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Fetal Lamb 3β,20α-Hydroxysteroid Oxidoreductase: Dual Activity at the Same Active Site Examined by Affinity Labeling with 16α-(Bromo[2'-^{14}C]acetoxy)progesterone†

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ABSTRACT: 3β,20α-Hydroxysteroid oxidoreductase was purified to homogeneity from fetal lamb erythrocytes. The M_r 35 000 enzyme utilizes NADPH and reduces progesterone to 4-pregnen-20α-ol-3-one [K_m = 30.8 μM and V_{max} = 0.7 nmol min^{-1} (nmol of enzyme)$^{-1}$] and 5α-dihydrotestosterone to 5α-androstane-3β,17β-diol [K_m = 74 μM and V_{max} = 1.3 nmol min^{-1} (nmol of enzyme)$^{-1}$]. 5α-Dihydrotestosterone competitively inhibits (K_i = 102 μM) 20α-reductase activity, suggesting that both substrates may be reduced at the same active site. 16α-(Bromoacetoxy)progesterone competitively inhibits 3β- and 20α-reductase activities and also causes time-dependent and irreversible losses of *both* 3β-reductase and 20α-reductase activities with the same pseudo-first order kinetic $t_{1/2}$ value of 75 min. Progesterone and 5α-dihydrotestosterone protect the enzyme against loss of the two reductase activities presumably by competing with the affinity alkylating steroid for the active site of 3β,20α-hydroxysteroid oxidoreductase. 16α-(Bromo[2'-^{14}C]acetoxy)progesterone radiolabels the active site of 3β,20α-hydroxysteroid oxidoreductase wherein 1 mol of steroid completely inactivates 1 mol of enzyme with complete loss of both reductase activities. Hydrolysis of the ^{14}C-labeled enzyme with 6 N HCl at 110 °C and analysis of the amino acid hydrolysate identified predominantly N^{π}-(carboxy[2'-^{14}C]methyl)histidine [His(π-CM)]. Digestion with trypsin of the ^{14}C-labeled enzyme, isolation of the radioactive peptides, and amino acid sequence analysis showed the modified amino acid to be in the sequence H_2N-Tyr-Val-Ala-Val-Met-Pro-Pro-Pro-Ile-Gly-Asp-His(π-CM)-Pro-Leu-Thr-Gly-Ala-Tyr-Tyr-COOH. The results suggest that (1) 5α-dihydrotestosterone and progesterone are reduced at one and the same active site of 3β,20α-hydroxysteroid oxidoreductase, (2) the ^{14}C-labeled peptides isolated from affinity labeling of 3β,20α-hydroxysteroid oxidoreductase with 16α-(bromo[2'-^{14}C]acetoxy)progesterone form part of the steroid-binding region at the active site, and (3) the D-ring of 16α-(bromo[2'-^{14}C]acetoxy)progesterone most likely proximates a His residue as the steroid binds to and reacts with the catalytically active site of fetal lamb 3β,20α-hydroxysteroid oxidoreductase.

Red blood cells from fetal lambs or calves convert progesterone to 4-pregnen-20α-ol-3-one (Nancarrow & Seamark 1968; Nancarrow et al., 1981a; Sharaf & Sweet, 1982). The daily production of progesterone by the pregnant ewe is about 2.7 mg/day, while 700 mL of fetal lamb's blood can theoretically metabolize more than 18 mg of progesterone/h (Short, 1959; Nancarrow & Seamark, 1968). Thus, the total metabolic capacity of fetal lamb blood exceeds the daily maternal output of progesterone by a factor of 160.

20α-Reductase activity in fetal lamb blood continues to increase throughout pregnancy and then rapidly disappears from the newborn (Nancarrow, 1983). 3β-Reductase activity accompanies 20α-reductase activity in fetal red blood cells from species in the family Bovidae (Nancarrow et al., 1981a,b; Sharaf & Sweet, 1982). To further study the nature of the 3β-reductase and 20α-reductase activities in fetal lamb blood, attempts were made to purify what was earlier believed to be two different enzymes. During isolation of 20α-hydroxysteroid oxidoreductase the enzyme was also found to possess 3β-reductase activity (Chen et al., 1987) like the enzyme isolated from bovine fetal blood (Nancarrow et al., 1981b; Sharaf &

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Sweet, 1982). Kinetic studies and affinity labeling experiments suggested that 3β-reductase and 20α-reductase share a single active site in fetal calf 3β,20α-hydroxysteroid oxidoreductase.

This paper describes an improved scheme for isolating 3β,20α-hydroxysteroid oxidoreductase from fetal lamb blood. The nature of the 3β- and 20α-reductase activities and the amino acid composition at the enzyme active site were examined by affinity labeling 3β,20α-hydroxysteroid oxidoreductase with 16α-(bromo[2′-^{14}C]acetoxy)progesterone.

Experimental Procedures

Reagents. Progesterone, 4-pregnen-20α-ol-3-one, 5α-dihydrotestosterone, and 5α-androstane-3β,17β-diol were purchased from Steraloids, Inc (Wilton, NH). Radioisotopes [4-^{14}C]progesterone (50 mCi/mmol) and 5α-[4-^{14}C]-androstan-17β-ol-3-one (58 mCi/mmol) were purchased from New England Nuclear and purified prior to use by chromatography on silica gel G plates (Eastman Kodak Co. no. 13181; developed with benzene/ethyl acetate, 3:1). Amino acids, Cibacron Blue–agarose, NADPH, calcium chloride, sodium phosphate (tribasic), Sephadex gels, and inorganic chemicals were from Sigma Chemical Co. (St. Louis, MO). Bromoacetic acid and dicyclohexylcarbodiimide were from Aldrich Chemical Co. (Milwaukee, WI). Organic solvents, HPLC-grade solvents, and dialysis tubing (SpectraPor No. 2) were from Fisher Scientific Co. (St. Louis, MO). Bromo[2-^{14}C]acetic acid (sp act. 17 mCi/mmol) was from Pathfinder Laboratories (St. Louis, MO).

