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Mechanism-Based Inactivation of Bovine Adrenal Cytochromes P450 C-21 and P450 17α by 17β-Substituted Steroids†

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ABSTRACT: A series of progesterone derivatives has been studied as potential inactivators of the bovine adrenocortical cytochromes P450, P450 17α, and P450 C-21. Replacement of the 21-methyl group of progesterone with a difluoromethyl group resulted in a selective inactivator of P450 C-21 in a reconstituted system. The loss of 21-hydroxylase activity caused by this compound exhibits a number of characteristics of mechanism-based inactivation including NADPH dependence, pseudo-first-order kinetics, saturability, irreversibility, and protection by substrate. In addition to the difluoro compound, 21,21-dichloroprogesterone, the acetylenic compound pregn-4-en-20-yn-3-one, and the olefinic compound pregna-4,20-dien-3-one all inactivate P450 C-21. In contrast, the only compound to inactivate the rabbit adrenal progesterone 21-hydroxylase is 21,21-dichloroprogesterone. In binding studies, the 21,21-dihalo steroids produce a greater maximal type I spectral shift of P450 C-21 than the two 17β-unsaturated steroids. The dihalo compounds inactivate P450 C-21 by both heme destruction and protein modification as shown by significant decreases in residual 21-hydroxylase activity and spectrally detectable P450 after incubation with P450 C-21 in a reconstituted system. Liquid chromatographic and mass spectral analyses of the organic extracts from these incubations showed that 21-pregnenoic acid is a major metabolite of the dihalo compounds with a partition ratio of 5 nmol of acid produced/nmol of P450 C-21 inactivated. This supports the hypothesis that inactivation proceeds in part through an acyl halide intermediate. In contrast, the acetylenic compound pregn-4-en-20-yn-3-one inactivates P450 C-21 mainly by protein modification, producing an NADPH-dependent irreversible type I spectral shift. The stoichiometry of inactivation is approximately 1.5 nmol of compound bound/nmol of enzyme inactivated, indicating selective modification of the enzyme at or near the substrate binding site.

Cytochromes P450 are a gene superfamily of hemoproteins that catalyze the oxidation of lipophilic substrates to more water-soluble products (Nebert et al., 1989). The numerous P450 forms in the liver are generally inducible by and carry out the metabolism of xenobiotics (Conney, 1986). In contrast, the P450 forms present in the adrenal gland are more limited in number and substrate specificity and utilize steroids as their endogenous substrates. Two key microsomal enzymes of the adrenal cortex are P450 C-21 (P450XXIA1)[1] and P450 17α (P450XVIIA1), which catalyze the hydroxylation of progesterone at the 21- and 17α-positions, respectively. The metabolic products of these reactions are important precursors in the production of mineralocorticoids, glucocorticoids, and sex hormones (Miller, 1988).

Although these and other P450s have been extensively studied at the biochemical and molecular level, the question of why certain P450 forms display superior catalytic activity and/or strict regioselectivity with a particular substrate has remained largely unanswered. Several approaches have been utilized by other laboratories to investigate the basis for differences in progesterone 21-hydroxylation by related P450s

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[1] The major cytochromes P450 referred to in this report, their steroid hydroxylase activities, and gene designations are as follows: P450 C-21 (P450XXIA1), an adrenal steroid 21-hydroxylase encoded by the bovine *CYP21A1* gene; P450 17α (P450XVIIA1), an adrenal steroid 17α-hydroxylase encoded by the bovine *CYP17* gene; PB-C (P450IIC6), a hepatic progesterone 21-hydroxylase encoded by the rat *CYP2C6* gene. Rabbit adrenal 21-hydroxylase will be referred to as such since the gene designation is presently unknown.

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or similarities in metabolism by P450 forms unrelated in primary structure. For human P450 C-21, a strong impetus for studies on the influence of minor changes in enzyme primary structure on steroid metabolism arose from analysis of the *CYP21A* gene of patients with impaired adrenal 21-hydroxylase activity. A single amino acid substitution in P450 C-21 at Ile^{172} or Arg^{356} has been shown to be one cause of steroid 21-hydroxylase deficiency (Chiou et al., 1990). In addition, rabbit hepatic P450IIC4 exhibits 95% amino acid sequence identity with the P450IIC5 form (Johnson et al., 1987) but displays a much lower efficiency for progesterone hydroxylation. The difference in K_m between the enzymes has recently been attributed to amino acid residue 113 (Johnson et al., 1990).

The objective of our study was to utilize mechanism-based inactivators to investigate determinants of the catalytic specificity of progesterone 21-hydroxylases of cow adrenal, rabbit adrenal, and rat liver. Mechanism-based inactivators, or suicide substrates, are compounds that are metabolized to reactive intermediates, which can then bind to the enzyme irreversibly, resulting in loss of activity (Rando, 1984). The requirement for metabolism imparts additional specificity compared with reversible inhibitors, which rely solely on binding. In addition, the inactivation may potentially involve modification of amino acid residues at or near the enzyme active site. These unique characteristics were exploited by examining a series of progesterone analogues as inactivators of three functionally similar (progesterone 21-hydroxylase activity) enzymes. This enabled us to address questions about the events and site of P450 catalysis that have not been investigated thus far.

Our experimental approach was to narrow the focus to specific structure–activity questions by first identifying an inactivator or inactivators of each of the three progesterone 21-hydroxylases. Our own recent work suggests that the replacement of the 21-methyl group of progesterone with a dihalomethyl moiety may confer upon a molecule the ability to selectively inactivate cytochromes P450 by virtue of the metabolic conversion of the steroid to an acyl halide intermediate (Halpert et al., 1988, 1989a). Both 21,21-dichloro- and 21,21-difluoroprogesterone were used to monitor not only the selectivity but also the efficiency of enzyme inactivation by measuring the production of free noninactivating metabolites during catalysis. Inclusion of two 17β-unsaturated compounds, 17β-vinylprogesterone[2] and 17β-ethynylprogesterone, was motivated by reports of P450 inactivation by olefins and acetylenes (Ortiz de Montellano et al., 1982; Ortiz de Montellano & Reich, 1984). More recently, P450 inactivation due at least in part to protein modification has been demonstrated for some terminal acetylenes (CaJacob et al., 1988; Hammons et al., 1989). By exploiting the similar function of these progesterone 21-hydroxylases, we have utilized 17β-substituted steroids to investigate important catalytic differences.

Materials and Methods

Materials. [4-^{14}C]Progesterone (57.2 mCi/mmol) and [7-^{3}H(N)]pregnenolone (10–25 Ci/mmol) were purchased from NEN (Boston, MA). Progesterone, deoxycorticosterone (21-hydroxyprogesterone), 17α-hydroxyprogesterone, dilauroyl-L-phosphatidylcholine, 3β-acetoxy-5-androsten-17-one, and NADPH were purchased from Sigma Chemical Co. (St. Louis, MO). Pyridinium chlorochromate (PCC), triethyl phosphonoacetate, *N*-chlorosuccinimide, platinum(IV) oxide, and 1-methyl-3-nitro-1-nitrosoguanidine were purchased from Aldrich Chemical Co. (Milwaukee, WI). Sodium cholate, sodium deoxycholate, and HEPES were purchased from Calbiochem-Behring (La Jolla, CA). Sepharose 4B was purchased from Pharmacia Fine Chemicals (Piscataway, NJ). Hydroxylapatite and the reagents for gel electrophoresis were purchased from Bio-Rad (Richmond, CA). Emulgen 913 was purchased from KAO-Atlas Chemicals (Tokyo, Japan). Methylene chloride was distilled from calcium hydride and stored over molecular sieves. The steroids 17β-ethynylprogesterone (pregn-4-en-20-yn-3-one), 17β-vinylprogesterone (pregna-4,20-dien-3-one), 21-chloro-21-fluoroprogesterone, 21,21-dichloroprogesterone, and 21,21-difluoroprogesterone were synthesized as described previously (Halpert et al., 1989b). [^{3}H]-17β-Ethynylprogesterone was tritiated from the cold compound to a specific activity of ~2.8 Ci/mmol as described previously (Tang & Peng, 1988). 21-Pregnenoic acid (3,20-dioxo-4-pregnen-21-oic acid) was generously provided by Dr. Ian R. Senciall of the Memorial University of Newfoundland, St. John's, Newfoundland, Canada. Proton magnetic resonance spectra were recorded at 250 MHz, and carbon magnetic resonance spectra were recorded at 62.9 MHz on a Bruker WM-250 NMR spectrometer with tetramethylsilane as an internal standard. Infrared spectra were recorded on a Beckman IR-33 spectrophotometer. Optical rotations were measured at 589 nm on a Rudolph Research Autopol III polarimeter. Mass spectral analyses were performed on a MAT-90 mass spectrometer (electron impact mode, 70 eV).

