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Study of 3α,20β-Hydroxysteroid Dehydrogenase with an Enzyme-Generated Affinity Alkylator: Dual Enzyme Activity at a Single Active Site†

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ABSTRACT: The substrate 17β-[(1*S*)-1-hydroxy-2-propynyl]-androst-4-en-3-one (β-HPA) and its enzyme-generated alkylating product 17β-(1-oxo-2-propynyl)androst-4-en-3-one (OPA) were synthesized to investigate the relationship between the 3α and 20β activities observed in commercially available cortisone reductase (EC 1.1.1.53) from *Streptomyces hydrogenans*. β-HPA, a substrate [apparent K_m = 145 μM; V_{max} = 63 nmol (min μg)$^{-1}$], when enzymatically oxidized by cortisone reductase to OPA, inactivates simultaneously the 3α and 20β activities in a time-dependent and irreversible manner following pseudo-first-order kinetics. OPA alone, an affinity alkylating steroid (K_I = 40.5 μM; k_3 = 1.8 × 10^{-2} s^{-1}), simultaneously inactivates 3α and 20β activities in a time-dependent and irreversible manner. At pH 7, the $t_{1/2}$ of enzyme inactivation for β-HPA (10 h) or OPA (41 min) is slower than at pH 9.2 (β-HPA, 16 min, and OPA, 3.3 min). Substrates (progesterone, 20β-hydroxypregn-4-en-3-one, and 5α-dihydrotestosterone), but not all steroids (20α-Δ^4-pregn-4-en-3-one and 17β-estradiol), protect against loss of both enzyme activities by β-HPA and OPA. The α isomer of HPA is not enzymatically oxidized and therefore does not cause inactivation of either 3α or 20β activity. Thus, β-HPA functions as a substrate for the enzymatic generation of a powerful affinity alkylator of cortisone reductase. Second, the identical change in both the 3α and 20β activities in all experimental conditions clearly results from dual enzyme activity at a single enzyme active site.

The oxidoreductase 20β-hydroxysteroid dehydrogenase (20β-HSD) (EC 1.1.1.53; trivial name, cortisone reductase) is officially named as specific for 20-ketopregnene steroids. Pocklington & Jeffrey (1968) observed reaction with 3-keto-androstane steroids and initiated several reports of 3-oxido-reductase activity (Gibb & Jeffery, 1971, 1972, 1973). These kinetic studies did not resolve whether catalysis occurred at one or at different active centers. Blomquist (1973) demonstrated comigration of the 3α and 20β activities in disc gel electrophoresis, thereby excluding a second enzyme as an explanation for two activities. Edwards & Orr (1978) reported that inactivation studies using haloacetoxy steroid derivatives showed interacting, perhaps even overlapping, 3- and 20-ketosteroid binding sites.

This report describes the inactivation of cortisone reductase, with the simultaneous loss of the 3α- and 20β-oxidoreductase activities when the substrate, 17β-[(1*S*)-1-hydroxy-2-

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propynyl]androst-4-en-3-one (β-HPA), is enzymatically converted to the affinity alkylating steroid, 17β-(1-oxo-2-propynyl)androst-4-en-3-one (OPA).

Experimental Procedures

Materials. Cortisone, progesterone, 17β-estradiol, 20β-hydroxypregn-4-en-3-one, 20α-hydroxypregn-4-en-3-one, and 17β-hydroxy-5α-androstan-3-one purchased from Sigma Chemical Co. had melting point values identical with reported standards and were chromatographically pure. Nucleotides (NAD^+ and NADH) and inorganic chemicals were obtained from Sigma Chemical Co. 20β-Hydroxysteroid dehydrogenase from *Streptomyces hydrogenans* (sp act. 29 units/mg) purchased from Sigma Chemical Co. was suitably homogeneous for use without further purification. Glass-distilled, deionized water was used for all aqueous solutions. Organic chemicals and solvents from Fisher Scientific Co. were reagent grade and were not distilled prior to use.