Synthesis and NMR Spectrum of 16α-(Bromoacetoxy)-progesterone. 16α-(Bromo[2′-^{14}C]acetoxy)progesterone was synthesized from 4-pregnen-16α-ol-3,20-dione and bromo[2-^{14}C]acetic acid according to our earlier reported procedure (Sweet et al., 1972). The final product was obtained in 70% yield, and the mp and infrared and ultraviolet spectra agreed with those of an authentic, nonradioactive sample of 16α-(bromoacetoxy)progesterone. The 300-MHz nuclear magnetic resonance (NMR) spectrum (Varian XL-300 spectrometer) of 16α-(bromo[2′-^{14}C]acetoxy)progesterone supports its earlier assigned molecular structure ($CDCl_3$ solution with tetramethylsilane internal standard; chemical shifts are reported in ppm): δ 5.75 (s, 1 H, H-4), 5.59 (t, 1 H, H-16β), 3.77 (s, 2 H, BrCH_2COO–), 2.72 (d, 1 H, H-17α), 2.18 (s, 3 H, 21-CH_3), 1.19 (s, 3 H, 19-CH_3), and 0.71 (s, 3 H, 18-CH_3). These data compare with those from the corresponding spectrum of 16α-acetoxyprogesterone (used in control incubations with 3β,20α-hydroxysteroid oxidoreductase during affinity labeling experiments): δ 5.75 (s, 1 H, H-4), 5.50 (t, 1 H, H-16β), 2.68 (d, 1 H, H-17α), 2.17 (s, 3 H, 21-CH_3), 2.01 (s, 3 H, CH_3COO–), 1.19 (s, 3 H, 19-CH_3), and 0.70 (s, 3 H, 18-CH_3).

Synthesis and NMR Spectra of Carboxymethylated Histidines. Synthesis of the reference compounds N^{τ}- and N^{π}-(carboxymethyl)histidine was accomplished by modification of an established method (Crestfield et al., 1963). Treating *N*-acetylhistidine with bromoacetic acid instead of iodoacetic acid provided similar yields and excluded formation of iodine during the workup. Isolation and desalting of *N*-(carboxymethyl)histidine derivatives were performed by the established method. The 300-MHz NMR spectra of the modified histidines in deuterium oxide [referred to sodium 3-(trimethylsilyl)-1-propanesulfonate] support the originally proposed structures for N^{τ}- and N^{π}-(carboxymethyl)histidine. Assignments of chemical shifts are referred to His analogues with established structures (Jones et al., 1987). N^{τ}-(Carboxymethyl)histidine NMR: δ 8.56 (s, 1 H, H-2), 7.25 (s, 1 H, H-5), 3.84 (t, J = 6 Hz, 1 H, H-α), 3.09 (d, J = 6 Hz, 2 H, H-β), and 1.85 (s, 2 H, N–CH_2COO). N^{π}-(Carboxymethyl)histidine NMR: δ 8.45 (s, 1 H, H-2), 7.18 (s, 1 H, H-5), 3.82 (dd, J = 5 and 8 Hz, 1 H, H-α), 3.19 (dd, J = 5 and 16 Hz, 1 H, H-β), 3.07 (dd, J = 8 and 16 Hz, 1 H, H-β′), and –0.05 (s, 2 H, N–CH_2COO). The elution order from the amino acid analyzer (below) of *N*-(carboxymethyl)-modified L-histidine is as follows: His(τ,π-diCM) (before Asp); His(π-CM) [after Cys(S-CM) and Ser and before Pro]; His(τ-CM) (after Gly, on Ala, and before Cys); followed by Phe and L-His.

Purification of Fetal Lamb 3β,20α-Hydroxysteroid Oxidoreductase. Fetal lamb blood was collected by heart puncture or from the jugular vein of the fetuses through hysterotomy of late-term (>100 days) pregnant ewes (under general anesthesia). The erythrocytes were washed twice with two volumes of cold 0.9% NaCl solution per volume of erythrocytes, centrifuged at 1500 rpm for 15 min, and then stored at −20 °C. Thawed cells were mixed with one volume of phosphate buffer (10 mM, pH 7.2) and centrifuged at 15000 rpm for 30 min, and the supernatant was subjected to ammonium sulfate fractionation (30%–60% saturation). The 30%–60% fraction (most of the enzyme plus large quantities of hemoglobin) was dialyzed against distilled water at room temperature. The retentate was lyophilized and then stored at −20 °C. The lyophilized material was resuspended in phospate buffer and purified according to the scheme summarized in Table I. Most of the hemoglobin was separated by chromatography in a column of Q-Sepharose (Figure 1). The 3β,20α-hydroxysteroid oxidoreductase recovered from the Q-Sepharose was further purified by affinity chromatography (Figure 2). Further purification of 3β,20α-hydroxysteroid oxidoreductase from the Cibacron Blue–agarose affinity chromatography step was accomplished with fast protein liquid chromatography (FPLC).

3α,20α-Hydroxysteroid oxidoreductase from affinity chromatography was dialyzed at 4 °C against 10 mM phosphate buffer, pH 7.0 (containing 0.5 mM 2-mercaptoethanol), concentrated to one-tenth the original volume in a dialysis bag packed in poly(ethylene glycol), and dialyzed against 10 mM phosphate buffer (containing 10% glycerol), pH 7.0. The retentate was injected into a Pharmacia FPLC system containing a Mono-Q column (conditioned with 2 N NaOH/2 N NaCl and then equilibrated with the above 10% glycerol/phosphate buffer). During chromatography, the column was eluted with a 0.0–600 mM NaCl gradient in 10% glycerol/phosphate buffer at a flow rate of 0.5 mL/min (under a pressure of 20 mPa). The major fractions of 3β,20α-hydroxysteroid oxidoreductase (eluting at approximately 150 mM NaCl) were pooled, concentrated in a dialysis bag [packed in poly(ethylene glycol)], and then stored at −20 °C.

Amino Acid Analysis. Acid hydrolysis of protein samples was conducted with constant-boiling 6 N HCl at 110 °C for 24 h in evacuated and sealed glass ampules. Determinations of cysteine and methionine were carried out by first oxidizing the protein with performic acid and then conducting amino acid analyses after the sample had been heated at 110 °C for 24, 48, or 72 h. Amino acid hydrolysates were analyzed in a Beckman (Fullerton, CA) Model 6300 amino acid analyzer interfaced with a Model 7000 data system. Beckman reagents and recommended conditions were employed for the analyses. The effluent was collected in 0.4-mL fractions. The amino acid composition of fetal lamb 3β,20α-hydroxysteroid oxidoreductase is shown in Table II.