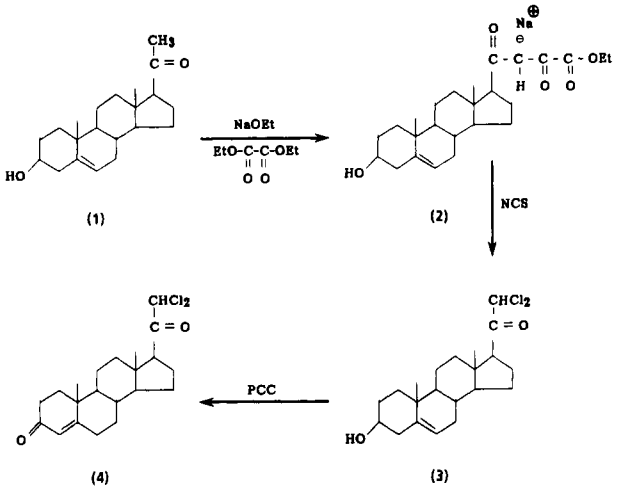

FIGURE 1: Scheme for the synthesis of 21,21-dichloroprogesterone (**4**) from pregnenolone (**1**). The overall product yield was 66%, which represents a significant improvement over a previous method (Halpert et al., 1988, 1989b). The experimental details are given under Materials and Methods.

Synthesis of [7-^{3}H(N)]-*21,21-Dichloroprogesterone* (**4**). This procedure utilizes a novel method for a relatively high yield preparation of the reaction intermediate 21,21-dichloropregnenolone (**3**) by the reaction of sodium 21-ethoxalylpregnenolone (**2**) with *N*-chlorosuccinimide (Figure 1). With this method, tritium-labeled **3** has been synthesized from [7-^{3}H(N)]pregnenolone (**1**) in 66% overall yield, a marked improvement over the 22% yield achieved by using the previous method (Halpert et al., 1988). For [7-^{3}H(N)]-21,21-di-

[2] Abbreviations: 17β-ethynylprogesterone, pregn-4-en-20-yn-3-one; 17β-vinylprogesterone, pregna-4,20-dien-3-one; 21-pregnenoic acid, 3,20-dioxo-4-pregnen-21-oic acid; etienic acid, 3-oxo-4-androstene-17β-carboxylic acid; PCC, pyridinium chlorochromate; NCS, *N*-chlorosuccinimide; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; SDS, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetic acid; HPLC, high-performance liquid chromatography.

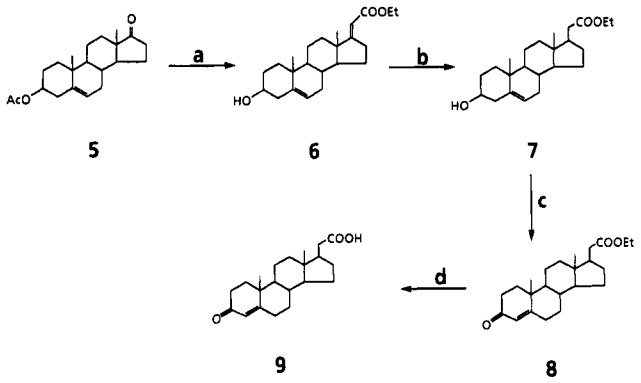

FIGURE 2: Scheme for the synthesis of 3-oxopregn-4-en-21-oic acid (**9**). Step a was as described previously (Wicha et al., 1977); step b included H_2 and platinum black and proceeded for 2 h; step c included aluminum isopropoxide, cyclohexanone, and toluene, with refluxing; step d was performed in sodium hydroxide and 95% alcohol for a duration of several hours.

chloropregnenolone, pregnenolone (126.6 mg, 0.4 mmol) was added to 2.5 mCi of [7-^{3}H(N)]pregnenolone, which had been dried under nitrogen. To this was added a solution of sodium (23 mg, 1 mmol) in ethanol (3 mL). After the addition of diethyl oxalate (0.3 mL), the solution was refluxed for 80 min, cooled on ice, and filtered with anhydrous ether (10 mL) to give 158.5 mg (95%) of **2**. To a well-stirred suspension of sodium salt (158.5 mg, 0.382 mmol) in methylene chloride (10 mL) was added NCS (77.3 mg, 0.58 mmol).[3] After 80 min, water (20 mL) was added, and the product was extracted with methylene chloride (15 mL). The extract was dried over $MgSO_4$, filtered, and concentrated in vacuo. The residue was chromatographed over silica gel (30 g) and eluted with ethyl acetate/hexanes (1:5) to give 102 mg (69%) of tritium-labeled **3**. The progesterone derivative **4** was synthesized from **3** by PCC oxidation with some modifications of a previous method (Halpert et al., 1989b). To a well-stirred suspension of PCC (162.5 mg, 0.75 mmol) in dry dichloromethane (10 mL) was added a solution of tritium-labeled **3** (126 mg, 0.327 mmol, from two separate syntheses) in dichloromethane (5 mL). After the solution was stirred for 3 h, anhydrous ether (80 mL) was added, and it was then filtered over a pad of SiO_2–$MgSO_4$. The filtrate was washed with water (15 mL), dried over $MgSO_4$, filtered, and concentrated in vacuo. The residue was dissolved in methanol (10 mL), and 3 drops of 6 N HCl was added. After 10 min, the solvent was removed under vacuum. The residue was column chromatographed over silica gel (30 g) and eluted with ethyl acetate/hexanes (1:6) to give tritium-labeled **4** (85 mg, 66% yield). The product was 96% pure as determined by HPLC and was purified further by HPLC to >98% pure on a 10 mm × 25 cm Altex Ultrasphere ODS 5-μm C18 column. The column was eluted at 1.5 mL/min with a mobile phase of 75% acetonitrile/25% mixture of 50% H_2O and 50% methanol. The retention time of the analyte was 20.75 min.

Synthesis of 3-Oxopregn-4-en-21-oic Acid (**9**). As shown in Figure 2, ethyl 3β-hydroxypregna-5,17-dien-21-oate (**6**) was prepared by a Wittig–Horner reaction as described previously (Wicha et al., 1977). Selective hydrogenation of **6** in ethanolic solution in the presence of freshly prepared platinum black catalyst gave a 92% yield of ethyl 3β-hydroxypregn-5-en-21-oate (**7**). Oxidation of **7** by PCC followed by isomerization with dilute HCl gave ethyl 3-oxopregn-4-en-21-oate (**8**) in 34% yield (Halpert et al., 1989b). However, the reaction yield is greatly increased to 83% by the Oppenauer oxidation of **7** to **8**. Hydrolysis of **8** with sodium hydroxide in 95% alcohol gave **9** in 94% yield.

Synthesis of Ethyl 3β-Hydroxypregn-5-en-21-oate (**7**). Platinum(IV) oxide (115 mg) in ethanol (30 mL) was freshly hydrogenated to platinum black at 20 psi for 20 min. To this solution was added **6** (1.51 g), the mixture was hydrogenated at 40 psi for 2 h and filtered to remove platinum, and the filtrate was then concentrated under reduced pressure to give 1.48 g of product (one spot as shown on TLC). Recrystallization from ethanol gave **7** (1.11 g, 73% yield, mp 108–110 °C). The mother liquor was concentrated and column chromatographed to give another 288 mg (19% yield): ^{1}H NMR ($CDCl_3$) δ 0.61 (s, 3 H, C18), 1.01 (s, 3 H, C19), 1.25 (t, 3 H, J = 7.1 Hz, ethyl), 2.32 (m, 2 H, C20), 3.52 (m, 1 H, C3), 4.11 (q, 2 H, J = 7.1 Hz, ethyl), 5.35 (d, 1 H, J = 5.2 Hz, C6); ^{13}C NMR ($CDCl_3$) δ 12.28, 14.12, 19.28, 20.67, 24.46, 28.00, 31.46, 31.76, 31.82, 35.16, 36.46, 37.10, 37.16, 41.79, 42.11, 46.69, 50.13, 55.45, 60.04, 71.54, 121.38 (C6), 140.71 (C5), 173.87 (C21); IR (KBr) 3310 (broad and strong, OH), 1720 (C=O); MS (rel intensity) 361.3 (M^+ + 1, 17), 360.3 (M^+, 67), 342.3 (96), 327.3 (63), 249.2 (53), 161.1 (100), 147.1 (64), 133.1 (56), 107.1 (79), 93.1 (52), 79.1 (36), 55.1 (28).

