Dual Activity at an Enzyme Active Site: 3β,20α-Hydroxysteroid Oxidoreductase from Fetal Blood†

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ABSTRACT: An enzyme exhibiting both 3β and 20α steroid reductase activities from calf fetal red blood cells was purified to homogeneity as judged by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. 3β,20α-Hydroxysteroid oxidoreductase (3β,20α-HSD) was found to be a single-stranded polypeptide with a molecular weight of 55 000 ± 1000 by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and Sephadex G-100 chromatography. The amino acid composition of 3β,20α-HSD was obtained. 17β-Hydroxy-5α-androstan-3-one and progesterone were substrates for the enzyme's 3β and 20α reductase activities, respectively, which required NADPH for both 3β [K_m = 9.4 μM; V_{max} = 2.4 nmol min^{-1} (nmol of enzyme)$^{-1}$] and 20α [K_m = 2.5 μM; V_{max} = 2.4 nmol min^{-1} (nmol of enzyme)$^{-1}$] reductase activities. 17β-Hydroxy-5α-androstan-3-one competitively inhibited (K_i = 35 μM) 20α reduction of progesterone. Incubating 3β,20α-HSD with 19-nortestosterone 17-bromoacetate at pH 7.0 and 25 °C caused simultaneous, time-dependent, and irreversible losses of 3β and 20α activities by a first-order kinetic process. Similar incubations with either of the 3β or 20α substrates present at concentrations equal to their respective K_m values practically doubled the time required for loss of 3β and 20α enzyme activites. These data lead us to conclude that the active site of 3β,20α-HSD contains 3β and 20α dual activity.

Previous experiments with affinity alkylating substrate analogues of microbial 3α,20β-hydroxysteroid dehydrogenase (3α,20β-HSD)[1] revealed that both 3α and 20β enzyme activities share the same active site (Sweet et al., 1980; Sweet & Samant, 1980a,b, 1981). These findings were independently verified (Strickler et al., 1980). Seeking other examples of dual enzyme activity directed at the 3 and 20 steroid positions, we wished to isolate the NADPH-dependent 20α-hydroxysteroid oxidoreductase which had been earlier detected in calf fetal blood. This 20α-hydroxysteroid oxidoreductase was intriguing, because by mid-pregnancy fetal blood in cattle and sheep contains sufficient activity to potentially convert the *daily* maternal output of the vital hormone progesterone. After parturition, this enzyme activity disappears (Nancarrow & Seamark, 1968; Seamark et al., 1972). A recent communication from our laboratory reported that partially purified hydroxysteroid oxidoreductase from bovine fetal red blood cells contained both 3β and 20α activities (Nancarrow et al., 1981). The present report describes the isolation and characterization of an enzyme from fetal blood with 3β- and 20α-hydroxysteroid oxidoreductase activity and exploration of the 3β and 20α activities by affinity alkylation.

Experimental Procedures

Materials

Progesterone, 20α-hydroxy-4-pregnen-3-one, 17-hydroxy-5α-androstan-3-one, and 5α-androstane-3β,17β-diol were purchased from Steraloids, Inc., Wilton, NH. The commerical steroids were found to be at least 95% pure by thin-layer chromatography. Dinucleotides NADP and NADPH, Sephadex G-25 and G-100, Cibacron blue–agarose, ultrapure ammonium sulfate, poly(ethylene glycol), and the protein standards ovalbumin, bovine serum albumin, phosphorylase *b*, β-galactosidase, myosin, alcohol dehydrogenase, and myoglobin were from Sigma Chemical Co., Inc., St. Louis, MO. Fetal red blood cells were obtained from local slaughterhouses by cardiac puncture. The serum was removed by centrifugation, and the red blood cells were washed several times with isotonic saline and then frozen for storage the day of collection. [4-^{14}C]Progesterone (110 mCi/mmol) and 17β-hydroxy-5α-[4-^{14}C]androstan-3-one (58 mCi/mmol) were purchased from New England Nuclear Corp., Boston, MA. 19-Nortestosterone 17-bromoacetate was synthesized according to a method previously reported by us (Clark et al., 1974). Organic solvents and inorganic salts were from Fisher Scientific Corp., St. Louis, MO.