Measuring 3β-Reductase Activity by a ^{14}C Radioisotope/TLC Method. Enzyme assays were conducted in du-

plicate or triplicate by carrying out incubations in 10 × 75 mm test tubes containing 1.0-mL solutions (0.1 M potassium phosphate buffer, pH 6.0; 25 °C) of 1.0 mM NADPH, 5α-[^{14}C]androstan-17β-ol-3-one (100 nmol; 100 000 cpm), and enzyme. The mixtures were incubated for 30 min at 37 °C in a Model H 2025-1 Temp-Blok heater (Cole-Parmer Co.) and then extracted with 1.5 mL of diethyl ether ethyl acetate (1:1). The organic extract was transferred to a test tube and concentrated to dryness at 37 °C (block heater) in a stream of dry air. The residue was dissolved in 50 μL of diethyl ether/ethyl acetate (1:1) and applied together with authentic 5α-androstan-17β-ol-3-one and 5α-androstane-3β,17β-diol to a 20 × 20 cm thin-layer chromatography (TLC) sheet of silica gel G. The chromatograms were developed with toluene/ethyl acetate (9:1.5), dried, and visualized with iodine vapor. Spots containing the steroids were removed from the TLC sheet and transferred to 7-mL vials containing 5.0 mL of ACS brand (Amersham Corp.) scintillation cocktail. Then the ^{14}C radioactivity in each sample was measured for 10 min in a Beckman Instruments (Palo Alto, CA) LS-330 liquid scintillation counter. Recovery of radioactivity was 85%. Prior to radioactivity measurement, the silica gel/scintillation cocktail mixture was allowed to stand in the dark at room temperature for at least 5 h to allow complete extraction of 5α-androstane-3β,17β-diol by the solvent. The identity of radioactive steroidal products of reductase activity was confirmed earlier by cocrystallization of the radioactivity with authentic steroids (Chen et al., 1987).

Measuring 20α-Reductase Activity by High-Performance Liquid Chromatography (HPLC). The advantages of HPLC over the ^{14}C isotope method for measuring the conversion of progesterone to 4-pregnen-20α-ol-3-one are greater precision, accuracy, rapidity, reproducibility, and avoidance of radioactive isotopes. 20α-Reductase activity assays were conducted similar to 3β-reductase activity assays (above) except for inclusion in the incubation mixture of radioactive progesterone (100 nmol/mL). The dried steroid residue from the diethyl ether/ethyl acetate extract was dissolved in 200 μL of HPLC-grade acetonitrile (Fisher Scientific Co.; redistilled by us from calcium hydride) and then injected in an HPLC system containing a LiChrosorb RP-18 column (4 × 250 mm). The column was eluted with acetonitrile at a flow rate of 2 mL/min (under 1000–1500 psi of pressure). The steroids were measured by a light absorption detector (240 nm). The molar extinction coefficients (at 240 nm) of progesterone and 4-pregnen-20α-ol-3-one are practically equal. Thus the ratios of the integrated peak areas were taken as the molar ratios of the two steroids. Results from the radioisotopic TLC and from the HPLC assay methods for measuring 20α-reductase activity were found to coincide exactly in the same enzyme preparations. Accordingly, the kinetic experiments involving 20α enzyme activity measurements were conducted by the more rapid HPLC method. Protein concentrations were determined by the measurement of optical density of solutions at 280 nm in a Beckman Model 25 spectrophotometer or by the method of Lowry et al. (1951).

K_i Determinations and Inactivation of 3β,20α-Hydroxysteroid Oxidoreductase with 16α-(Bromo[2′-^{14}C]acetoxy)progesterone. 16α-(Bromoacetoxy)progesterone was tested for reversible inhibition of 3β,20α-hydroxysteroid oxidoreductase. The assay conditions were similar to those employed for measuring 3β-reductase activity by the radioisotope method for 20α-reductase activity by HPLC, described above. To 3β,20α-hydroxysteroid oxidoreductase (1.0 μM) in 1.0-mL solutions (0.1 M potassium phosphate buffer, pH 6.0; 25 °C) containing NADPH (1.0 mM) were added appropriately varying concentrations of progesterone or 5α-[4-^{14}C]-androstan-17β-ol-3-one and 16α-(bromoacetoxy)progesterone. Dixon plots of the kinetic data provided K_i values for 16α-(bromoacetoxy)progesterone of 32 μM for 3β-reductase activity (radioisotope/TLC method) and of 58 μM for 20α-reductase activity (HPLC method).

Kinetic studies on inactivation of the enzyme were carried out with various concentrations of nonradioactive 16α-(bromoacetoxy)progesterone and 3β,20α-hydroxysteroid oxidoreductase (1.0 μM) in 3.00 mL of potassium phosphate buffer (0.1 M, pH 6.0; at 25 °C). The assay conditions were similar to those described above, employing radioisotope/TLC (3β-reductase activity) and HPLC (20α-reductase activity) methods. Control incubations contained the enzyme and concentrations of 16α-(acetoxy)progesterone corresponding to that of 16α-(bromoacetoxy)progesterone in the enzyme inactivating mixtures. To measure changes in the activity of 3β,20α-hydroxysteroid oxidoreductase, assays of 20α-reductase activity were carried out by the HPLC method. Radioactive 16α-(bromo[2′-^{14}C]acetoxy)progesterone (100 μM; by adding 250 μL of a 1.2 mM solution in ethanol) was added to a solution of 3β,20α-hydroxysteroid oxidoreductase (1.0 μM) in 2.75 mL of potassium phosphate buffer (50 mM, pH 7.0) at 25 °C. The control incubation mixture contained 3β,20α-hydroxysteroid oxidoreductase (1.0 μM) and 16α-acetoxyprogesterone (100 μM; by adding 250 μL of a 1.2 mM solution in ethanol) in 3.00 mL of phosphate buffer. After the desired degree of inactivation (i.e., affinity radioalkylation) of the the enzyme had been attained, a 50-fold molar excess of 2-mercaptoethanol was added to the incubation mixture (to arrest the inactivation reaction). Then the resulting mixture was dialyzed with frequent changes of dialyzate until the radioactivity in the dialyzate reached background values. The samples were frozen, lyophilized, and stored at −20 °C.

Purification and Sequencing of ^{14}C-Radiolabeled Tryptic Peptides. The lyophilized, radioactively labeled samples were resuspended in a minimum volume of 0.1 M ammonium bicarbonate buffer (pH 8.0; adjusted to 0.05 N in NaOH). After the samples had been allowed to stand overnight at room temperature (to hydrolyze the steroid ester linkage), guanidine hydrochloride was added (6 M final concentration), the solution was adjusted to pH 8 with 1.0 N NaOH, and the resulting mixture was stirred for 30 min. Sufficient 2-mercaptoethanol was added to provide a 10-fold molar excess of the mercaptide relative to the amount of protein, and the mixture was stirred under nitrogen for 20 min. Iodoacetic acid (10-fold molar excess relative to the 2-mercaptoethanol) and an amount of 1.0 N NaOH equivalent to the acid were added to the mixture, and stirring under nitrogen was resumed at room temperature for 15 min. An excess of 2-mercaptoethanol was added to arrest the reaction, and the resulting mixture was dialyzed against four changes of dialyzate (0.1 M ammonium bicarbonate, pH 8.0; dialyzate/retentate, 10:1 v/v). Trypsin-TPCK (4% w/w) was added to the retentate (modified enzymic protein) during three 4-h intervals. Following the last addition, the samples were allowed to undergo digestion overnight at 37 °C. Then they were frozen and lyophilized, and the residual digest was washed twice with small portions of water to remove any remaining bicarbonate salt.