Synthesis of Ethyl 3-Oxopregn-4-en-21-oate (**8**). A solution of **7** (610 mg, 1.69 mmol), cyclohexanone (7.2 mL), and toluene (30 mL) was distilled to 3 mL of solvent. To this solution was added aluminum isopropoxide (650 mg, 1.7 mmol). After being refluxed for 50 min, the solution was cooled and acidified by the addition of 1 N HCl. The product was extracted with ethyl acetate twice (30 mL, 20 mL). The combined extracts were washed with brine, dried over $MgSO_4$, filtered, and concentrated in vacuo. Cyclohexanone was further removed under vacuum. The residue was column chromatographed over silica gel (ethyl acetate/hexanes, 1:6.5) to give 506 mg (83% yield) of **8**: mp 111–113 °C (recrystallized from MeOH–H_2O); ^{1}H NMR ($CDCl_3$) δ 0.64 (s, 3 H, C18), 1.17 (s, 3 H, C19), 1.24 (t, 3 H, J = 7.1 Hz, ethyl), 2.32 (m, 2 H, C20), 4.10 (q, 2 H, J = 7.1 Hz, ethyl), 5.71 (br s, 1 H, C4); ^{13}C NMR ($CDCl_3$) δ 12.38, 14.11, 17.28, 20.63, 24.32, 27.99, 31.87, 32.78, 33.85, 35.20, 35.55, 35.61, 36.96, 38.54, 41.87, 46.61, 53.86, 54.60, 60.07, 123.74 (C4), 171.20 (C5), 171.44 (C21), 199.40 (C3); IR (KBr) 2950, 1710, 1650, 1600, 1450, 1300; MS (rel intensity) 359.2 (M^+ + 1, 11), 358.2 (M^+, 39), 316.2 (45), 235.2 (22), 161.1 (18), 147.1 (45), 124.1 (100), 107.1 (27), 93.1 (23), 91.1 (30), 55.1 (14).

Synthesis of 3-Oxopregn-4-en-21-oic Acid (**9**). To a solution of **8** (210 mg, 0.58 mmol) in 95% alcohol (6 mL) was added sodium hydroxide (115 mg). After it was stirred for 7.5 h, the solvent was removed in vacuo. The residue was dissolved in water (50 mL), acidified with 1 N HCl, and filtered. The crude product was then column chromatographed over silica gel (dichloromethane/methanol, 8:1) to give 181 mg (94% yield) of **9**: mp 193–195 °C (recrystallized from acetone–water); ^{1}H NMR (acetone-d_6) δ 0.70 (s, 3 H, C18), 1.24 (s, 3 H, C19), 2.34 (m, 2 H, C20), 5.63 (s, 1 H, C4); IR (KBr) 1720, 1635, 1600, 1250; MS (rel intensity) 331.2 (M^+ + 1, 10), 330.2 (M^+, 38), 315.2 (13), 288.2 (69), 245.2 (45), 207.1 (18), 147.1 (58), 145.1 (21), 134.1 (39), 124.1 (100), 105.1

[3] This reaction was optimized by using methylene chloride as the reaction solvent (51% yield). Other solvents used and the respective yields of **3** were as follows: ether, 36%; tetrahydrofuran, 35%; benzene, 28%; and ethanol, trace amount. In a separate experiment, a 65% yield of **3** was achieved by using 1.6 equiv of NCS, whereas the addition of 2.0 and 3.5 equiv of NCS gave increasing amounts of 21,21,21-trichloropregnenolone.

(32), 91.1 (58), 79.1 (32), 67.1 (29), 55.1 (37).

Microsome Preparation and Enzyme Purification. Fresh bovine adrenal glands were obtained from the local meat packers. The inner medullary region was dissected away from the cortex and discarded, and the cortical region was then scraped from the capsule and saved. Adrenocortical microsomes were then prepared as described previously for rat liver microsomes (Halpert et al., 1983). The protein concentration (Lowry et al., 1951) and the cytochrome P450 content (Omura & Sato, 1964) were determined by standard methods. Rat liver P450IIC6 (PB-C) (Graves et al., 1987), rat liver NADPH–cytochrome P450 reductase (Halpert et al., 1985), and rat liver cytochrome b_5 (Halpert et al., 1989a) were purified as described previously. One unit of reductase is defined as the amount that reduces 1 μmol of cytochrome *c* per minute when assayed in 300 mM potassium phosphate buffer (pH 7.7) at 25 °C.

Inactivation of P450 C-21: Loss of Enzyme Activity and P450. Experiments to compare the residual spectrally detectable P450 with residual enzymatic activity after the incubation of P450 C-21 with 17β-substituted steroids were performed as follows. The 1-mL incubation mixtures contained cytochrome P450 (1 nmol/mL), NADPH–cytochrome P450 reductase (2 units/nmol of P450), 30 μg/mL dilauroyl-L-phosphatidylcholine, 50 mM HEPES buffer (pH 7.6), 15 mM $MgCl_2$, 0.1 mM EDTA, and 25 μM inhibitor added in 1% methanol. The samples were preincubated at 37 °C for 2 min before the addition of NADPH (1 mM final concentration). The reaction was allowed to proceed for 10 min at 37 °C before the tubes were placed on ice. The material was transferred to dialysis bags and dialyzed against 4 × 1 L of 10 mM Tris–acetate (pH 7.4) containing 0.1 mM EDTA and 20% (V/V) glycerol. Following dialysis, the spectrally detectable P450 concentration was determined from the absorbance at 417 nm by using an extinction coefficient of 118 mM^{-1} cm^{-1}. A 0.05-nmol aliquot of the material was also assayed for residual progesterone 21-hydroxylase activity. Fresh NADPH–cytochrome P450 reductase (0.30 unit) was added to these incubations on the basis of the highest P450 value determined for either the +methanol or −NADPH control samples after dialysis. This reconstituted system (0.1-mL total volume) contained 30 μg/mL dilauroyl-L-phosphatidylcholine, 50 mM HEPES buffer (pH 7.6), 15 mM $MgCl_2$, 0.1 mM EDTA, and 25 μM [^{14}C]progesterone added in 1% methanol. The material was preincubated for 2 min at 37 °C before the addition of NADPH. After 2 min, the reaction was stopped by the addition of 50 μL of tetrahydrofuran. A 50-μL aliquot was spotted on a thin-layer chromatography plate and developed three times in chloroform/ethyl acetate (93:7) (McCarthy & Waterman, 1988). The hydroxylated progesterone metabolite(s) was (were) identified by autoradiography, scraped into a scintillation vial and counted, and the residual progesterone hydroxylase activity was calculated. The R_f values for the 17α-hydroxy- and 21-hydroxyprogesterone metabolites were 0.40 and 0.47, respectively.

Binding of 17β-Substituted Steroids to P450 C-21. A cuvette containing 0.92 μM P450 C-21 in ω-amino-*n*-octyl-Sepharose column elution buffer [100 mM potassium phosphate (pH 7.25), 20% glycerol, 0.1 mM EDTA, 0.4% sodium cholate, and 0.16% Emulgen 913] was placed in a Beckman DU-7 spectrophotometer and allowed to equilibrate in the cell to 25 °C with a thermostated circulator. Increasing concentrations of the inhibitors were added in 1 μL of methanol, the mixture was stirred, and the sample was scanned from 500 to 380 nm. The $\Delta A_{390-420}$ was recorded at each inhibitor concentration and plotted vs [S]. The concentration of steroid that produced half of the maximal shift (K_S) and the maximal change in absorbance (ΔA_{max}) for each steroid was determined by using nonlinear regression analysis and a one-site saturation model.