Methods. The substrate 17β-[(1*S*)-1-hydroxy-2-propynyl]androst-4-en-3-one (β-HPA), its α isomer, 17β-[(1*R*)-1-hydroxy-2-propynyl]androst-4-en-3-one, and the corresponding alkylating steroid 17β-(1-oxo-2-propynyl)-androst-4-en-3-one (OPA) were synthesized as reported by Covey (1979).[1]

High-pressure liquid chromatography was performed on a Waters Associates instrument with a 3.9 mm × 30 cm μBondapak C_{18} column eluted at 2 mL/min with methanol/water. Eluted compounds were detected by a Waters Associates Model 450 variable wavelength detector and displayed on an Omniscribe Model B-5000 strip chart recorder. The detector response was not calibrated for each compound. Enzyme assays for 20β activity were prepared with the following solutions added to a final volume of 1.0 mL in matched 1 cm path length cuvettes: 0.75 mL of 0.05 M potassium phosphate buffer, pH 6.5; 0.1 mL of NADH in distilled water (1.4×10^{-3} M); 0.1 mL of cortisone in ethanol (1.8×10^{-3} M). The reaction was initiated by the addition of 0.05 mL of the different enzyme incubation mixtures. Enzyme assays for 3α activity required 0.60 mL of 0.05 M potassium phosphate buffer, pH 6.5, 0.1 mL of NADH in distilled water (1.4×10^{-3} M), 0.1 mL of dihydrotestosterone in ethanol (1.0×10^{-3} M), and initiation with 0.2 mL of the enzyme incubation mixture. The slope of the initial linear decrease in absorbance at 340 nm (due to the oxidation of NADH) as a function of time was used to calculate enzyme activity. Assays were performed in duplicate at 25 °C in a Beckman Acta II recording spectrophotometer. Kinetic data were fitted by least mean squares with a Texas Instruments SR-52 programmable calculator.

Results

The substrate (β-HPA), which is in itself not an alkylating agent, and the enzyme-catalyzed steroid oxidation which generates within the enzyme active site a potent alkylating agent (OPA) are illustrated in Figure 1.

Stability of β-HPA and OPA. β-HPA and OPA dissolved in ethylene glycol monoethyl ether (EGME, 1 mM) or incubated at 25 °C in 0.05 M sodium carbonate buffer, pH 9.2 (10 μM), and 0.05 M 0.05 potassium phosphate buffer, pH 7.0 (10 μM), were examined for stability. The buffer incubations contained either progesterone (10 μM) or 21-hydroxypregn-4-ene-3,20-dione (10 μM) as an internal standard. For analysis by high-pressure liquid chromatography (LC), 0.1-mL aliquots from the EGME and phosphate buffer incubation were applied directly to the LC column. From the carbonate buffer incubation, 2 mL was removed and acidified to pH 6.7 with 0.05 mL of glacial acetic acid, and 0.1 mL of this solution was applied to the LC instrument. The column was eluted with 65% methanol/35% water, and the steroids were quantitated from their absorbance peaks at 245 nm. In buffered solutions observed over 3 h, (1) new compounds resultant from the spontaneous degradation of β-HPA and OPA were not observed, (2) spontaneous conversion of β-HPA to OPA was not detected, and (3) the ratio of the test steroid to the internal standard was constant.

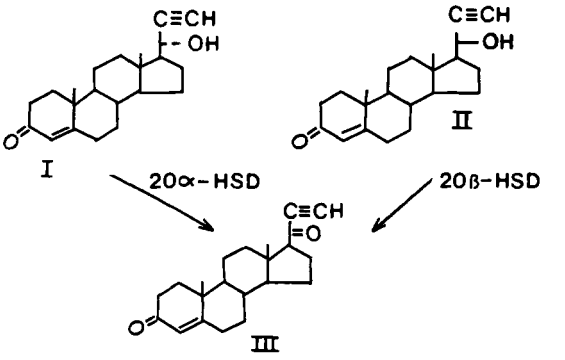

FIGURE 1: Enzymatic formation of an affinity alkylator from stereospecific substrates. 17β-[(1*R*)-1-Hydroxy-2-propynyl]androst-4-en-3-one (I) and 17β-[(1*S*)-1-hydroxy-2-propynyl]androst-4-en-3-one (II) are catalyzed by 20α- and 20β-hydroxysteroid dehydrogenase to the alkylating steroid, 17β-(1-oxo-2-propynyl)androst-4-en-3-one (III).