After the above lyophilized ^{14}C-labeled peptides had been resuspended in 500 μL of 0.05% aqueous trifluoroacetic acid, the mixture was injected in a high-performance liquid chromatograph system containing a LiChrosorb RP-18 column. Peptides were eluted with a gradient of 0.05% trifluoroacetic

Table I: Purification of Fetal Lamb 3β,20α-Hydroxysteroid Oxidoreductase

process	total protein (g)	total enzyme (milliunits)[a]	sp act. (microunits/mg)	recovery per step (%)	purification factor
A.S.[b]	50.0	1100	22		1
Q-Sepharose[c]	0.587	946	1610	86 [14][f]	73 [11][f]
affin chrom[d]	0.085	567	6667	60 [55]	303 [160]
FPLC	0.041	397	9600	70 [80]	436 [320]
				36[e] [6]	

[a] 1 unit = 1 μmol of substrate reduced per min (Nancarrow et al., 1981; Chen et al., 1987). [b] The fraction between 30% and 60% saturation of ammonium sulfate provided most of the crude starting material. [c] Q-Sepharose separated the enzyme from practically all of the hemoglobin. [d] Cibacron Blue–agarose affinity chromatography step. [e] Overall recovery of enzyme. [f] Data from Table I in Chen et al. (1987).

acid/water with a limit buffer of 0.05% trifluoroacetic acid/acetonitrile, at a rate of 0.25% limit buffer. The effluent was monitored at 214 nm while fractions were collected at 0.5-min intervals (1.0 mL/min), and radioactivity was measured in each fraction. Two major ^{14}C-labeled peptides contained 85% of the total radioactivity (Figure 6). The isolated peptides were rechromatographed in the RP-18 column after the starting buffer had been adjusted to pH 7.5 with dilute triethylamine. The recovered ^{14}C-labeled peptides were lyophilized, and the residues were washed with two small volumes of water. Peptides were sequenced on an Applied Biosystems Inc. (San Jose, CA) Model 477A or 470A protein sequencer using automated Edman degradation chemistry. Determinations were run with a precycled program with Polybrene (Abbott Laboratories) and had 85–95% repetitive yields. Each lyophilized peptide was dissolved in 50 μL of methanol and converted to a phenylthiohydantoin (PTH) derivative. Amino acid identification was performed in a Hewlett-Packard 1084B high-performance liquid chromatograph equipped with an Altex/PTH column, eluted with a gradient of sodium acetate (0.3 M) and methanol/acetonitrile (17:3). Aliquots (5–10 μL) were removed for radioactivity measurements. In different experiments, peptides corresponding to B and C (Figure 6) were prepared for hydrolysis with 6 N HCl at 110 °C as described under Amino Acid Analysis.

Results

Purification of 3β,20α-Hydroxysteroid Oxidoreductase. The purification scheme for isolation of 3β,20α-hydroxysteroid oxidoreductase from fetal lamb blood is summarized in Table I. Ammonium sulfate (30%–60% saturation) fractionation of the lysate from red blood cells provided initial purification of the enzyme. The ammonium sulfate fractionated material was dialyzed, and the retentate was chromatographed by salt gradient elution in a Q-Sepharose column that separated the hemoglobin from 3β,20α-hydroxysteroid oxidoreductase (Figure 1). Affinity chromatography (Figure 2) and then finally FPLC chromatography provided homogeneous 3β,20α-hydroxysteroid oxidoreductase, judged as a single protein component by disc gel electrophoresis and analytical FPLC. On the basis of the ammonium sulfate fractionated starting material used, the overall recovery of homogeneous enzyme was 36%. Earlier, a 6% overall recovery of enzyme was obtained when calcium phosphate gel adsorption was used to separate hemoglobin from 3β,20α-hydroxysteroid oxidoreductase (Chen et al., 1987). Samples of homogeneous fetal lamb 3β,20α-hydroxysteroid oxidoreductase treated with 6 N HCl at 110 °c for 24–72 h provided hydrolysates that were subjected to amino acid analysis (Table II).

The 3β- and 20α-reductase activities were found to be in a constant ratio of about 1:1.3 throughout purification of the enzyme. The specific activity (20α-reductase) of the isolated 3β,20α-hydroxysteroid oxidoreductase is 9.6 milliunits/mg, with kinetic parameters as follows: 3β-reductase (5α-dihydrotestosterone), K_m = 74 μM and V_{max} = 1.3 nmol min^{-1} (nmol of enzyme)$^{-1}$; 20α-reductase (progesterone), K_m = 30.8 μM and V_{max} = 0.7 nmol min^{-1} (nmol of enzyme)$^{-1}$. 5α-Dihydrotestosterone is a competitive inhibitor of 20α-reductase activity (by the HPLC assay) with a K_i value of 102 μM. Competitive inhibition data were derived from Dixon plots of

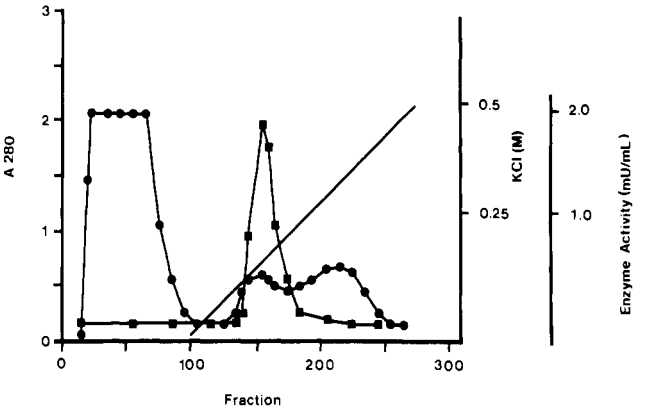

FIGURE 1: Purification of fetal lamb 3β,20α-hydroxysteroid oxidoreductase by ion-exchange chromatography with Q-Sepharose. After the sample with phosphate buffer (10 mM, pH 7.2) had been loaded, the column was washed with the same phosphate buffer until the bulk of protein (hemoglobin) was eluted. After fraction 100, the column was eluted with a linear gradient of 0–0.5 M KCl in the same phosphate buffer. Illustrated are the profiles of elution of protein measured by optical absorbance at 280 nm (●), enzyme activity (■), and a linear gradient of ionic strength (—).