Analysis of 17β-Substituted Steroid Metabolites by HPLC. These experiments were carried out under the same conditions described for the incubations to compare the loss of enzyme activity with the decrease in spectrally detectable P450. However, instead of dialyzing the sample after the 10-min incubation, the reaction mixture was acidified by addition of 1 N HCl and extracted (2 × 1 mL) with ethyl acetate. The organic extract was then dried, and when the inhibitor was a 21,21-dihalo steroid, diazomethane was added and the solvent removed under a stream of nitrogen. The sample was taken up in methanol, and an aliquot was analyzed by HPLC on a 10 mm × 25 cm Altex Ultrasphere ODS 5-μm C18 reverse-phase column. For the 21,21-dihalo steroids, the column was eluted with 75:25 (v/v) acetonitrile/50% methanol and 50% H_2O mixture at a flow rate of 1.5 mL/min, and the column eluent was monitored at 254 nm. For 17β-ethynylprogesterone, the mobile phase was 85% methanol/15% water, and the eluent was monitored at 240 nm. For [^{3}H]-21,21-dichloroprogesterone and [^{3}H]-17β-ethynylprogesterone, the HPLC eluent was also collected in fractions and the amount of metabolite quantitated by scintillation counting. For 21,21-difluoroprogesterone, the amount of metabolite was quantitated on the basis of the absorbance at 254 nm by using the parent compound as an internal standard.

Results

Purification of Bovine Adrenal Cytochromes P450 17α and P450 C-21. Bovine adrenal cytochromes P450 17α and P450 C-21 were purified according to the method of Kominami et al. (1980). After elution of approximately 12 bed volumes from the ω-amino-*n*-octyl-Sepharose column with a linear gradient of 0–0.16% Emulgen 913, a mixture of cytochromes P450 17α and P450 C-21 was collected as pool 1 (Figure 3, lane 2). As the elution gradient continued, P450 C-21 eluted as the major protein and was pooled separately. Detergent in the elution buffer was removed by hydroxylapatite chromatography (Graves et al., 1987). Cytochrome P450 C-21 was considered pure on the basis of a single protein band upon SDS–polyacrylamide gel electrophoresis, as shown in Figure 3, lane 1, and the absence of progesterone 17α-hydroxylase activity when assayed in a reconstituted system. Purified P450 C-21 has a specific cytochrome P450 content of 19 nmol/mg before detergent removal and a progesterone 21-hydroxylase activity of 27 nmol of 21-hydroxyprogesterone produced min^{-1} (nmol of P450 C-21)$^{-1}$ after detergent removal. The progesterone 17α- and 21-hydroxylase activities of the pool 1 sample were 14.0 and 8.0 nmol of the respective hydroxylated product min^{-1} (nmol of P450)$^{-1}$.

Selectivity of P450 C-21 and P450 17α Inactivation by 17β-Substituted Steroids. Previous work from our laboratory has shown that replacement of the 17β-methylketo group of progesterone with a dihalomethyl, vinyl, or ethynyl group could target certain P450 forms of rat liver, rabbit liver, and rabbit adrenal for inactivation (Halpert et al., 1989a,b). A variety of 17β-substituted steroids were therefore tested for their ability to inactivate bovine adrenal P450 17α and P450 C-21 in a reconstituted system. For this purpose, pool 1 from the enzyme purification procedure was used since it contained both P450 17α and P450 C-21 (Figure 3, lane 2). The time-dependent loss of progesterone 17α- and 21-hydroxylase activities

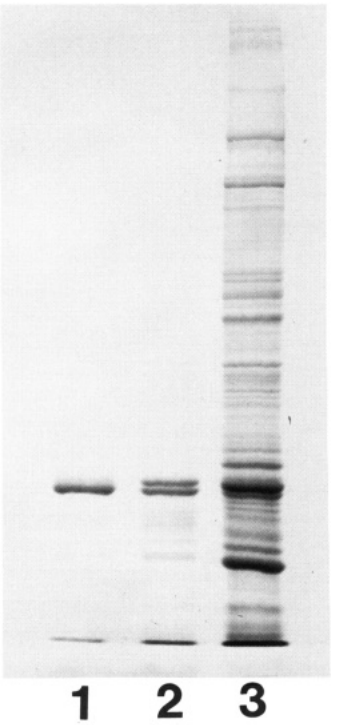

FIGURE 3: SDS–polyacrylamide gel electrophoresis of microsomes and fractions from the purification of bovine adrenocortical P450 17α and P450 C-21. Samples were electrophoresed on a 0.75 mm thick 7.5% gel and stained with Coomassie blue (Laemmli, 1970). The samples are as follows: lane 1, 1 μg of purified P450 C-21; lane 2, 1 μg of pool 1 material showing approximately equal amounts of P450 17α and P450 C-21; lane 3, 10 μg of bovine adrenocortical microsomal protein.

caused by the inhibitors in the presence of NADPH was measured. The effect of 25 μM 21,21-difluoroprogesterone on these enzymes is shown in Figure 4. With P450 C-21, the difluoro compound causes a time-dependent loss of enzyme activity corresponding to enzyme inactivation and a reversible (competitive) inhibition component due to the presence of unmetabolized inhibitor during the progesterone hydroxylase assay, represented by a decrease in the y intercept. In contrast, this compound reversibly inhibits but does not inactivate P450 17α. The selectivity of the difluoro compound was supported by larger scale experiments where residual enzyme activity was measured at a single time point. Only 49% of the residual progesterone 21-hydroxylase activity remained after the P450 17α/P450 C-21 mixture (as pool 1) was incubated with 25 μM 21,21-difluoroprogesterone and NADPH for 10 min and the sample dialyzed to remove any free inhibitor. However, all of the progesterone 17α-hydroxylase activity remained (data not shown).

Rate constants for inactivation of P450 17α and P450 C-21 by the 17β-substituted steroids are given in Table I. Of the five compounds tested, the two 17β-unsaturated steroids and 21,21-dichloroprogesterone inactivate P450 17α. However, all of the compounds except 21,21-dichloropregnenolone inactivate P450 C-21. This compound was considered a negative control since pregnenolone itself is not an endogenous substrate for P450 C-21. Table I shows that 17β-vinylprogesterone was the only steroid to inactivate P450 17α more rapidly than P450 C-21 (k_{inact} = 0.12 min^{-1} and 0.06 min^{-1}, respectively).

Inactivation of Rabbit Adrenal Progesterone 21-Hydroxylase by 17β-Substituted Steroids. The ability of these progesterone derivatives to inactivate the rabbit adrenal progesterone 21-hydroxylase was studied to explore differences between this enzyme and the homologous bovine adrenal P450.

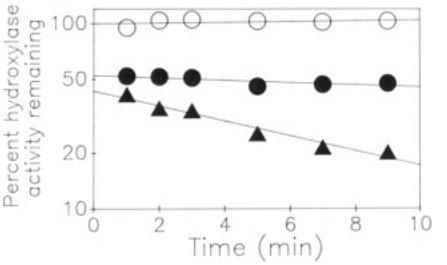

FIGURE 4: Effect of preincubation with 25 μM 21,21-difluoroprogesterone on progesterone 21-hydroxylase (▲) and progesterone 17α-hydroxylase (●) activity. For the sake of clarity, only the 21-hydroxylase methanol control (○) is shown. Purified P450 C-21 and P450 17α (as a mixture in pool 1) were assayed for residual progesterone hydroxylase activity after incubation with inhibitor and NADPH in a reconstituted system. The incubation conditions were 0.05 nmol/mL P450, 0.30 unit/mL NADPH–cytochrome P450 reductase, 30 μg/mL dilauroyl-L-phosphatidylcholine, 50 mM HEPES buffer (pH 7.6), 15 mM $MgCl_2$, 0.1 mM EDTA, and 1 mM NADPH. Any inhibitor was added in a final concentration of 1% methanol. The proteins were reconstituted in a small, concentrated volume for 5 min at room temperature before addition to the incubation mixture. The mixture was preincubated at 37 °C for 2 min, and the reactions were started by the addition of NADPH and allowed to proceed for up to 9 min. An 80-μL aliquot was removed at various times and added to 20 μL of [^{14}C]progesterone in buffer. The reaction proceeded for an additional 1.5 min before being quenched with 50 μL of tetrahydrofuran. One-third of each sample was spotted on a thin-layer chromatography plate [Baker silica gel, 250 μm, Si 250F (19c)], and the plate was developed three times in chloroform/ethyl acetate (93:7) (McCarthy & Waterman, 1988). The lines shown were drawn by linear regression analysis of the natural logarithm of the residual activity as a function of time. The 100% values for the 21- and 17α-hydroxylase activities were 14.2 and 7.9 nmol of 21-hydroxy and 17α-hydroxyprogesterone formed min^{-1} nmol^{-1}, respectively.