Methods

Enzyme Assays. Enzyme 20α activity was assayed by adding solutions of the appropriate substrate and cofactor to 0.5 mL of crude (or appropriately diluted purified) enzyme solutions in 0.01 M potassium phosphate buffer, pH 6.0. The solutions added to 0.5 mL of enzyme solution were 1.4 mL of 0.01 M phosphate buffer, pH 6.0, at 25 °C, 0.020 mL of progesterone (10 mM in nonradioactive steroid and 100 000 cpm of ^{14}C-containing steroid) in ethanol, and 0.1 mL of NADPH (0.5 mM) in 0.01 M phosphate buffer, pH 6.0, at 0 °C. The resulting mixture was incubated at 37 ± 0.1 °C (Temp-Blok Model H 2025-1 module heater, Scientific Products) for 30 min. Then 1.5 mL of ether–ethyl acetate (1:1 v/v) was vigorously shaken (Vortex) with the incubation mixture; the organic extract was transferred from the aqueous layer (lower layer) to a test tube and concentrated to dryness (37 °C) in a stream of dry air. The residue was dissolved in 0.060 mL of ether–ethyl acetate (1:1) and spotted on a 20 cm × 20 cm thin-layer chromatography (TLC) sheet of silica gel G containing a fluorescent indicator (Eastman no. 6060). A reference mixture of progesterone and 20α-hydroxy-4-preg-

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[1] Abbreviations: 3α,20β-HSD, 3α,20β-hydroxysteroid dehydrogenase; 3β,20α-HSD, 3β,20α-hydroxysteroid oxidoreductase; NADP(H), nicotinamide adenine dinucleotide 2′-phosphate oxidized (reduced); TLC, thin-layer chromatography; $NaDodSO_4$, sodium dodecyl sulfate; Ca-P gel, calcium phosphate gel; EGME, ethylene glycol monomethyl ether; NTBA, 19-nortestosterone 17-bromoacetate.

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nen-3-one was spotted on the same TLC sheet. The TLC plate was developed with benzene–ethyl acetate (3:1) in a Gelman chamber, dried, and visualized under ultraviolet light (254 nm) to locate the appropriate components. The appropriate spots were cut from the TLC plate and shaken with 10 mL of ACS brand scintillation cocktail (from Amersham Corp.) and then shaken 1 h later and counted for 10 min in a Beckman LS 320 scintillation spectrometer. Enzyme assays for 3β activity were carried out under similar conditions with 17β-hydroxy-5α-androstan-3-one (0.020 mL of a 10 mM ethanol solution containing 100 000 cpm of steroid) instead of progesterone as the substrate, and 5α-androstane-3β,17β-diol as the product reference compound. It was necessary to allow the appropriate spots from the 3β assay (which were transferred from the TLC plate in the scintillation cocktail) to stand overnight prior to counting. 5α-Androstane-3β,17β-diol takes several hours to be extracted by toluene from silica gel G. Unequivocal identification of the C-21 and C-19 steroid products of the 3β and 20α enzyme activities has been previously reported by us (Nancarrow et al., 1981).

Protein concentrations were determined by optical density measurements at 280 nm in a Beckman Model 25 spectrophotometer or by the procedure of Lowry et al. (1951) with bovine serum albumin as a standard.

Enzyme Purification. Details of the preliminary enzyme purification including the first calcium phosphate (Ca-P) gel adsorption (Figure 1) were recently described by us (Nancarrow et al., 1981). The subsequent purification of 3β,20α-HSD (80–90% purity) was accomplished by affinity chromatography on Cibacron blue–agarose with a mixture of NADPH and progesterone during recovery of the enzyme (Figure 2). Polyacrylamide disc gel electrophoresis (7.5% acrylamide) of this 3β,20α-HSD product revealed that it contained 10–20% of a contaminating protein. Ca-P gel adsorption of 3β,20α-HSD and washing the gel with 20 mM phosphate buffer removed the contaminant. The final, homogeneous protein was recovered from the Ca-P gel with 100 mM phosphate buffer and used for amino acid analysis (Table I). The 3β,20α-HSD enzyme from the 40–60% ammonium sulfate fractionation of the Ca-P gel purified preparation was used for molecular weight determination of 3β,20α-HSD by gel filtration and for affinity alkylation. The scheme for purification of 3β,20α-HSD from red blood cells to homogeneity is represented in Figure 1.