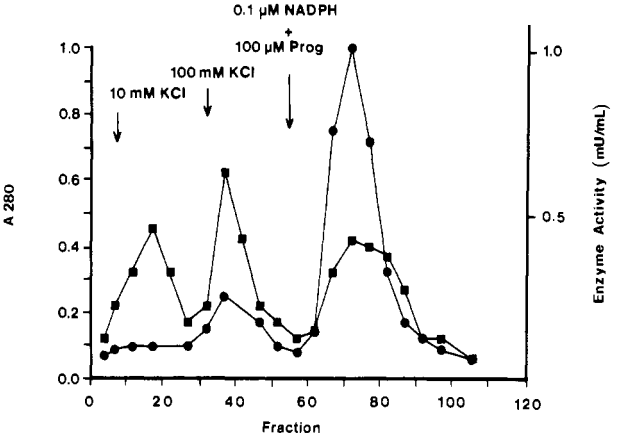

FIGURE 2: Purification of fetal lamb 3β,20α-hydroxysteroid oxidoreductase by affinity chromatography with Cibacron Blue–agarose. The material from chromatography with Q-Sepharose (Figure 1) was further purified by affinity chromatography with Cibacron Blue–agarose. A typical elution profile is illustrated with plots of optical absorbance of protein measured at 280 nm (■) and enzyme activity (●) emerging from the column. The vertical arrows indicate elutions of the chromatographic column with various concentrations of KCl solutions. A mixture of NADPH (1.0 mM) and progesterone (100 μM) in phosphate buffer (10 mM containing 5% ethanol, pH 7.2) produced elution of 3β,20α-hydroxysteroid oxidoreductase.

Table II: Amino Acid Composition of Fetal Lamb 3β,20α-Hydroxysteroid Oxidoreductase

amino acid	no. of residues[a]	amino acid	no. of residues[a]
Asx	30	Met[b]	3
Thr	18	Ile	6
Ser	22	Leu	24
Glx	36	Tyr	5
Pro	19	Phe	10
Gly	112	Lys	14
Ala	23	His	13
Cys[b]	4	Arg	15
Val	27		

[a] Number of each amino acid residue per mole of enzyme protein from analysis of the 6 N HCl digest from duplicate analyses. [b] From performic acid oxidized samples.

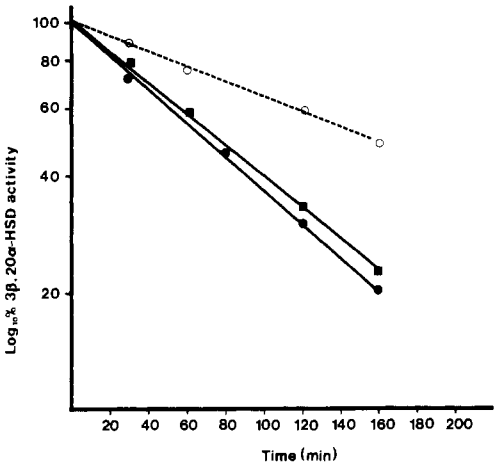

FIGURE 3: Inactivation by 16α-(bromoacetoxy)progesterone of fetal lamb 3β,20α-hydroxysteroid oxidoreductase and protection of the enzyme by its substrates. 3β,20α-Hydroxysteroid oxidoreductase (1.0 μM) was incubated with 16α-(bromoacetoxy)progesterone (100 μM) in 3.00 mL of potassium phosphate buffer (5% ethanol; pH 7.0; 25 °C). Control incubations contained 16α-acetoxyprogesterone (100 μM) instead of the corresponding 16α-(bromoacetate). Plots of the $\log_{10}$ (% of 3β-reductase activity) (●) and $\log_{10}$ (% of 20α-reductase) (■) activity versus time provided the tracings shown. Both 3β- and 20α-reductase activities disappeared at practically equal rates. Repeating the reaction in the presence of progesterone (30 μM) provided a tracing (○) in which the slope (rate of enzyme inactivation) was reduced by half; 5α-dihydrotestosterone (100 μM) similarly caused the slope due to enzyme inactivation to be reduced by half (not shown).

20α-reductase velocities as a function of the concentrations of 5α-dihydrotestosterone and progesterone when the two steroids, NADPH, and 3β,20α-hydroxysteroid oxidoreductase were coincubated. Similarly, 16α-(bromoacetoxy)progesterone is a competitive inhibitor of both 3β-reductase (K_i = 32 μM; ^{14}C isotopic/TLC assay) and 20α-reductase (K_i = 58 μM; HPLC assay) activities of 3β,20α-hydroxysteroid oxidoreductase.

Kinetics and Stoichiometry of Enzyme Inactivation and Amino Acids Modified with 16α-(Bromo[2′-^{14}C]acetoxy)-progesterone. Measurements of the 20α-reductase activity (HPLC method or 3β-reductase by isotope/TLC method) in an incubation mixture of 3β,20α-hydroxysteroid oxidoreductase (1.0 μM) and nonradioactive 16α-(bromoacetoxy)progesterone (100 μM) revealed that both 3β- and 20α-reductase activities were lost in a time-dependent and irreversible manner by pseudo-first-order kinetics (Figures 3–5). Adding progesterone (30 μM) or 5α-dihydrotestosterone (100 μM) to the enzyme inactivating mixture partially protected 3β,20α-hydroxysteroid oxidoreductase against loss of its two activities, evidenced by increases in the $t_{1/2}$ values from 75 min (unprotected, k_{app} = 0.0092 min^{-1}) to 155 min (protected, k_{app} = 0.0045 min^{-1}). Thus the presence of 5α-dihydrotestosterone

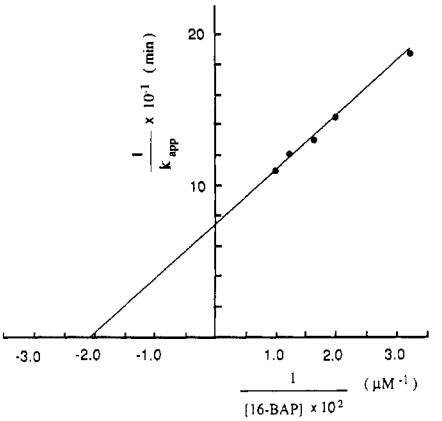

FIGURE 4: Dependence of the pseudo-first-order rate constant from inactivation of fetal lamb 3β,20α-hydroxysteroid oxidoreductase on the concentration of 16α-(bromoacetoxy)progesterone. 3β,20α-Hydroxysteroid oxidoreductase was incubated with various concentrations (30–120 μM) of 16α-(bromoacetoxy)progesterone (16-BAP) as described in the legend to Figure 3. Extrapolation of a double-reciprocal plot (Kitz & Wilson, 1962) of the pseudo-first-order rate constants ($1/k_{app}$) versus the inhibitor concentration (1/[16-BAP]) provided values for K_i (48 μM) and for the limiting inactivation rate constant, k_{inact} (0.014 min^{-1}).