Table I: Rate Constants for Inactivation of Adrenocortical Cytochromes P450 by 17β-Substituted Steroids[a]

compd	concn (μM)	k_{inact} (min^{-1}) 17α-hydroxylase	k_{inact} (min^{-1}) 21-hydroxylase
methanol (control) (n = 4)		0.00	0.00
21,21-dichloroprogesterone	2.5	0.09	0.13
21,21-difluoroprogesterone	25	0.02	0.10
21,21-dichloropregnenolone	25	0.01	0.01
17β-ethynylprogesterone	25	0.09	0.27
17β-vinylprogesterone	100	0.12	0.06

[a] A mixture of cytochromes P450 C-21 and P450 17α (pool 1) was incubated with various 17β-substituted steroids in a reconstituted system as described in the legend to Figure 4. Rate constants for inactivation were calculated by linear regression analysis of the natural logarithm of the residual progesterone hydroxylase activity as a function of time. The values given represent the mean of duplicate experiments.

Although all of the progesterone derivatives studied inactivate bovine adrenal P450 C-21 (Table I), only 21,21-dichloroprogesterone rapidly inactivates the rabbit adrenal form. The rate constants for inactivation of the rabbit adrenal 21-hydroxylase by the 17β-substituted steroids (25 μM inhibitor) were methanol (control), 0.01 min^{-1}; 21,21-dichloroprogesterone, 0.19 min^{-1}; 21,21-difluoroprogesterone, 0.04 min^{-1}; 17β-ethynylprogesterone, 0.03 min^{-1}; and 21-chloro,21-fluoroprogesterone, 0.05 min^{-1}. It is interesting to note that, by simply replacing a 21-chloro atom with a fluorine, the 21,21-dihalo steroid is essentially rendered unable to inactivate the rabbit enzyme.

Characterization of 21,21-Difluoroprogesterone Inactivation of P450 C-21. Because of the unique ability of 21,21-difluoroprogesterone to inactivate P450 C-21 but not P450 17α, several experiments were conducted to illustrate that this compound can be rigorously categorized as a mechanism-based inactivator of P450 C-21. First, the rate of enzyme inactivation

Table II: Type I Binding of Progesterone and 17β-Substituted Steroids to Purified Bovine Adrenal P450 C-21: K_s and ΔA_{max} Values

compd	K_s (μM)	$\Delta A_{max390-420}$
progesterone	1.2	0.079
21,21-dichloroprogesterone	15.0	0.081
21,21-difluoroprogesterone	4.9	0.079
pregna-4,20-dien-3-one (17β-vinylprogesterone)	3.5	0.066
pregn-4-en-20-yn-3-one (17β-ethynylprogesterone)	8.2	0.038

was measured at inhibitor concentrations of 5, 10, 25, and 100 μM. The rate constants for inactivation were 0.14 min^{-1}, 0.17 min^{-1}, 0.20 min^{-1}, and 0.22 min^{-1}, respectively. This analysis showed that loss of progesterone 21-hydroxylase activity is saturable (Walsh, 1984). The maximal rate constant for inactivation by 21,21-difluoroprogesterone was determined by nonlinear regression analysis to be 0.22 min^{-1}, and the inhibitor concentration required for half-maximal inactivation (K_I) was 3.2 μM. Two additional characteristics of P450 C-21 inactivation by 21,21-difluoroprogesterone were also studied. The rate constant for P450 C-21 inactivation was significantly decreased (0.04 min^{-1} vs 0.16 min^{-1}) when excess unlabeled substrate (100 μM progesterone) was included in an incubation with 25 μM inhibitor. However, the addition of glutathione did not protect against P450 C-21 inactivation (k_{inact} = 0.14 min^{-1}) (data not shown). This may indicate that, once formed, the reactive (acyl halide) intermediate does not diffuse from the enzyme active site before reacting with the enzyme.

Binding of 17β-Substituted Steroids to P450 C-21. Progesterone interacts with purified P450 C-21 to form a type I optical difference spectrum, a finding that is indicative of substrate binding to low-spin ferric P450 (Jefcoate, 1978; Kominami et al., 1980). Therefore, the affinity of the 17β-substituted steroids for P450 C-21 and the extent of the spectral shift produced were examined as a measure of the reversible binding of these compounds compared with progesterone. Figure 5 shows the type I spectral shifts produced when increasing concentrations of 21,21-difluoroprogesterone and 17β-ethynylprogesterone were added to P450 C-21. 21,21-Difluoroprogesterone (Figure 5A) produces a greater type I spectral shift than does 17β-ethynylprogesterone (Figure 5B). For each compound, the change in absorbance at 390 and 420 nm was measured and plotted versus the concentration of inhibitor to determine (1) the concentration that produced a half-maximal change in absorbance (K_S) and (2) the maximal shift in absorbance (ΔA_{max}) for each compound. The results are shown in Table II. The dihalo compounds produce essentially the same ΔA_{max} as progesterone and have K_S values

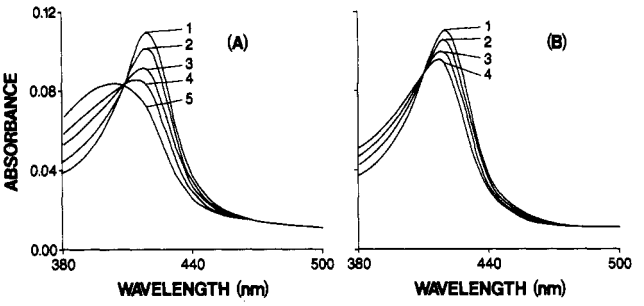

FIGURE 5: Type I binding spectra produced by the titration of purified P450 C-21. Shown are spectra obtained with (panel A) 21,21-difluoroprogesterone and (panel B) 17β-ethynylprogesterone. Curve 1 shows the spectrum before addition of the steroid. In panel A, curves 2–5 show the spectra produced upon the addition of 1.0, 3.5, 8.5, and 96.0 μM 21,21-difluoroprogesterone, respectively. Curves 2–4 in panel B show the spectra produced upon the addition of 3.5, 21.0, and 146.0 μM 17β-ethynylprogesterone. The concentration of P450 was 0.92 μM. Other conditions are given under Materials and Methods.

in the low micromolar range, indicating a high affinity for the enzyme. However, both 17β-unsaturated steroids have significantly lower ΔA_{max} values, suggesting that the 20-keto oxygen may be important in the binding of 17β-substituted steroids to P450 C-21.

P450 C-21 Inactivation by Heme and Protein Modification. The type I spectral shift shown in Figure 5 represents reversible binding of the inhibitor to the enzyme prior to metabolism. In contrast, the ultimate inactivation event should be metabolism dependent and irreversible and can occur by modification of the protein and/or the heme moiety of the enzyme. Both types of inactivation result in loss of enzyme activity, but only inactivation by heme modification will presumably produce a decrease in spectrally detectable cytochrome P450. Therefore, three of the 17β-substituted steroids were incubated with P450 C-21 in a reconstituted system in the presence or absence of NADPH, dialyzed after the incubation to remove any free inhibitor, and examined for residual P450 levels and progesterone 21-hydroxylase activity. The results are shown in Table III. In each case, inactivation was shown to be NADPH dependent and irreversible. There was a 62% decrease in the remaining P450 after incubation of P450 C-21 with 25 μM 21,21-dichloroprogesterone, indicating that heme destruction is the predominant pathway of inactivation for this compound. However, a 78% decrease in residual enzyme activity was noted for 17β-ethynylprogesterone compared with only a 25% drop in spectrally detectable P450, indicating that protein modification is favored. Interestingly, when the dialyzed sample that had been incubated with 17β-ethynylprogesterone plus NADPH (Figure 6, scan 2) was compared