Affinity Chromatography and Isolation of 3β,20α-HSD. The 3β,20α-HSD extract from the first Ca-P gel purification was used in the next step. The extract was diluted with 10 mM phosphate buffer, pH 6 (1:1 v/v) (which we shall refer to as the *diluted Ca-P gel extract* below). Sufficient ammonium sulfate was added to the resulting solution to provide 40% saturation at 4 °C; the resulting mixture was centrifuged at 25000*g* for 30 min at 4 °C. The pellet, containing a small amount of enzyme activity, was discarded. The supernatant was decanted, adjusted to 60% ammonium sulfate saturation at 4 °C, allowed to stand for 45 min, and then centrifuged at 25000*g* for 30 min. The 3β,20α-HSD (40–60% ammonium sulfate) pellet was dissolved in a minimum volume of 10 mM phosphate buffer, pH 6.0, and then dialyzed overnight at 4 °C against a large volume of the same buffer. The retentate was slowly percolated through a column (0.9 cm × 50 cm) containing Cibacron blue–agarose (15 mL) which had been previously equilibrated with 10 mM phosphate buffer, pH 6.0. Then the enzyme–Cibacron blue–agarose was transferred to a beaker containing a magnetic stirrer and sequentially washed at 4 °C in a batchwise manner. The blue–agarose was washed 3 times with volumes (each equal to the volume of the *diluted Ca-P gel extract*) of potassium phosphate buffers, pH 6.0, containing in ascending salinity (a) 0.2 M phosphate (0.1 M NaCl), (b) 0.4 M phosphate (0.2 M NaCl), (c) 0.6 M phosphate (0.3 M NaCl), and (d) 0.8 M phosphate (0.4 M NaCl). Each wash was conducted with continuous stirring for 20 min. The resulting mixture was centrifuged for 5 min at 1500 rpm to separate the washings from the enzyme–Cibacron blue–agarose. These batchwise phosphate buffer–NaCl washes, as specified, were critical for efficient removal of extraneous protein. 3β,20α-HSD was recovered from the Cibacron blue–agarose matrix by stirring it with 3 volumes (each equal to one-third the volume of the *diluted Ca-P extract*) of a mixture of 2 mM potassium phosphate buffer, pH 6.0, containing progesterone (20 mM in ethanol, 0.050 mL/10 mL of buffer), NADPH (0.050 mM), and 2-mercaptoethanol (0.05 mM). The combined extracts were dialyzed overnight at 4 °C against 10 mM potassium phosphate buffer, pH 6.0, containing 0.5 mM 2-mercaptoethanol. The retentate was adsorbed on a volume of Ca-P gel equal to half the volume of the *diluted Ca-P gel extract*. The Ca-P gel was washed 3 times with 20 mM potassium phosphate buffer, pH 6.0. Then the homogeneous 3β,20α-HSD was extracted from the Ca-P gel by stirring it for 30 min with 3 volumes (each equal to half the volume of the diluted Ca-P extract) of 100 mM potassium phosphate buffer, pH 6.0 (containing 50 mM NaCl). The extracts were separated from the Ca-P gel by centrifugation at 1500 rpm for 5 min at 4 °C. The combined extracts were concentrated by placing them in a dialysis bag and packing it in solid poly(ethylene glycol) at 4 °C. The final product was a protein with both 3β and 20α reductase enzyme activities which migrated as a single band both on 7.5% polyacrylamide gel electrophoresis (panel A, Figure 3) and with sodium dodecyl sulfate ($NaDodSO_4$–polyacrylamide gel electrophoresis) (panel B, Figure 3).

Molecular Weight Subunit Determination and Amino Acid Composition of 3β,20α-HSD. Subunit and molecular weight determination of the homogeneous 3β,20α-HSD was obtained by $NaDodSO_4$–polyacrylamide gel electrophoresis (7.5% polyacrylamide) according to a method by Laemmli (1970) using a mixture of homogeneous 3β,20α-HSD heated at 90 °C for 5 min with 3% sodium dodecyl sulfate and 0.5 mM 2-mercaptoethanol and calibrated against a mixture containing 1 mg each of the standards ovalbumin (M_r 45 000), bovine serum albumin (M_r 66 200), phosphorylase *b* (M_r 97 400), β-galactosidase (M_r 116 250), and myosin (M_r 200 000). A single band due to 3β,20α-HSD (stained with Coomassie brilliant blue) migrated at a position coinciding with M_r 50 000–55 000. The molecular weight of native 3β,20α-HSD was determined by gel filtration on a column of Sephadex G-100 (0.9 cm × 90 cm) eluted with 0.1 M phosphate buffer, pH 6.0, containing 0.5 M sodium chloride, and at 25 °C. The fractionated eluate was assayed for protein content and 20α reductase enzyme activity. Construction of a calibration curve with alcohol dehydrogenase (M_r 80 000), ovalbumin (M_r 45 000), and myoglobin (M_r 18 000) showed that 3β,20α-HSD activity emerged from the Sephadex G-100 column in a single region coinciding with a molecular weight of 55 000. This value agreed with that from $NaDodSO_4$–polyacrylamide gel electrophoresis analysis of 3β,20α-HSD. These experiments were each repeated 3 times. Thus, 3β,20α-HSD is a single-stranded polypeptide with a molecular weight of approximately 55 000. Homogeneous 3β,20α-HSD (0.1 mg) was heated with 6 N HCl in a sealed tube at 110 °C for 24 h. The amino acid digest was concentrated in vacuo to dryness, the residue was