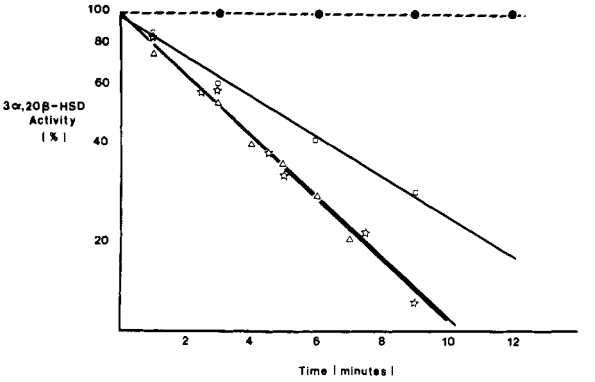

FIGURE 2: Inactivation of 3α,20β-hydroxysteroid dehydrogenase by 17β-(1-oxo-2-propynyl)androst-4-en-3-one and protection by 20β-hydroxypregn-4-en-3-one. Enzyme (0.2 or 0.4 nmol) dissolved in 2.0 mL of 0.5 M carbonate buffer, pH 9.2, was incubated at 25 °C with substrate (20 or 40 nmol in 0.02 mL of ethylene glycol monoethyl ether) alone or in addition with 20β-hydroxypregn-4-en-3-one (100 nmol in 0.02 mL of ethylene glycol monoethyl ether). Identical control incubations containing 20β-hydroxypregn-4-en-3-one were assayed for both enzyme activities (closed circles). At various time intervals 0.05 or 0.2 mL was assayed for 3α- (open star) and 20β-hydroxysteroid dehydrogenase (open triangle) activities and the protective effects of 20β-hydroxypregn-4-en-3-one (open square). The percentage of enzyme activity is plotted on a logarithmic scale along the ordinate, and the time of incubation is a linear scale along the abscissa. The values are the mean of at least duplicate experiments.

Enzyme Inactivation by OPA. 20β-HSD (10 μg/mL) dissolved in 2.0 mL of 0.05 M sodium carbonate buffer, pH 9.2, was incubated at 25 °C with a 100-fold molar excess of OPA in EGME. A solution containing identical concentrations of enzyme and 20β-hydroxypregn-4-en-3-one in the same buffer served as a control. Enzyme assays of aliquots removed from the incubation mixture over time show inactivation

[1] The stereochemical descriptors of chirality were erroneously reversed in this reference. The nomenclature has been corrected in this publication.

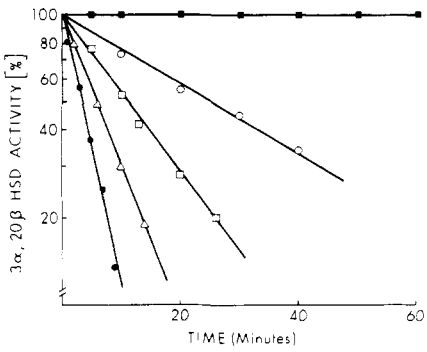

FIGURE 3: Inactivation of 3α,20β-hydroxysteroid dehydrogenase activity by various concentrations of 17β-(1-oxo-2-propynyl)-androst-4-en-3-one. Enzyme (0.2 nmol) dissolved in 2.0 mL of 0.05 M carbonate buffer, pH 9.2, was incubated at 25 °C with substrate [20 nmol (closed circle), 10 nmol (open triangle), 5 nmol (open square), and 2 nmol (open circle)] in 0.02 mL of ethylene glycol monoethyl ether. Identical control incubations (closed squares) contained 20β-hydroxypregn-4-en-3-one. At various time intervals, aliquots were removed to assay enzyme activities as described under Methods. The percentage of enzyme activity is plotted on a logarithmic scale along the ordinate, and time is a linear scale along the abscissa. The values are the mean of at least duplicate experiments.