Table III: Loss of Spectrally Detectable Cytochrome P450 and Progesterone 21-Hydroxylase Activity after Incubation of Bovine Adrenal P450 C-21 with 17β-Substituted Steroids in a Reconstituted System[a]

treatment	[P450] (nmol/mL)	residual act.[b]
methanol, +NADPH	0.71 (0.78, 0.63)	32.6 (36.0, 29.1)
25 μM 21,21-dichloroprogesterone, -NADPH	0.67 (0.63, 0.70)	29.1 (26.1, 32.1)
25 μM 21,21-dichloroprogesterone, +NADPH	0.28 (0.25, 0.30) (38%)[c]	4.6 (3.4, 5.7) (15%)
25 μM 21,21-difluoroprogesterone, -NADPH	0.72 (0.74, 0.69)	31.5 (33.0, 29.9)
25 μM 21,21-difluoroprogesterone, +NADPH	0.53 (0.56, 0.49) (73%)	12.2 (11.9, 12.4) (39%)
25 μM 17β-ethynylprogesterone, -NADPH	0.80 (0.84, 0.76)	32.9 (34.6, 31.1)
25 μM 17β-ethynylprogesterone, +NADPH	0.55 (0.56, 0.54) (75%)	6.8 (5.9, 7.6) (22%)

[a] The details are given under Materials and Methods. The P450 values are the mean of single analyses of two independent samples and are given in parentheses. The enzyme activities given in parentheses are the mean of triplicate determinations of two individual samples. [b] Activity is given in nmol of 21-hydroxyprogesterone produced min^{-1} (nmol of P450)$^{-1}$. [c] These numbers represent the percentage of the mean of the +inhibitor, -NADPH, and +methanol, +NADPH controls (n = 8).

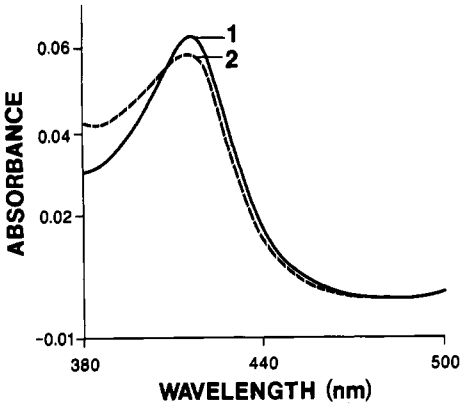

FIGURE 6: Irreversible type I spectral shift produced by the binding of 17β-ethynylprogesterone metabolite(s) to purified bovine adrenal P450 C-21. Scan 1 is the control sample where 0.5 nmol of P450 C-21 was incubated in a reconstituted system with 25 μM 17β-ethynylprogesterone in the absence of NADPH. Scan 2 is from an identical incubation where NADPH was added. After the incubation, each sample was extensively dialyzed against buffer containing 10 mM Tris–acetate (pH 7.4), 1 mM EDTA, and 20% glycerol before the spectrophotometric determination. Both samples were scanned, and the absorbance at the isosbestic point from Figure 5B (A_{410}) was noted. Sample 1 was then diluted with buffer to match the A_{410} of sample 2.

Table IV: Determination of the Stoichiometry of Inactivation of P450 C-21 by [^{3}H]-17β-Ethynylprogesterone[a]

	P450 C-21[b] (nmol)	sample − blank (dpm)	^{3}H compd bound[c] (nmol)	P450 inact (%)	^{3}H compd bound/P450 inact[d] (nmol/nmol)
sample 1	0.039	1138	0.050	85	1.51
sample 2	0.039	1107	0.048	85	1.45

[a] P450 C-21 was incubated in a reconstituted system containing NADPH–cytochrome P450 reductase, HEPES buffer, phospholipid, 50 μM [^{3}H]-17β-ethynylprogesterone, and NADPH for 10 min at 37 °C. Residual progesterone 21-hydroxylase activity was determined on an aliquot of the dialyzed incubation mixture as described under Materials and Methods. A 50-μL aliquot was spotted on the preadsorbent area of a thin-layer chromatography plate and developed twice in methanol and once in hexane. The loading zone was then scraped into a scintillation vial, 0.5 mL of 1 N sodium hydroxide added, and the sample allowed to sit overnight. The material was then neutralized with an equimolar amount of 1 N hydrochloric acid, 2 mL of methanol was added to prevent emulsification, scintillation cocktail was added, and the sample was counted. [b] Amount of enzyme applied to preadsorbent loading zone. [c] Based on a specific activity of 2.29×10^4 dpm/nmol. [d] Obtained by first dividing the nmol of compound bound by the amount of P450 and then dividing this result by 0.85 on the basis of the amount of enzyme inactivated.

with the corresponding minus NADPH control at the same P450 concentration (Figure 6, scan 1), an irreversible type I spectral shift was observed. To our knowledge this is the first example of an irreversible type I spectral shift of a purified P450 produced by a mechanism-based inactivator. [^{3}H]-17β-Ethynylprogesterone was used to determine the stoichiometry of binding of this compound to P450 C-21. The enzyme was incubated in a reconstituted system with NADPH and [^{3}H]-17β-ethynylprogesterone as described in Table IV. An aliquot of the incubation material was spotted on the preadsorbent zone of a thin-layer chromatography plate and developed in methanol and hexane to remove any unbound inhibitor (Halpert et al., 1980). Scintillation counting of the protein-bound loading zone material enabled calculation of a stoichiometry of inactivation of approximately 1.5 nmol of ^{3}H compound bound/nmol of P450 C-21 inactivated. Also, separation of P450 C-21 from NADPH–cytochrome P450 reductase by electrophoresis yielded no evidence of a ^{3}H label associated with the reductase protein band, and no decrease

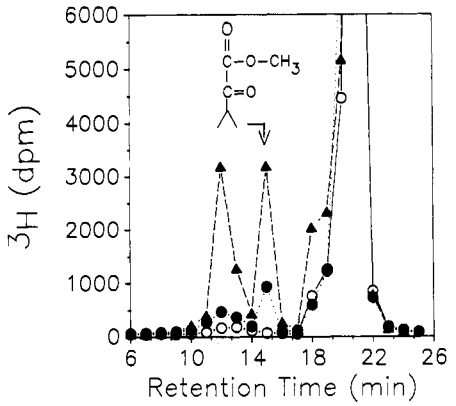

FIGURE 7: Reverse-phase HPLC of the organic extract from the incubation of 25 μM [^{3}H]-21,21-dichloroprogesterone with bovine P450 C-21 (▲, dashed line) and rat hepatic P450IIC6 (●, dotted line). Cytochrome P450 C-21 (0.2 nmol) was incubated in a reconstituted system containing cytochrome P450 reductase (0.4 unit), phospholipid, and HEPES buffer for 2 min at 37 °C in a total volume of 0.5 mL. Incubation conditions were identical for P450IIC6 except that cytochrome b_5 (0.4 nmol) was added. The reactions were started by the addition of NADPH and allowed to proceed for 10 min. The reaction was stopped by returning the sample to ice. Hydrochloric acid (1 N, 0.1 mL) was added, and the sample was extracted with two 1-mL additions of ethyl acetate and removal of 0.75 mL of the organic layer each time. The organic phase was dried under nitrogen and derivatized with diazomethane reagent in ether, and the ether was removed under nitrogen. The sample was taken up in 150 μL of methanol, and a 50-μL aliquot was injected onto an Altex 5-μm, 10-mm, 25-cm C18 column. The column was eluted with 75%–25% acetonitrile/50% methanol and 50% water mixture at a flow rate of 1.5 mL/min, and 1-min fractions were collected. The fractions were monitored by ultraviolet spectroscopy (254 nm, data not shown) and by liquid scintillation counting. The arrow shows the location of the methyl ester of the 21-pregnenoic acid standard. The results for the blank incubation tube (NADPH, no protein) are represented by the open circles and solid line.

in cytochrome *c* reductase activity was observed (data not shown).