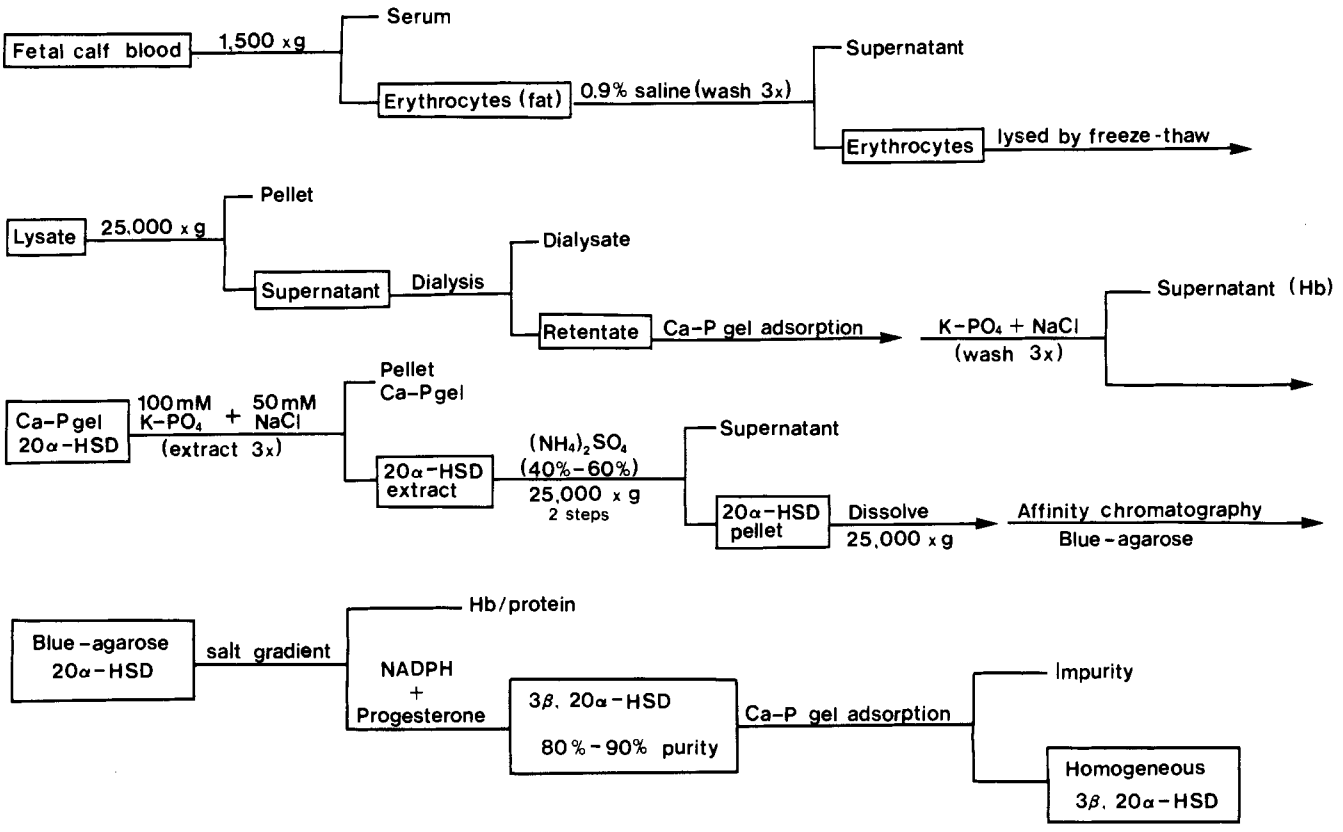

FIGURE 1: Isolation of 3β,20α-hydroxysteroid oxidoreductase (3β,20α-HSD). The schematic flow diagram represents the sequence of steps used for isolation of 3β,20α-HSD from bovine fetal red blood cells. The entire process can be carried out within 2 weeks. Abbreviations: 20α-HSD, 3β,20α-HSD; Ca-P, calcium phosphate; Hb, hemoglobin; blue–agarose, Cibacron blue–agarose. The process can be interrupted after any step and the 3β,20α-HSD mixture frozen (−20 °C). The frozen enzyme is stable indefinitely. The purification process is described under Methods.

dissolved in 1.0 mL of sodium citrate buffer, and 0.05-mL aliquots were placed in an amino acid analyzer. The amino acid composition calculated for an M_r 55 000 polypeptide is summarized in Table I.