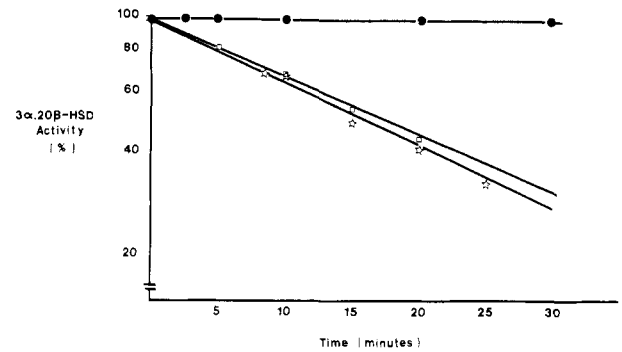

FIGURE 4: Inactivation of 3α,20β-hydroxysteroid dehydrogenase by 17β-[(1*S*)-1-hydroxy-2-propynyl]androst-4-en-3-one. Enzyme (0.2 nmol) dissolved in 2.0 mL of 0.05 M carbonate buffer, pH 9.2, was incubated at 25 °C with substrate (20 nmol in 0.02 mL of ethylene glycol monoethyl ether) and NAD^+ (0.3 μmol in 0.1 mL of distilled water). Identical control incubations containing 20β-hydroxypregn-4-en-3-one in place of the β-HPA substrate were assayed for both enzyme activities (closed circles). At various time intervals, 0.05 or 0.2 mL was assayed for 3α- (open star) and 20β-hydroxysteroid dehydrogenase (open rectangle) activities. The percentage of enzyme activity is plotted on a logarithmic scale along the ordinate, and the time of incubation is a linear scale along the abscissa. The values are the mean of at least duplicate experiments.

follows pseudo-first-order kinetics ($t_{1/2}$ of 3.3 min) with identical loss of both the 3α and 20β enzyme activities (Figure 2). When a fivefold molar excess (protector steroid/inactivator steroid) of 20β-hydroxypregn-4-en-3-one (shown in Figure 2), progesterone, 5α-dihydrotestosterone was added to the inactivation reaction, the rate of enzyme inactivation was slower. Substrates, by competing with OPA for the active site, protect 20β-HSD against inactivation.

When identical incubations are performed in 0.05 M potassium phosphate buffer, pH 7.0, the 3α and 20β activities again simultaneously decrease in a time-dependent and irreversible manner following pseudo-first-order kinetics with a $t_{1/2}$ of 41 min.

Incubations of enzyme (10 μg/mL) and differing concentrations of OPA were used to effect inactivation (Figure 3), to further substantiate that OPA was an active site directed inhibitor of 20β-HSD. This family of curves was then analyzed as suggested for irreversible inhibitors by Kitz & Wilson (1962):

$$E + I \underset{k_2}{\overset{k_1}{\rightleftharpoons}} E{\cdot}I \xrightarrow{k_3} E\text{–}I \qquad (1)$$

A k_{app} is determined from the slopes of the ln (% activity) vs. time plots in Figure 3, and the double-reciprocal plot, $1/k_{app}$ vs. 1/[inhibitor], is linear ($r^2 = 0.999$). By use of the formula

$$\frac{1}{k_{app}} = \frac{K_I}{k_3[I]} + \frac{1}{k_3}$$

$K_I = 40.53$ μM and $k_3 = 1.8 \times 10^{-2}$ s^{-1}.

Substrate Characteristics of β-HPA. Double-reciprocal plots of the oxidation of β-HPA by 20β-HSD were analyzed. The kinetic data were obtained with enzyme concentrations of 1 and 0.5 μg in 0.05 M potassium phosphate buffer, pH 7.0, added to a cuvette containing 0.75 mL of 0.05 M sodium carbonate buffer, pH 9.2, NAD^+ (0.14 μmol) in 0.1 mL of distilled water, and steroid in different concentrations (0.1–0.01 μmol) in 0.1 mL of EGME. β-HPA is a substrate for this enzyme with apparent K_m and V_{max} values of 1.45×10^{-4} M and 63 nmol (min μg)$^{-1}$, respectively.

Enzymatic Conversion of β-HPA to OPA. 20β-HSD (0.2 nmol) dissolved in 2.0 mL of 0.05 M carbonate buffer, pH 9.2, was incubated at 25 °C with 20 nmol of β-HPA (dissolved in 0.02 mL of EGME) and 0.3 μmol of NAD^+. Identical control incubations were prepared without the cofactor. After 1 min of incubation, 0.1 mL was removed and applied directly to the LC instrument. The column was eluted with methanol/water (70:30), and the steroids were detected by their absorbance at 245 nm. β-HPA (retention time 9 min) was 53% converted to OPA (retention time 10 min), and no other reaction products were detected.