Metabolism of 17β-Substituted Steroids by 21-Hydroxylases. The evidence presented thus far strongly suggests that metabolism of the dihalomethyl or acetylenic group of the 17β-substituted steroids is responsible for the inactivation of progesterone 21-hydroxylases. Our hypothesis was that 21-hydroxylase inactivation by the dihalo steroids proceeds as follows: hydroxylation of the 21-carbon to give a halohydrin (–CO–CX_2OH) followed by spontaneous dehydrohalogenation to yield an acyl halide (–CO–CO–X) intermediate that could bind irreversibly to the P450. Therefore, to test this hypothesis and to address differences observed in the ability of the 21,21-dihalo steroids to inactivate bovine P450 C-21 and rat liver P450IIC6, we conducted more rigorous metabolism studies. The rate constant for enzyme inactivation reflects not only the rate of conversion of the inactivator to reactive intermediate(s) but also the number of turnovers per inactivation event, the so-called partition ratio (Rando, 1984; Nagahisa et al., 1983). An alternative fate of the acyl halide intermediate would be the addition of water to give the soluble metabolite 21-pregnenoic acid (–CO–CO-OH). Therefore, quantitation of the amount of 21-pregnenoic acid produced during 21,21-dihalo steroid metabolism by 21-hydroxylases relative to the amount of enzyme inactivated will yield the partition ratio and a measure of the efficiency of enzyme inactivation.

Incubation of the 21,21-dihalo steroids with P450 C-21 and analysis of the organic extract for soluble metabolites showed that one major metabolite formed had a retention time of approximately 15.0 min after the sample was derivatized with

diazomethane (Figure 7). This metabolite was tentatively identified as 21-pregnenoic acid on the basis of identical chromatographic behavior as the 21-pregnenoic acid standard in both the methylated and nonmethylated forms and on a requirement for acidification prior to extraction. Approximately 5 nmol of acid was formed from 21,21-dichloro- or 21,21-difluoroprogesterone/nmol of P450 C-21 inactivated. As a comparison, we also measured the partition ratio for 21,21-dichloroprogesterone-mediated inactivation of the rat liver 21-hydroxylase P450IIC6. Since this enzyme has a much lower progesterone 21-hydroxylase activity than P450 C-21 (0.8 versus 26 nmol min^{-1} $nmol^{-1}$) but a greater rate constant for inactivation (Halpert et al., 1989b) compared with P450 C-21, an extremely low partition ratio was suspected. HPLC studies (Figure 7, dotted line) showed that 1 nmol of 21-pregnenoic acid was formed/nmol of P450IIC6 inactivated.[4] For positive identification of this metabolite, 21,21-dichloroprogesterone was incubated with P450 C-21, and the derivatized organic extract for the incubation was separated by HPLC. The 15-min metabolite was collected and analyzed by mass spectroscopy. This analyte displayed mass spectral signals at m/z 358 (M^+, 2.9), 299 (100), 271 (46), 253 (28), and 147 (22) and a fragmentation pattern consistent with the 21-pregnenoic acid standard: m/z 358 (M^+, 1.7), 299 (100), 271 (44), 253 (27), and 147 (22). The molecular ion signal (M^+) of m/z 358, although weak as is characteristic for steroids, agrees with the molecular weight for the methyl ester of the standard 21-pregnenoic acid. The base peak at m/z 299 represents loss of a methoxycarbonyl fragment (CH_3OCO) from the 21-methyl ester. Subsequent loss of the 20-keto fragment (m/z = 28) yields the m/z 271 fragment. The remaining major peaks at m/z 253 and m/z 147 result from the fragmentation of the steroid nucleus. The mass spectrum of the metabolite with a retention time of 12 min (Figure 7) contained a molecular ion at m/z 316, the molecular weight of etienic acid. This C-20 steroid could result from further metabolism of pregnenoic acid as has been reported for the stepwise conversion of progesterone to 21-hydroxyprogesterone to etienic acid (Dey & Senciall, 1978).

Similar metabolism studies were carried out with the acetylenic compound 17β-ethynylprogesterone and P450 C-21. Recently CaJacob et al. (1988) proposed a scheme for the mechanism-based inactivation of a rat hepatic lauric acid ω-hydroxylase by an acetylenic fatty acid analogue. This involved the metabolism of the compound to a ketene intermediate, which could bind to the enzyme or undergo hydrolysis to a carboxylic acid. Therefore, 3-oxopregn-4-en-21-oic acid ($-CH_2-COOH$) was synthesized as a standard for the expected metabolite of 17β-ethynylprogesterone. Purified P450 C-21 was incubated with the ^3H compound, and the organic extract was analyzed by HPLC. The results showed that 3-oxopregn-4-en-21-oic acid is formed at a ratio of approximately 4 nmol/nmol of P450 C-21 inactivated (data not shown). However, this is not the major metabolite, and the total partition ratio is approximately 30 nmol of soluble metabolites produced/nmol of P450 C-21 inactivated.

Discussion

This report documents the ability of 17β-substituted steroids to inactivate bovine adrenal P450 C-21 and P450 17α. In addition, the rabbit adrenal and rat hepatic (P450IIC6) progesterone 21-hydroxylases were included in some experiments to explore differences in selectivity or to investigate the metabolic basis for differences in rate constants of inactivation. The 17β-substituted steroids that retain the steroid nucleus of progesterone were considered to be excellent candidates for this study since these four enzymes normally metabolize progesterone in a specific fashion.

Of the two 21,21-dihalo and two 17β-unsaturated steroids tested, 21,21-difluoroprogesterone is the only compound to inactivate bovine adrenal P450 C-21 but not P450 17α. Chemically this compound most closely resembles progesterone due to the similar steric, electronic, and hydrophobic properties of fluorine and hydrogen (Topliss & Martin, 1975). In terms of reversible binding to P450 C-21 the difluoro compound mimics progesterone as evidenced by K_S and ΔA_{max} values. In addition, the K_I value for inactivation of P450 C-21 by 21,21-difluoroprogesterone and the K_m for 21-hydroxylation of progesterone (Lorence et al., 1989) are both in the low micromolar range. With regard to P450 17α, inhibition studies show that 21,21-difluoroprogesterone competes favorably with progesterone for binding. The lack of enzyme inactivation suggests that this enzyme, as well as P450 C-21, retains its regioselectivity with the difluoro compound.

In contrast to the bovine adrenal enzyme, the presumably homologous rabbit adrenal 21-hydroxylase is not inactivated by 21,21-difluoroprogesterone. The rabbit enzyme is also refractory to inactivation by 21-chloro-21-fluoroprogesterone but is inactivated by the dichloro compound. This latter observation is yet another example from our laboratory of replacement of a fluorine atom for a chlorine rendering a compound ineffective as a P450 inactivator (Halpert et al., 1989b, 1990). Based on an analogy with dihalomethane metabolism (Kubic & Anders, 1975; Stevens et al., 1979), the probable rate-limiting step in 21,21-dihalosteroid metabolism is the abstraction of a hydrogen atom to form a carbon-centered radical. The ease of carbon–hydrogen bond cleavage increases with the decreasing electronegativity of the halide substituent (F > Cl > Br) (Macdonald, 1982). The ability of the halogen atom(s) at the 21-carbon to dictate progesterone 21-hydroxylase inactivation may thus be explainable by the oxidation potential of the individual enzyme. This potential includes an intrinsic value, common to all P450s, and a factor characteristic of the individual P450 active site (Macdonald, 1989). Our data suggest that differences in the enzyme active site result in a greater oxidative capacity of the bovine enzyme compared with the rabbit enzyme.

The structurally unrelated progesterone 21-hydroxylases of rat liver and bovine adrenal were used to determine whether the rate of inhibitor metabolism is the sole determinant of enzyme inactivation. Although we have previously documented progesterone hydroxylase inactivation by several dihalo steroids, we now provide direct evidence that metabolism proceeds, at least in part, through an acyl halide intermediate. This was accomplished by mass spectral identification of the expected hydrolysis product, 21-pregnenoic acid. This metabolite was produced at a ratio of 5 nmol of acid/nmol of P450 C-21 inactivated, regardless of whether the substrate was the dichloro or difluoro compound. In an identical experiment with the dichloro compound, the rat liver progesterone 21-hydroxylase (P450IIC6) produced approximately 1 nmol of acid/nmol of P450 inactivated. This extremely low partition ratio partially explains why P450IIC6, which metabolizes progesterone slowly [0.8 nmol of 21-hydroxyprogesterone min^{-1} $(nmol\ of\ P450)^{-1}$], can be inactivated by 21,21-dichloroprogesterone with a rate constant of 0.22 min^{-1} (Halpert et al., 1989b).

[4] Calculation of the total soluble metabolites yielded partition ratios of 12 nmol of metabolite/nmol of P450 C-21 inactivated and 2 nmol of metabolite/nmol of P450IIC6 inactivated.