Affinity Alkylation of 3β,20α-HSD with 19-Nortestosterone 17-Bromoacetate (NTBA). To a solution of 3β,20α-HSD (0.1 mg) in 7.75 mL of 0.05 M potassium phosphate buffer, pH 6.0, at 25 °C was added 2.25 mL from a solution of NTBA (12 mM) in ethylene glycol monomethyl ether (EGME), which provided a final concentration of 0.15 mM NTBA. The control was prepared from 7.75 mL of a solution containing 3β,20α-HSD (0.1 mg) to which was added 2.25 mL of EGME containing 19-nortestosterone 17-acetate (12 mM). Every half hour or hour, two 0.5-mL aliquots were withdrawn from the reaction and control mixtures and assayed for 20α enzyme activity. In separate experiments, similar incubation mixtures of 3β,20α-HSD were assayed for 3β activity. Assays for 20α activity were conducted by adding to a 0.5-mL aliquot from the incubation solution 0.5 mL of a solution containing 22.5 mg of NADPH, 0.019 mL of 2-mercaptoethanol, and 0.5 mL of a 10 mM solution of progesterone (containing 100 000 cpm of [^{14}C]progesterone) in ethanol added to 19.5 mL of 0.1 M phosphate buffer, pH 6.0 (kept chilled). The resulting assay mixture was incubated at 37 °C for 30 min and then processed further as an assay for 20α enzyme activity, described under *Enzyme Assays* above. Affinity alkylation experiments in which 3β activity was measured were similarly conducted, except that an equivalent concentration of 17β-hydroxy-5α-androstanone was used as the substrate instead of progesterone in the assay mixtures (carried out as described under *Enzyme Assays* above). Incubations of 3β,20α-HSD with NTBA (0.15 mM) under conditions similar to those described above, were conducted with either progesterone (2.5 μM) or 17β-hydroxy-5α-androstan-3-one (9.4 μM) in the incubation mixtures. The results from the affinity alkylation experiments are represented in Figure 4.

Results

Purification and Isolation of 3β,20α-Hydroxysteroid Oxidoreductase (3β,20α-HSD). Isolation of 3β,20α-HSD from bovine fetal red blood cells is schematically represented in Figure 1. Beginning with 250 mL of packed red blood cells, we isolated 1–2 mg of homogeneous enzyme. The entire process can be conducted within 2–3 weeks. During purification, the intermediate preparations of 3β,20α-HSD in 0.1 M phosphate buffer, pH 6.0, were frozen for storage at −20 °C. After these preparations were thawed, they were found to retain the original enzyme activity, even after months of storage. Lyophilization of similar preparations led to complete loss of enzyme activity in a few days. However, 3β,20α-HSD, which was precipitated with a 40–60% saturation of ammonium sulfate (4 °C) and then frozen, retained the original enzyme activity.

Affinity Chromatography. The first Ca-P gel adsorption step was found to separate 3β,20α-HSD from practically all of the copious amounts of hemoglobin, the major component in the crude mixture derived from the lysed fetal calf red blood cells. The 3β,20α-HSD extracted from the Ca-P gel was found to be stable indefinitely during storage at −20 °C. This material was used for affinity chromatography. Affinity chromatography with Cibacron blue–agarose provided an efficient means of obtaining 3β,20α-HSD in 80–90% purity (Figure 2). During the batchwise, salt gradient washing of the enzyme–Cibacron blue–agarose complex, very little release of 3β,20α-HSD activity from the blue matrix was detected.

