al., 1986). Although conditions were found (low [NAD]) in which glutathione did *not* provide complete protection, the enzymatically generated ketone was also shown to inactivate only after its release and return to the active site of free enzyme. In both cases, NADH and NAD protect the enzymes from inactivation by the respective steroidal ketones. On the basis of these observations, we propose that *only a dehydrogenase that forms kinetically significant concentrations of binary complexes with steroid upon dissociation of NADH from the ternary complex could be susceptible to true suicide inactivation by Michael acceptors*. For such enzymes, NADH could dissociate from the evolutive ternary complex (E·K·NADH), leaving reactive ketone in the binary complex (E·K) wherein inactivation might occur *before* release of ketone.

Although the kinetic mechanism of E_2-HSD is random, the enzyme appears to form binary complexes more readily with steroids (E_1/E_2) than with cofactors (NAD/NADH) (Betz, 1971). We reasoned that a mechanism-based inactivator of

[6] At pH 7.4, ketone **2** inactivates E_2-HSD more slowly than at pH 9.2 (K_i = 8.1 μM, limiting $t_{1/2}$ = 28 min). Incubation of alcohol **1** (10–100 μM) with E_2-HSD and 200 μM NAD at pH 7.4 afforded 20–25% loss of activity after 3 h (data not shown in either case).

this enzyme might inactivate prior to release of the inactivating species (a "true" suicide substrate) if that species (ketone) possessed sufficiently high affinity and reactivity. Alcohol **1** seemed a good candidate for a suicide substrate since ketone **2** has excellent affinity (K_i = 2.8 μM) and reactivity (limiting $t_{1/2}$ = 12 min) toward E_2-HSD. Our data, however, suggest that inactivation occurs after release of ketone **2** and its return to the active site. Admittedly, faster inactivation by other acetylenic ketones has been observed, raising the possibility that the orientation of ketone **2**'s reactive moiety and the attacking enzymic nucleophile is not optimal for inactivation before dissociation.

Since dehydrogenase reactions involve pairs of both substrates and cofactors, and since these enzymes catalyze equilibrations that are pH-dependent, studies involving mechanism-based inactivators of dehydrogenases entail inherent complications. These two studies [see Covey et al. (1986)] suggest that the affinity and reactivity of the substrate–product pair are not the sole determinants of the inactivation mechanism. Rather, the manner in which the enzyme binds cofactor and the order in which the evolutive ternary complex preferentially dissociates appear to be significant factors controlling the inactivation process. Thus far, we have not demonstrated that a mechanism-based inactivator of a hydroxysteroid dehydrogenase can inactivate the enzyme before its release and return to the active site (a true suicide substrate). On the basis of our results, we predict that suicide inactivation would more likely occur with an enzyme (i.e., E_2-HSD) that forms a significant amount of binary enzyme–steroid complexes.

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