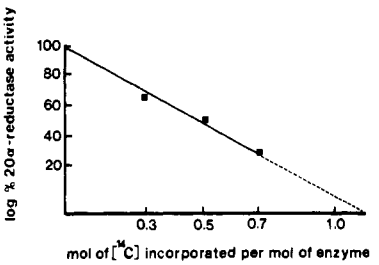

FIGURE 5: Stoichiometry of enzyme inactivation with 16α-(bromo-[2′-^{14}C]acetoxy)progesterone and fetal lamb 3β,20α-hydroxysteroid oxidoreductase. Inactivation of 3β,20α-hydroxysteroid oxidoreductase with 16α-(bromo[2′-^{14}C]acetoxy)progesterone (17 mCi/mmol; conditions specified in Figure 3) was periodically measured, and the ^{14}C radioactivity that became covalently attached to the enzyme (i.e., not dialyzed) was measured. A plot of enzyme activity versus incorporation of ^{14}C radioactivity provided the tracing shown. Extrapolating 2α-reductase activity to 0% indicates that 1 mol of 16α-(bromo[2′-^{14}C]acetoxy)progesterone completely inactivates 1 mol of 3β,20α-hydroxysteroid oxidoreductase. Similar results (not shown) are obtained for loss of 3β-reductase activity versus incorporation of ^{14}C radioactivity.

in the enzyme protection experiment at a concentration equal to its K_i value for competitive inhibition of 20α-reductase reduced the apparent pseudo-first-order rate constant by half. Finally, plotting variations in the rates of inactivation of 3β,20α-hydroxysteroid oxidoreductase versus varying concentrations (30–100 μM) of 16α-(bromoacetoxy)progesterone indicated that enzyme inactivation involves a saturation process (Figure 4). Extrapolation of a double-reciprocal plot (Kitz & Wilson, 1962) of the data provided a limiting rate constant for enzyme inactivation (k_{inact} = 0.014 min^{-1}) and a K_i value (48 μM) consistent with those from experiments in which 16α-(bromoacetoxy)progesterone acts as a competitive inhibitor of 3β- and 20α-reductase activities.

16α-(Bromo[2′-^{14}C]acetoxy)progesterone was incubated with 3β,20α-hydroxysteroid oxidoreductase under conditions that provided inactivation of the enzyme (Figure 3) and produced the results summarized in Figure 5. Incorporation of ^{14}C radioactivity in the enzyme plotted as a function of loss of 3β- and 20α-reductase activities revealed that 1 mol of 16α-(bromo[2′-^{14}C]acetoxy)progesterone completely inactivates 1 mol of 3β,20α-hydroxysteroid oxidoreductase. Samples

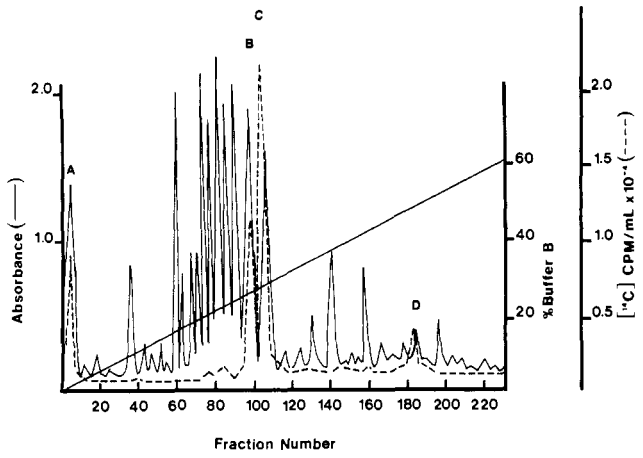

FIGURE 6: Isolation of ^{14}C-labeled peptides by HPLC from 3β,20α-hydroxysteroid oxidoreductase that was affinity radioalkylated with 16α-(bromo[2′-^{14}C]acetoxy)progesterone. Details of ^{14}C-labeled peptide isolation are described under Experimental Procedures. The major peptides (B and C) contained 85% of the total radioactivity. The amino acid sequence of the peptides is listed in Table III.

of the ^{14}C-labeled 3β,20α-hydroxysteroid oxidoreductase were hydrolyzed with 6 N HCl at 110 °C and then subjected to amino acid analysis. The ^{14}C-modified amino acids, constituting more than 80% of the total radioactivity in the hydrolysate, were identified in an amino acid analyzer as His(π-CM) and His(τ-CM) by their comigration with the authentic materials (Experimental Procedures). More than 75% of the total radioactivity in the CM-modified histidines comigrated with His(π-CM).

Isolation and Amino Acid Sequence of ^{14}C-Labeled Peptides from Affinity Radioalkylation of 3β,20α-Hydroxysteroid Oxidoreductase. Following trypsin digestion of 3β,20α-hydroxysteroid oxidoreductase that was affinity radioalkylated with 16α-(bromo[2′-^{14}C]acetoxy)progesterone, the ^{14}C-labeled peptides were separated by HPLC (Figure 6). The two peptides in fractions 87 and 101 (B and C, Figure 6) constituted 85% of the radioactivity. Each of these fractions was rechromatographed by HPLC prior to amino acid sequence analysis. The pregradient, minor fraction (A) could not be resolved by subsequent chromatography, and the low level of radioactivity in the last fraction (D) prevented its detection after rechromatography. Following the second chromatography (50% recovery), each major peptide was subjected to amino acid sequence analysis. After each cycle of the amino acid sequenator, the exiting amino acid was collected, and the radioactivity was measured. In different experiments, peptides corresponding to B and C (Figure 6) were prepared for hydrolysis with 6 N HCl at 110 °C. The radioactivity from these samples comigrated with standard His(π-CM) [no radioactivity was found in the His(τ-CM) fraction]. The amino acid sequence of the ^{14}C-labeled peptides is shown in Table III.