Recovery of 3β,20α-HSD from the blue matrix by extrac-

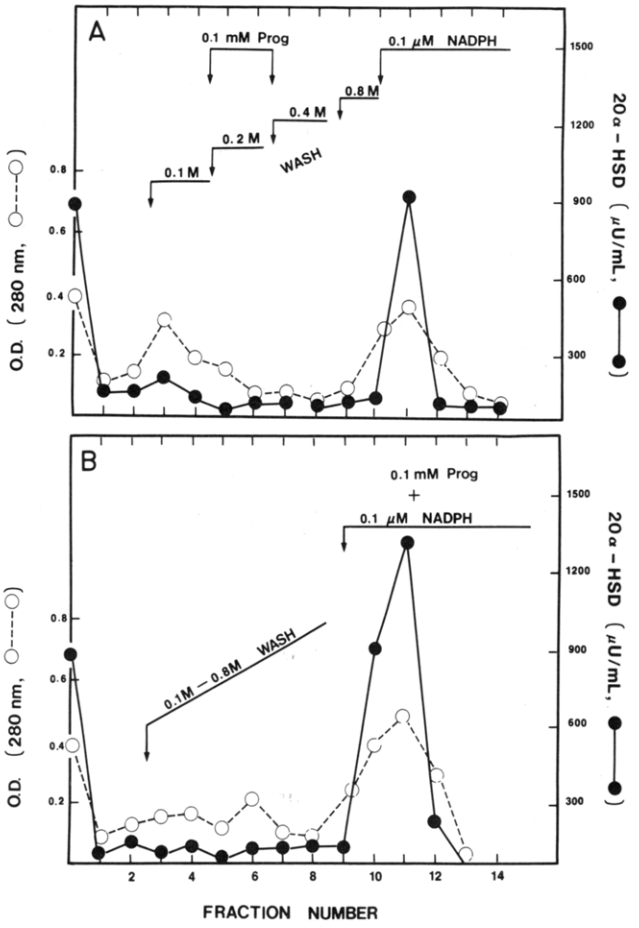

FIGURE 2: Affinity chromatography of 3β,20α-hydroxysteroid oxidoreductase with Cibacron blue–agarose. Panels A and B represent typical results from batchwise processing of 3β,20α-HSD–Cibacron blue–agarose after application of the partially purified enzyme from the Ca-P gel and 40–60% ammonium sulfate fractionation steps (Figure 1). Panel A includes results from recovery with 0.1 μM NADPH of 28% of the bound 3β,20α-HSD after a final, 0.8 M salt wash and also the results of an unsuccessful attempt to remove 3β,20α-HSD with 0.1 mM progesterone during the batchwise wash procedure. Panel B shows results following adsorption of 3β,20α-HSD in an amount equal to that of panel A. Following the salt gradient wash, over 60% of the 3β,20α-HSD (80–90% purity) was recovered by batchwise extraction with buffer containing *both* NADPH (0.1 μM) and progesterone (0.1 mM). Each of the experiments was repeated at least 3 times. Experimental details are given under Methods.

tion with NADPH (0.1 μM) containing buffers removed less than half of the matrix-bound enzyme compared with the extraction carried out with an NADPH (0.1 μM) and progesterone (0.1 mM) mixture (represented in Figure 2). Evidently, progesterone enhances the binding of NADPH to the active site of 3β,20α-HSD and shifts the binding equilibrium of the enzyme from the immobilized Cibacron blue due to NADPH in the buffered solution. The batchwise washing and enzyme recovery process was found to be superior to the corresponding column process. The batchwise process was more convenient, more rapid, more efficient, and more reproducible than the column method. Sodium dodecyl sulfate–polyacrylamide disc gel electrophoresis revealed that the 3β,20α-HSD obtained after Cibacron blue–agarose affinity chromatography contained 10–20% of a protein impurity. The impurity was removed by a final Ca-P gel adsorption purification step which provided homogeneous 3β,20α-HSD, evidenced by its single band on $NaDodSO_4$–polyacrylamide gel electrophoresis analysis (a photograph of a typical gel is shown in Figure 3).

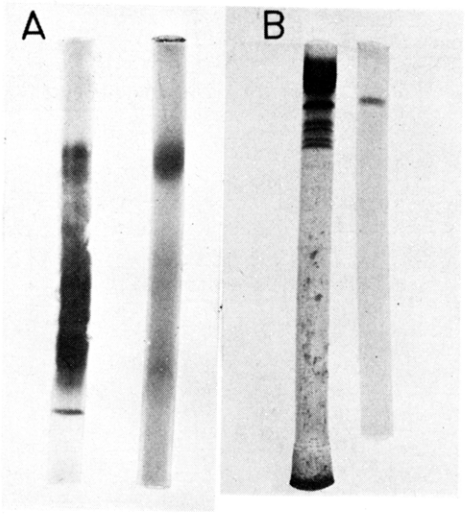

FIGURE 3: Polyacrylamide gel electrophoresis of homogeneous 3β,20α-hydroxysteroid oxidoreductase. Panel A compares a gel from polyacrylamide gel electrophoresis of homogeneous 3β,20α-HSD (single band) with that of crude enzyme freed of hemoglobin during the first Ca-P gel purification (Figure 1). The homogeneous protein contained both 3β and 20α activities and was subjected to amino acid analysis (Table I). Panel B compares a gel from sodium dodecyl sulfate–polyacrylamide gel electrophoresis of homogeneous 3β,20α-HSD (single band) with that of the crude enzyme from Ca-P gel purification. Following each polyacrylamide gel electrophoresis the gels were stained with Coomassie brilliant blue and destained at 75–90 °C with 10% acetic acid–water.

Table I: Amino Acid Composition of 3β,20α-Hydroxysteroid Oxidoreductase

amino acid	% composition[a]	no. of residues[b]
Asp	7.87 (1.03)	32
Thr	5.06 (0.75)	24
Ser	17.58 (4.23)	92
Glu	16.66 (1.06)	62
Pro	3.46 (1.63)	16
Gly	17.58 (0.20)	129
Ala	8.58 (1.31)	53
Val	3.47 (0.58)	16
Ile	1.52 (0.22)	7
Leu	3.55 (1.10)	15
Tyr	2.36 (0.77)	7
Phe	1.95 (0.22)	7
His	1.72 (0.21)	6
Lys	6.54 (0.33)	25
Arg	2.16 (0.22)	7

[a] Percent composition (±SEM) is based on the integrated areas under the peaks in the elution profiles from three amino acid analyses of the 6 N HCl hydrolysates of homogeneous 3β,20α-HSD. [b] Number of residues of each amino acid in a single-stranded polypeptide is calculated (within ±10%) from the percent composition, from the molecular weight of the amino acids, and from the molecular weight of 55 000 ± 1000 found for 3β,20α-HSD (described under Results).