Cortisone Reductase Inactivation When β-HPA Is Enzymatically Oxidized to OPA. 20β-HSD (10 μg/mL) dissolved in 2.0 mL of 0.05 M sodium carbonate buffer, pH 9.2, was incubated with excess NAD^+ and β-HPA substrate (molar ratio of substrate/enzyme, 100:1). A control incubation containing identical concentrations of enzyme, cofactor, and 20β-hydroxypregn-4-en-3-one in the same buffer served as a control. Serial enzyme assays show inactivation follows pseudo-first-order kinetics with a $t_{1/2}$ of 16 min and indistinguishable rates of loss for both the 3α and 20β enzyme activities (Figure 4). When a fivefold molar excess (protector steroid/inactivator steroid) of steroid hormone was included in the incubation mixture, 20β-hydroxypregn-4-en-3-one protected the enzyme against inactivation while progesterone, 17β-estradiol, and 20α-hydroxypregn-4-en-3-one had no effect on the rate of activity loss (Figure 5).

Incubations containing 2-mercaptoethanol, equimolar in concentration to β-HPA, had an ~33% slower rate of enzyme inactivation ($t_{1/2}$ of 21 min) compared to untreated control incubations. A 100-fold molar excess of 2-mercaptoethanol completely protected against inactivation of 20β-HSD.

When identical incubations were performed in potassium phosphate buffer, pH 7.0, parallel loss of both enzyme activities again was observed. The time-dependent and irreversible inactivation at pH 7.0 followed pseudo-first-order kinetics, the markedly slower rate gave a $t_{1/2}$ of 10 h, and substrates protected against inactivation.

Stereospecificity of the Enzyme for β-HPA. Incubations of 20β-HSD in sodium carbonate buffer, pH 9.2, were identical with the above described conditions except that the substrate α isomer, 17β-[(1*R*)-1-hydroxy-2-propynyl]androst-4-en-3-one, was substituted for β-HPA. No enzyme inactivation, relative to control incubations containing substrate, was observed over 24 h.

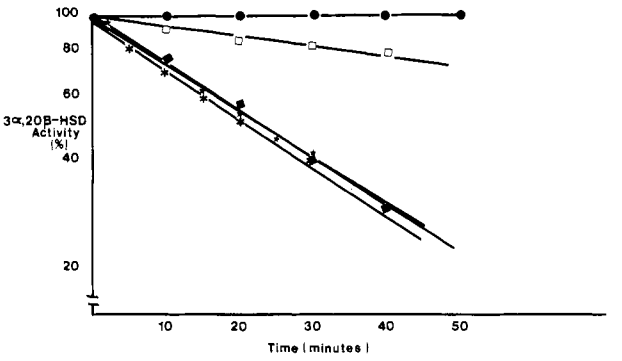

FIGURE 5: Inactivation of 3α,20β-hydroxysteroid dehydrogenase by 17β-[(1*S*)-1-hydroxy-2-propynyl]androst-4-en-3-one and protection by usual substrates. Enzyme (0.2 nmol) dissolved in 2.0 mL of 0.05 M carbonate buffer, pH 9.2, was incubated at 25 °C with β-HPA substrate (20 nmol in 0.02 mL of ethylene glycol monoethyl ether) and NAD^+ (0.3 μmol in 0.1 mL of distilled water) in addition with 100 nmol of 20α-hydroxypregn-4-en-3-one (asterisk), 17β-estradiol (closed star), progesterone (closed diamond), or 20β-hydroxypregn-4-en-3-one (open square). Control incubations (closed circle) contained 20β-hydroxypregn-4-en-3-one in place of the β-HPA substrate. At various time intervals, each mixture was assayed for enzyme activity as described under Methods. The percentage of enzyme activity is plotted on a logarithmic scale along the ordinate, and time is a linear scale along the abscissa. The values are the mean of at least duplicate experiments.

Discussion

Several laboratories have recognized both 3α- and 20β-oxidoreductase activity in the enzyme cortisone reductase from *Streptomyces hydrogenans*. The coexistence of these activities in commercially available crystalline enzyme suspension (Blomquist, 1973) and in enzyme carefully purified to address the dual activity problem (Edwards & Orr, 1978) has been convincingly demonstrated. Whether these activities were catalyzed at one, or more than one, site remained unclear.