The second major objective of this work was to determine whether the changes in the 17β-substituent not only alter the selectivity and rate constant for enzyme inactivation but also dictate whether inactivation occurs by protein or heme modification. Olefins and acetylenes are the most thoroughly studied suicide substrates of cytochrome P450 (Ortiz de Montellano & Reich, 1986), producing enzyme inactivation presumably by N-alkylation of the heme prosthetic group (Ortiz de Montellano & Reich, 1984). Recently, however, several groups have reported P450 inactivation by terminal acetylenes where the loss of enzyme activity could not be fully explained by the loss of P450 chromophore, thus suggesting inactivation by protein acylation (CaJacob et al., 1988; Hammons et al., 1989). CaJacob et al. (1988) proposed that addition of the activated oxygen to the internal carbon of terminal acetylenes leads to heme alkylation, while addition at the terminal carbon leads to protein modification. These results may partially explain why the 17β-substituted steroid that inactivates P450 C-21 mainly by protein modification is the terminal acetylene 17β-ethynylprogesterone. This could be interpreted as inactivation proceeding through addition of the activated oxygen to the terminal (C-21) carbon with the production of a ketene capable of protein acylation. The observed loss of some of the enzyme chromophore might then indicate that the strict regioselectivity of the enzyme for attack at the 21-carbon of progesterone is compromised when the 17β-methylketo group of progesterone is replaced with an acetylenic group, leading to some internal carbon (C-20) attack and heme alkylation. Binding studies also show that the interaction of the acetylenic compound with P450 C-21 is clearly different from that of progesterone, as evidenced by the 50% lower ΔA_{max} value. Covalent binding of the acetylenic compound to the protein may be at or near the enzyme active site, as suggested by the irreversible type I binding spectrum produced.

In addition to protein acylation, the proposed ketene intermediate can undergo addition of water to form an acid. The expected metabolite, 3-oxopregn-4-en-21-oic acid, is formed at a ratio of approximately 4 nmol/nmol of P450 C-21 inactivated. This can be compared with the ratio of 2 nmol of acid formed from 10-undecynoic acid/nmol of lauric acid ω-hydroxylase inactivated (CaJacob, 1988). In addition to 3-oxopregn-4-en-21-oic acid, several other unidentified 17β-ethynylprogesterone metabolites are formed by P450 C-21. This leaves open the possibility that 17β-ethynylprogesterone inactivates P450 C-21 by an as yet undescribed metabolic pathway. Future experiments will investigate the process of P450 C-21 inactivation and the identity of the amino acid(s) modified by 17β-ethynylprogesterone.

Acknowledgments

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Registry No. **1**, 145-13-1; **2**, 120142-44-1; **3**, 117038-60-5; **4**, 117038-59-2; **6**, 21929-90-8; **7**, 81882-50-0; **8**, 55470-59-2; **9**, 132565-34-5; NCS, 128-09-6; NADPH, 53-57-6; EtOCOCOOEt, 95-92-1; 21,21-difluoroprogesterone, 1995-21-7; 17β-ethynylprogesterone, 21321-89-1; 17β-vinylprogesterone, 14508-15-7; 21-chloro-21-fluoroprogesterone, 120027-34-1; 21-pregnenoic acid, 2681-57-4; progesterone, 57-83-0; cytochrome P450, 9035-51-2; steroid 17α-hydroxylase, 9029-67-8; steroid 21-hydroxylase, 9029-68-9.

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Steady-State Kinetic Mechanism of Rat Tyrosine Hydroxylase†

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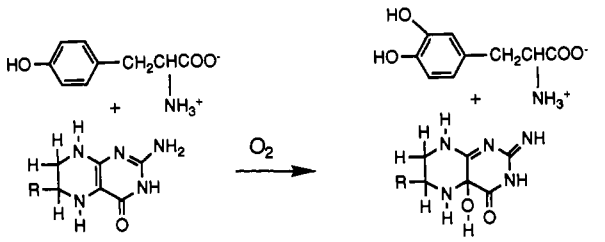
ABSTRACT: The steady-state kinetic mechanism for rat tyrosine hydroxylase has been determined by using recombinant enzyme expressed in insect tissue culture cells. Variation of any two of the three substrates, tyrosine, 6-methyltetrahydropterin, and oxygen, together at nonsaturating concentrations of the third gives a pattern of intersecting lines in a double-reciprocal plot. Varying tyrosine and oxygen together results in a rapid equilibrium pattern, while the other substrate pairs both fit a sequential mechanism. When tyrosine and 6-methyltetrahydropterin are varied at a fixed ratio at different oxygen concentrations, the intercept replot is linear and the slope replot is nonlinear with a zero intercept, consistent with rapid equilibrium binding of oxygen. All the replots when oxygen is varied in a fixed ratio with either tyrosine or 6-methyltetrahydropterin are nonlinear with finite intercepts. 6-Methyl-7,8-dihydropterin and norepinephrine are competitive inhibitors versus 6-methyltetrahydropterin and noncompetitive inhibitors versus tyrosine. 3-Iodotyrosine, a competitive inhibitor versus tyrosine, shows uncompetitive inhibition versus 6-methyltetrahydropterin. At high concentrations, tyrosine is a competitive inhibitor versus 6-methyltetrahydropterin. These results are consistent with an ordered kinetic mechanism with the order of binding being 6-methyltetrahydropterin, oxygen, and tyrosine and with formation of a dead-end enzyme–tyrosine complex. There is no significant primary kinetic isotope effect on the V/K values or on the V_{max} value with [3,5-$^2H_2$]tyrosine as substrate. No burst of dihydroxyphenylalanine production is seen during the first turnover. These results rule out product release and carbon–hydrogen bond cleavage as rate-limiting steps.

Tyrosine hydroxylase catalyzes the hydroxylation of tyrosine to form dihydroxyphenylalanine (DOPA)[1] (Scheme I), the first step in the biosynthesis of catecholamine neurotransmitters (Kaufman & Kaufman, 1985). Besides tyrosine, the physiological substrates for the enzyme are molecular oxygen and tetrahydrobiopterin; a 4a-hydroxytetrahydropterin is the other product (Dix et al., 1987; Haavik & Flatmark, 1987). Tyrosine hydroxylase is known to be an iron protein; the active form contains one ferrous iron atom per subunit (Dix et al., 1985; Fitzpatrick, 1989). The role of the iron is unknown, but attempts to replace it with other metals have not been successful (Fitzpatrick, 1989).

The kinetic mechanism of bovine tyrosine hydroxylase has been studied by several workers, with contradictory results. Thus, Ikeda et al. (1966), using a protease-treated enzyme, reported that varying 6,7-dimethyltetrahydropterin and tyrosine together resulted in a series of parallel lines in a double-reciprocal plot. They concluded that the enzyme used a ping-pong mechanism involving a reduced enzyme intermediate. However, Kaufman and Fisher (1974) reported unpublished experiments in which an intersecting line pattern was obtained with the same substrates and concluded that the mechanism involved a quaternary complex of enzyme with all three substrates. Oka et al. (1981) reported that varying tetrahydrobiopterin and oxygen or tetrahydrobiopterin and tyrosine together gave intersecting line patterns, but varying

Scheme I



tyrosine and oxygen together gave parallel lines. In all cases, the double-reciprocal plots were concave down. In contrast, Bullard and Capson (1983) found intersecting line patterns with all three substrate pairs, with no sign of curvature. On the basis of product inhibition studies, these authors concluded that either tyrosine or tetrahydrobiopterin could bind to the enzyme first but that oxygen must bind after tetrahydrobiopterin. Recently, we have reported that tyrosine must be able to bind after the tetrahydropterin and that both DOPA and 5-deazatetrahydropterin, two of the inhibitors used by Bullard and Capson (1983), can bind to multiple forms of the bovine enzyme (Fitzpatrick, 1988).

The difficulty of obtaining significant amounts of purified tyrosine hydroxylase has undoubtedly contributed to the contradictory results reported to date. We have recently ex-

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[1] Abbreviations: MES, 2-(*N*-morpholino)ethanesulfonic acid; DOPA, dihydroxyphenylalanine; 6-MePH$_4$, 6-methyltetrahydropterin; 6-MePH$_2$, 6-methyl-7,8-dihydropterin.

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