Molecular Weight and Amino Acid Composition of 3β,20α-HSD. Homogeneous 3β,20α-HSD was subjected to $NaDodSO_4$–polyacrylamide gel electrophoresis and also to Sephadex G-100 chromatography. Measured against two different groups of known protein standards, 3β,20α-HSD was found by $NaDodSO_4$–polyacrylamide gel electrophoresis to be a single-stranded polypeptide of M_r 55 000 consistent with the results from chromatography of 3β,20α-HSD on Sephadex G-100. The procedures are under Methods.

Samples of homogeneous 3β,20α-HSD were hydrolyzed with 6 N HCl at 110 °C for 24 h. After the hydrolysate was concentrated to dryness, the mixture of amino acids was dissolved in citrate buffer and subjected to amino acid analysis.

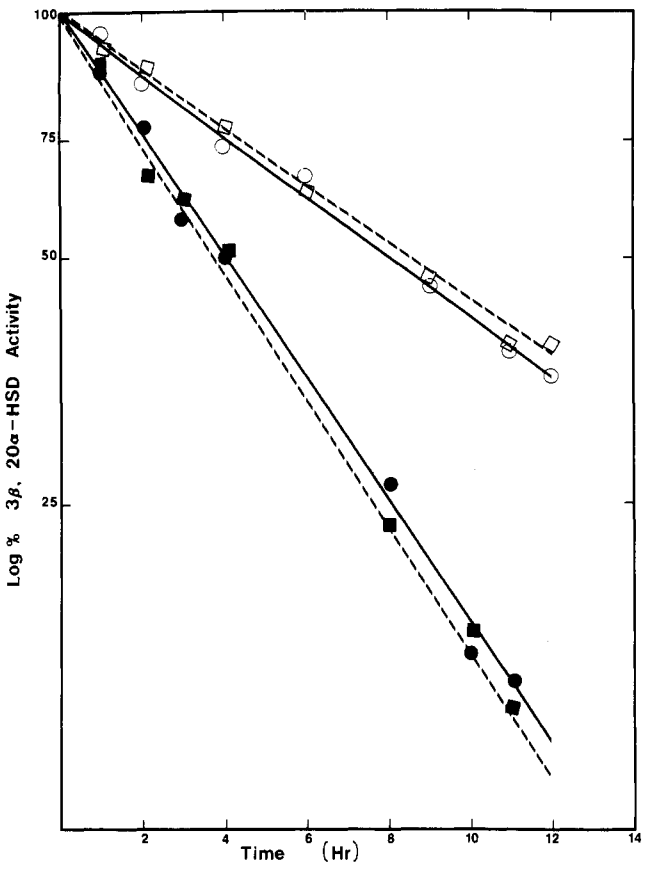

FIGURE 4: Affinity alkylation of 3β,20α-hydroxysteroid oxidoreductase with 19-nortestosterone 17β-bromoacetate. Incubations of 3β,20α-HSD (0.1 mg) with NTBA (0.15 mM) caused loss of both 3β (■) and 20α (●) reductase activities by the time-dependent, irreversible, and first-order kinetic process represented here. When progesterone (2.5 μM) or 17β-hydroxy-5α-androstan-3-one (9.4 μM) was present in the incubation mixture, slower losses of 3β (□) and 20α (○) activities were observed.

The results from this analysis are presented in Table I. These results were consistent from three separate analyses derived from two different, homogeneous, enzyme preparations.

3β- and 20α-Hydroxysteroid Oxidoreductase Activities and Inhibition of Enzymatic Activity. Reductase activity of purified 3β,20α-HSD was found to proceed optimally at pH 5.5–6.0 in 0.1 M phosphate buffer at 37 °C with an enzyme saturating concentration of 0.5 mM NADPH as cofactor. When the concentrations of either progesterone (20α activity) or 17β-hydroxy-5α-androstan-3-one under standard assay conditions were varied, double-reciprocal plots of the resulting data indicated the enzyme kinetic parameters to be the following: K_m value of 2.5 μM (V_{max} = 8.85 nM min^{-1} nmol^{-1}) for progesterone; K_m value of 9.4 μM (V_{max} = 2.4 nM min^{-1} nmol^{-1}) for 17β-hydroxy-5α-androstan-3-one. 20α reductase activity of purified 3β,20α-HSD was assayed with NADPH (0.5 mM), [4-^{14}C]progesterone (at 21.2 or 63 μM) as a fixed substrate, and 17β-hydroxy-5α-androstan-3-one (21.2–106 μM) as a variable inhibitor. Dixon plots of the kinetic data showed 17β-hydroxy-5α-androstan-3-one to be a competitive inhibitor of 20α activity with a K_i value of 35 μM.