Edwards & Orr (1978) attempted to resolve the characteristics of substrate binding from an analysis of enzyme inactivation by bromoacetoxy, affinity alkylating analogues of androstane and pregnene steroids. The resultant complex kinetics, vs. the results reported here and the previous reports that 20β-hydroxysteroid dehydrogenase inactivation by bromoacetoxy steroids follows pseudo-first-order kinetics [Sweet et al. (1978); Strickler et al. (1975) and cited references], likely resulted from an unfortunate choice of incubation conditions. At pH 9.0, the ester linkage in the affinity alkylating substrates described by Edwards and Orr will rapidly hydrolyze and the resultant mixture of alkylating substrate, protecting hydroxysteroid, and free bromoacetic acid would give complex kinetic interactions (Sweet & Samant, 1980). Furthermore, enzyme inactivated by the affinity alkylating analogue 6β-bromoacetoxyprogesterone has been shown to reactivate (Sweet, 1976) in the incubation conditions selected by Edwards and Orr. Thus, although we agree with their conclusion that "the binding sites for the two activities do not act independently", their methodology has muddied this unclear issue.

β-HPA is not in itself an inactivating agent. However, following correct binding within the enzyme active site, enzyme catalysis converts this compound into OPA, an alkylating product. This characteristic of correct alignment for active site generation of the alkylating inhibitor makes β-HPA a useful substrate to address the question of one or more active sites. In the present case, β-HPA does not suffer from the liabilities of previously used affinity alkylating ester-bridged analogues: pH-limited conditions of study and possible nonspecific alkylating capability.

The substrate, β-HPA (by oxidation to OPA), and OPA alone both inactivate cortisone reductase in a time-dependent and irreversible manner following pseudo-first-order kinetics. The stereospecificity for β-HPA was proven since the enzyme failed to utilize the α-HPA isomer. The pattern of inactivation is independent of pH, while the rate of inactivation ($t_{1/2}$ is rapid at pH 9.2 compared to pH 7.0) is pH dependent. The pH maximum for 20β-HSD-catalyzed oxidation is 9.2 with a rapid decline in rate as the pH falls below 8.0 (Betz, 1968; Blomquist, 1973). At pH 7.0, the oxidation of excellent substrates for 20β-HSD is too slow to accurately define the reaction kinetics. Thus, the slow inactivation by β-HPA oxidation at pH 7.0 ($t_{1/2}$ of 10 h) compared to pH 9.2 ($t_{1/2}$ of 16 min) is consistent with this well-recognized pH effect on enzyme catalysis. At pH 7.0, the $t_{1/2}$ for 20β-HSD inactivation by OPA is 41 min. OPA inactivates 20β-HSD at a relatively rapid rate compared to other affinity alkylating steroids studied with this enzyme in buffer at pH 7.0: 16α-bromoacetoxyprogesterone, $t_{1/2}$ = 4 h (Sweet, 1971); 21-bromoacetylaminoprogesterone, $t_{1/2}$ = 48 h (Sweet et al., 1978); 2α-bromoacetoxyprogesterone, $t_{1/2}$ = 4.6 h (Strickler et al., 1975). The more rapid rate of inactivation by OPA in buffer at pH 9.2 ($t_{1/2}$ of 3.3 min) presumably results from conformational changes of the protein in solution combined with increased reactivity of nucleophilic amino acids within the enzyme active site.

Kinetic analyses of β-HPA as an enzyme substrate (possible since the rate of catalysis is much faster than the rate of product alkylation) and OPA as an irreversible inhibitor indicate that these steroids bind at the active site of cortisone reductase. Furthermore, the protection conferred by both pregnene and androstane substrates against inactivation by β-HPA and OPA is consistent with competition for a single active site. This protection is specific for 20β-HSD substrates: 17β-estradiol and 20α-hydroxypregn-4-en-3-one do not inhibit enzyme inactivation, showing that protection is not a nonspecific effect. The failure of progesterone to protect against inactivation *under the stated conditions* (Figure 5) is consistent with cofactor ordered binding (Betz & Warren, 1968) and an inability to simultaneously bind an oxidized cofactor (NAD^+) and steroid—an abortive complex.