Table III: Amino Acid Sequence Analysis of ^{14}C-Labeled Tryptic Peptides[a]

	amino acid HPLC peptide[b]			amino acid HPLC peptide[b]	
cycle	B	C	cycle	B	C
1	Tyr(N)	Tyr	11	Asx	Asx
2	Val	Val	12	[His(π-CM)][c]	[His(π-CM)]
3	Ala	Ala	13	Pro	Pro
4	Val	Val	14	Leu	Leu
5	Met	Met	15	Thr	Thr
6	Pro	Pro	16	ND[d]	Gly
7	Pro	Pro	17		Ala
8	Pro	Pro	18		Tyr
9	Ile	Ile	19		Tyr
10	Gly	Gly	20		ND

[a] Approximately 200 pmol of peptide was sequenced. [b] Refers to Figure 6. [c] Radioactivity found in the sequence cycle, identified by amino acid analysis of 6 N HCl hydrolysate from a sample of the peptide. [d] ND, not determined due to peptide washout.

Discussion

Enzyme recovery problems accompanied initial attempts to isolate 3β,20α-hydroxysteroid oxidoreductase from fetal lamb blood. Isolating 3β,20α-hydroxysteroid oxidoreductases from blood requires its separation from copious amounts of hemoglobin, constituting about 80% of the protein in erythrocytes (Nancarrow et al., 1981b). Separation of bovine 3β,20α-hydroxysteroid oxidoreductase from hemoglobin was first attempted by adsorbing the enzyme on calcium phosphate gel. This was the only method known to provide *any* separation of hemoglobin from an enzyme when fetal calf 3β,20α-hydroxysteroid oxidoreductase was isolated from fetal bovine blood (Sharaf & Sweet, 1982). During its recovery from calcium phosphate gel, losses of 80% of the fetal lamb 3β,20α-hydroxysteroid oxidoreductase were rather typical (Chen et al., 1987). However, a 6-fold increase in yield was achieved (Table I) by chromatographing fetal lamb 3β,20α-hydroxysteroid oxidoreductase in a column of Q-Sepharose (Figure 1) instead of applying the calcium phosphate gel adsorption step.

Affinity chromatography with Cibacron Blue–agarose (Figure 2) followed by preparative FPLC provided good yields of homogeneous 3β,20α-hydroxysteroid oxidoreductase. Enzymic protein from the final FPLC step was judged to be homogeneous by analytical FPLC, disc gel electrophoresis, and terminal amino acid sequence analysis. Fetal lamb 3β,20α-hydroxysteroid oxidoreductase is a single-stranded protein of M_r 35 000 possessing both 3β- and 20α-reductase activities. Kinetic characteristics of 3β,20α-hydroxysteroid oxidoreductase with the substrates 5α-dihydrotestosterone (3β-reductase) or progesterone (20α-reductase) are summarized under Results. 5α-Dihydrotestosterone acts as a competitive inhibitor when progesterone is the substrate for 20α-reductase activity, suggesting that 3β- and 20α-reductase activities may share the same active site.

To fix the origin(s) of 3β- and 20α-reductase activities, a series of affinity labeling experiments was performed with 16α-(bromoacetoxy)progesterone and its radioactive bromo-[2′-^{14}C]acetate analogue. 16α-(Bromoacetoxy)progesterone caused time-dependent, irreversible, and simultaneous loss of 3β- and 20α-reductase activities of fetal lamb 3β,20α-hydroxysteroid oxidoreductase (Figure 3). The present enzyme inactivation kinetics and other characteristics associated with loss of enzyme activity conform to established criteria of affinity labeling (Sweet & Murdock, 1987): (1) 16α-(bromoacetoxy)progesterone is a competitive inhibitor of 3β- and 20α-reductase activities of 3β,20α-hydroxysteroid oxidoreductase, and it therefore binds to the enzyme's catalytically active site; (2) 16α-(bromoacetoxy)progesterone causes losses of both 3β- and 20α-reductase activities (Figure 3) by an active site saturation process (Figure 4); (3) loss of both reductase activities can be instantly arrested by addition of 2-mercaptoethanol (viz. reaction with the bromoacetoxy group); (4) bromoacetic acid or ethyl bromoacetate does not cause loss of enzyme activity; (5) the natural substrates protect the enzyme against inactivation, reducing the rate of enzyme inactivation by competing against 16α-(bromoacetoxy)progesterone; (6) the stoichiometry of enzyme inactivation is a small whole number [i.e., 1 mol of 16α-(bromo[2′-^{14}C]acetoxy)-

Table IV: Rates of Enzyme Inactivation with Isomers of (Bromoacetoxy)progesterone

configuration of bromoacetoxy	k_{app} (min^{-1})[a]	modified amino acid	ref
6β	0.01	HS-Cys	Arias et al., 1973
6β	0.02	HS-Cys	Sweet, 1976
17	0.02[b]	HS-Cys	Onoda et al., 1987
16α	0.004	*N*-imid-His	Sweet et al., 1972, 1978
21	0.004[c]	*N*-imid-His	Ganguly & Warren, 1971
2α	0.002	MeS-Met	Strickler et al., 1975
11α	0.001	MeS-Met	Strickler et al., 1975
17	0.0005	HOOC-R[d]	Sweet & Samant, 1980b

[a] Apparent pseudo-first-order rate constants (k_{app}) were calculated from corresponding $t_{1/2}$ values for inactivation of bacterial 3α,20β-hydroxysteroid dehydrogenase (or as indicated) under similar conditions of 0.05 M phosphate buffer (pH 7.0; 4%–5% ethanol) at 25 °C, with the (bromoacetoxy)progesterone at initial concentrations of 30–60 μM. [b] 17-(Bromoacetoxy)progesterone- (30 μM; 5 μM, k_{app} = 0.004 min^{-1}) inactivated neonatal porcine cytochrome P-450. [c] For cortisol 21-(iodoacetate) (60 μM), 21-(bromoacetoxy)progesterone gave similar results (Sweet & Warren, 1972). [d] The bromo[2′-^{3}H]acetoxy group formed a second ester linkage (R = Glu or Asp).

progesterone completely inactivates 1 mol of 3β,20α-hydroxysteroid oxidoreductase (Figure 5)]; (7) the stoichiometry of inactivation agrees with the isolated, major radioactive peptide (Figure 6) containing a single modified amino acid (i.e., His, Table III).

Modified histidine was identified in the hydrolysate from the ^{14}C-labeled enzyme that was treated with 6 N HCl. Similarly, His(π-CM) was identified in the amino acid sequence of the ^{14}C-labeled peptides from the affinity radioalkylated fetal lamb 3β,20α-hydroxysteroid oxidoreductase (Table III; Figure 6). Histidine has been modified at the active sites of other steroid oxidoreductases, namely, bacterial 3α,20β-hydroxysteroid dehydrogenase (Sweet et al., 1972, 1978; Sweet & Samant, 1980b; Tanimoto et al., 1982), bacterial 3(17)β-hydroxysteroid dehydrogenase (Levy et al., 1987), and human placental estradiol 17β-dehydrogenase (Inano & Tamaoki, 1983; Murdock et al., 1988). Strong hydrogen bonding between the imidazole nitrogen of an active site histidine and the NH of a steroidal A-ring-fused pyrazole was considered to promote inhibition of 3(17)β-hydroxysteroid dehydrogenase. This hypothesis was further supported by the irreversible inhibition of 3(17)β-hydroxysteroid dehydrogenase with diethyl pyrocarbonate (Levy et al., 1987). Diethyl pyrocarbonate has been reported to inhibit bacterial 3α,20β-hydroxysteroid dehydrogenase by carbethoxylation of histidine at or near the enzyme active site (Tanimoto et al., 1982). The location by X-ray crystallographic analysis of histidine at the catalytic sites of malate dehydrogenase (Birktoft et al., 1982) and lactate dehydrogenase (Parker & Holbrook, 1977) suggests that this amino acid participates in hydrogen transfer between the reactive oxygen on a substrate molecule and the cofactor. In the present work, modification of histidine by affinity labeling fetal lamb 3β,20α-hydroxysteroid oxidoreductase is consistent with the presence of this amino acid at the catalytic sites of oxidoreductases in general and steroid-specific oxidoreductases in particular.

It is interesting that 16α-(bromoacetoxy)progesterone produces similar rates of inactivation with fetal lamb 3β,20α-hydroxysteroid oxidoreductase and bacterial 3α,20β-hydroxysteroid dehydrogenase. Variations in the pseudo-first-order rate constants for inactivation of bacterial 3α,20β-hydroxysteroid dehydrogenase with isomers of (bromoacetoxy)progesterone span 2 orders of magnitude (Table IV). The magnitude of the pseudo-first-order rate constant appears to follow the order of nucleophilic activity (R–SH

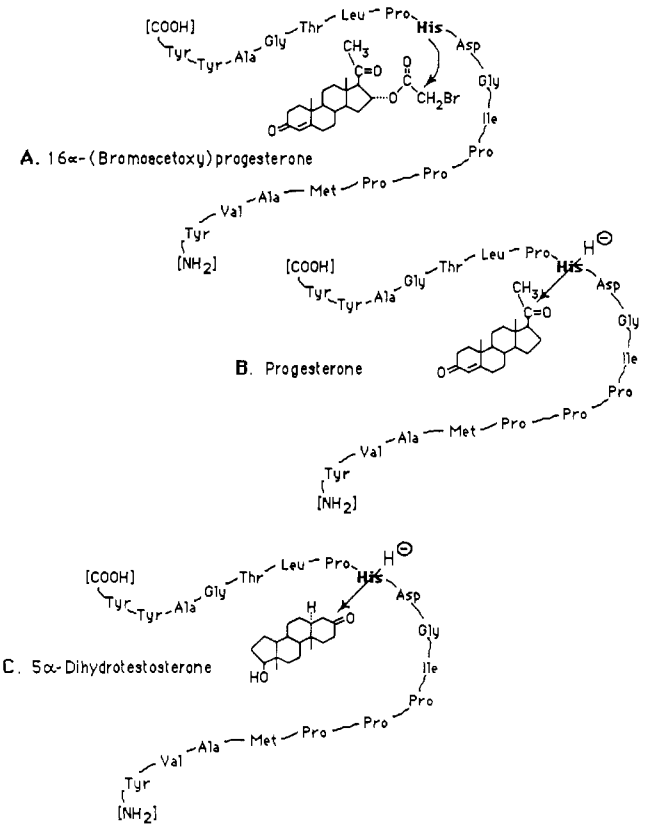

FIGURE 7: Schematic diagrams of affinity alkylation and 3β- or 20α-reductase activity at the active site of fetal lamb 3β,20α-hydroxysteroid oxidoreductase. The amino acid sequence is represented according to the data in Table III. Panel A represents an alkylation reaction between a His residue at the active site and 16α-(bromo-[2′-^{14}C]acetoxy)progesterone. Panels B and C represent 20α-reductase and 3β-reductase activities, respectively.

greatest and R–COOH least; Wells, 1963) of the functional groups on the different amino acids that are modified by the bromoacetoxy reagent group. Fetal lamb 3β,20α-hydroxysteroid oxidoreductase and bacterial 3α,20β-hydroxysteroid dehydrogenase undergo similar rates of inactivation with 16α-(bromoacetoxy)progesterone, and a histidine residue is modified in both enzymes. These results suggest that the steroid-binding regions at the active sites of the two enzymes may have similar structural and topographical features.

Dual activity at the active site of steroid-specific enzymes appears to be a pattern in nature. 3α,20β-Hydroxysteroid oxidoreductase from *Streptomyces hydrogenans* (Sweet & Samant, 1980a,b; Strickler et al., 1980), 3β,20α-hydroxysteroid oxidoreductase from fetal calf blood (Sharaf & Sweet, 1982), and the present 3β,20α-hydroxysteroid oxidoreductase from fetal lamb blood each exhibit *dual* reductase activity at the same active site. Thus the same active site of a bacterial or mammalian hydroxysteroid oxidoreductase can reduce the 3-keto group on the A-ring of 5α-dihydrotestosterone or the 20-keto group of the side chain on the D-ring of progesterone. Two models of steroid binding have been proposed to account for dual activity at the same active site. One of the models requires opposite modes of steroid binding to the same region, adjacent to a cofactor binding region at the same active site (Sweet & Samant, 1980b, 1981).

Two laboratories independently studying different enzymes advanced the hypothesis that steroids can bind in opposite ways to the same enzyme active site (Bevins et al., 1980; Sweet & Samant, 1980b). Pollack and co-workers affinity alkylated Asp-38 at the active site of bacterial 3-oxo-Δ^5-steroid isomerase with steroidal A-ring 3β-oxirane and D-ring 17β-oxirane,

suggesting that the enzyme binds steroids in two opposite orientations (Bevins et al., 1980; Kayser et al., 1983; Bounds & Pollack, 1987). Affinity alkylation experiments with human placental estradiol 17β-dehydrogenase further support the opposite-orientation hypothesis. Both estrone 3-bromoacetate and estradiol 17-bromoacetate react with the same histidine residue of the three that have been identified in the amino acid sequence of peptides from the active site of human placental estradiol 17β-dehydrogenase, suggesting that estradiol 17-bromoacetate binds the "wrong way" to the enzyme relative to natural substrates (Murdock et al., 1988).

The model represented in Figure 7 suggests that steroids can undergo 3β- or 20α-reductase activity by binding in opposite ways to one and the same catalytically active site in fetal lamb 3β,20α-hydroxysteroid oxidoreductase.

Acknowledgments

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