It is clear that the rate of enzyme catalysis greatly excedes the rate of irreversible alkylation. Were this not true, kinetic analysis of β-HPA as a substrate would be difficult. Furthermore, if each turnover resulted in an alkylation event, then it would not have been possible to demonstrate enzymatic conversion of β-HPA to OPA by using LC. Since the alkylating steroid product is undoubtedly liberated into solution, it is equally possible that alkylation occurs in situ as the alkylator is generated or that liberated alkylator reenters the active site to effect inactivation. The slower rate of inactivation when the equimolar scavenger nucleophile 2-mercaptoethanol is present, presumably because it reacts with the alkylator and precludes interaction with the protein, superficially supports the return of the alkylator and inactivation by an "affinity alkylation" rather than a "suicide substrate" mechanism. However, the active site is not a dry island surrounded by a solvent ocean, and thus the scavenger molecule may be as much a part of the active-site environment as is the buffer, the cofactor, and the amino acids. In this setting, in situ generated alkylator may react with either the 2-mercaptoethanol or the protein, and since the 2-mercaptoethanol is a strong nucleophile, it may be preferred. Finally, it is relevant that bromoacetoxyprogesterone affinity alkylating steroids have measurable kinetic constants of similar magnitude to those of β-HPA (Strickler et al., 1975). This is a clear indication that

the simple occupation of the active site of 20β-HSD by an alkylator does not always (and more likely rarely) lead to covalent bond formation.

The 3α and 20β activities were lost simultaneously and at an identical rate during cortisone reductase inactivation by OPA, both when it was incubated directly and when it was enzymatically generated from β-HPA. That this coidentity persisted during inactivation in different pH buffers, in the presence of competing substrate steroids, in the presence of nonprotective steroids, and in 24-h incubations offers persuasive evidence that the 3α and 20β activities result from catalysis within the same active site on a single protein and further justifies naming this enzyme 3α,20β-hydroxysteroid dehydrogenase.

These studies do not indicate whether the active site performs dissimilar stereospecific oxidation/reduction reactions because (1) two cofactor sites, one adjacent to the A ring and one adjacent to the D ring, could proximate an active site wherein all steroids identically align or (2) a single steroid binding site might proximate a single cofactor region and the androstane steroids undergo "wrong way binding" (i.e., the steroid is reversed and rotated 180°) relative to pregnene steroids within the active center. This question may be answered by studies designed to map the amino acids which proximate affinity alkylating analogues of androstane and pregnene steroids as they align within the active site. Finally, the observed inactivation may arise from two mechanisms when β-HPA in the presence of cofactor is enzymatically oxidized to OPA. The enzyme-generated alkylating product could covalently bond to a nucleophilic amino acid within the active site, or the alkylating steroid could modify the cofactor and thereby prevent dissociation of either from the binding region. The synthesis of radiolabeled β-HPA would be one approach to answer this question.

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Active Site of Bovine Galactosyltransferase: Kinetic and Fluorescence Studies†

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ABSTRACT: Bovine galactosyltransferase has been shown to have two metal binding sites. The functional properties of these metal binding sites have been established by using kinetic, spectroscopic, and affinity chromatographic approaches. Metal site I, which is involved in maintaining the structural integrity of the protein, must be liganded prior to other substrates binding and prior to a second metal binding to site II, which is shown to be associated with UDP-galactose binding. Both metal sites can bind a variety of metals; however, calcium and its fluorescent analogue europium bind only to site II. Fluorescent resonance energy transfer measurements between europium in site II and cobalt in site I indicate a distance of 18 ± 3 Å between the two sites. Chemical modification studies with *S*-mercuric-*N*-dansylcysteine indicate that one (of a total of three exposed sulfhydryl groups) can be specifically dansylated and that this sulfhydryl group is in or near the UDP-galactose binding site. Resonance energy transfer measurements between this introduced sulfhydryl group and cobalt in metal site I give a distance of 19 ± 3 Å between these points, consistent with the interpretation that the UDP-galactose binding site, which is associated with metal site II, is located some distance from the structural metal site (site I).

Galactosyltransferase catalyzes the transfer of galactose from UDP[1]-galactose to *N*-acetylglucosamine, which may be either the free monosaccharide or in glycosidic linkage in glycoproteins, forming β1→4 galactosides (Hill & Brew, 1975). The transferase, in the presence of α-lactalbumin, will also utilize glucose as an acceptor substrate producing lactose. Together the transferase and α-lactalbumin form lactose synthase (Brew & Hill, 1975).

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[1] Abbreviations used: dansyl, 8-dimethylaminonaphthalene-1-sulfonyl chloride; GlcNAc, *N*-acetylglucosamine; UDP, uridine diphospho or phosphate; EDTA, ethylenediaminetetraacetic acid.

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