Affinity Alkylation of 3β,20α-HSD with 19-Nortestosterone 17-Bromoacetate (NTBA). The results from affinity alkylation of 3β,20α-HSD with NTBA are represented in Figure 4. The experimental details are described under Methods. Measurements of both 3β and 20α activities during incubation of 3β,20α-HSD with NTBA showed that the two activities decreased at practically equal rates by a first-order kinetic process. During 24 h of incubation, 3β,20α-HSD in the control mixture retained over 70% of its original activity. When progesterone or 17β-hydroxy-5α-androstan-3-one was present in incubation mixtures at concentations equal to their respective K_m values together with 3β,20α-HSD and NTBA, the $t_{1/2}$ values for 3β and 20α inactivation were approximately doubled (Figure 4). Evidently, the two substrates for 3β,20α-HSD protected the enzyme against inactivation from NTBA by competing with the affinity alkylating steroid for the same active site.

Discussion

Steroid-specific enzyme activity in plants and animals has attracted wide attention for decades. Recently, several laboratories observed the occurrence of multiple, steroid hydroxylase/lyase (Nakajin et al., 1981), oxidoreductase (Tobias & Strickler, 1981; Rodway & Rahman, 1978; Heyns & de Moor, 1974), and aromatase (Thompson & Siiteri, 1974a,b; Kelley et al., 1977) enzyme activities in purified enzyme preparations. Some enzymes were isolated and found to contain two distinctly different activities (Nakajin et al., 1981; Tobias & Strickler, 1981). Classical kinetic methods were employed to learn more about the relationship between the activities. Most recently, affinity alkylation techniques were used to locate the origin of 3α and 20β reductase activities in a steroid-specific enzyme.

Affinity alkylation experiments with microbial 3α,20β-hydroxysteroid dehydrogenase first required the synthesis of several affinity alkylating, steroid substrate analogues and nucleotide cofactor analogues of the enzyme. Then a series of experiments showed that both 3α and 20β activities share the same active site (Sweet et al., 1980; Sweet & Samant, 1980a,b, 1981). With these affinity alkylating techniques now firmly established, they were used in the present study to investigate mammalian 3- and 20-oxidoreductase activities, reported earlier to coexist in fetal calf red blood cells (Nancarrow & Seamark, 1968). For accomplishment of this, the 3β,20α-hydroxysteroid oxidoreductase (3β,20α-HSD) had to be separated from the large amounts of hemoglobin that are released when the red blood cells are lysed.

Bovine fetal red blood cells are waste materials from the fetal calf serum industry which derives its blood from the beef packing industry. Therefore, an inexhaustible and inexpensive source of the enzyme 3β,20α-HSD is currently available. After the red blood cells were washed several times with isotonic saline solution, the resulting material can be frozen, and the enzyme activity remains intact indefinitely. In fact, at any stage during the enzyme purification (Figure 1), the intermediate preparations can be stored frozen for weeks or months with retention of enzyme activity.

The major problem during purification of 3β,20α-HSD was removal of the copious amounts of hemoglobin. Ammonium sulfate fractionation of 3β,20α-HSD was practically hopeless. An exhaustive series of fractionation experiments with ammonium sulfate failed to provide working quantities of enzyme free of hemoglobin from the lysed red blood cells (Nancarrow et al., 1981). The breakthrough in separating the hemoglobin from 3β,20α-HSD was in using Ca-P gel adsorption. Conditions were found under which 3β,20α-HSD was firmly adsorbed to Ca-P gel while most of the hemoglobin remained in the buffer.

Throughout the purification of 3β,20α-HSD, 3β activity was compared with 20α activity. Both the 3β and 20α activities were measured with the appropriate radioactive steroid substrates (requiring TLC separation of the substrates from the products of reductase activity). Precise kinetic measurements

were important for comparing 3β and 20α activities which relied on quantitative recovery of the steroids from the silica gel of the TLC plates. However, 5α-androstane-3β,20α-diol and 17β-hydroxy-5α-androstan-3-one (3β substrate) were extracted from silica gel G by the toluene of the scintillation cocktail more slowly than were 20α-hydroxy-4-pregnen-3-one (20α product) and progesterone (20α substrate). This problem was overcome by allowing the appropriate TLC spots to stand overnight to be extracted completely by the scintillation cocktail prior to measurement of the radioactivity.

The results from affinity chromatography of 3β,20α-HSD with Cibacron blue–agarose are interesting. With both progesterone and NADPH present in a buffer during recovery of 3β,20α-HSD from the Cibacron blue–agarose matrix, over twice the enzyme was recovered as compared to with NADPH alone (Figure 2). Progesterone without NADPH did not remove measurable amounts of 3β,20α-HSD from the blue matrix (panel A, Figure 2). Cibacron blue coupled to agarose is known to selectively bind proteins containing adenosine mono- or dinucleotide binding sites (Heyns & de Moor, 1974; Bohme et al., 1972). Therefore, 3β,20α-HSD must bind to the blue matrix through its NADP(H)-cofactor binding region at the enzyme active site. During removal of 3β,20α-HSD from the blue matrix by NADPH, the enhancement of this process by progesterone must reflect an increased affinity between the enzyme and the cofactor. The oxidized cofactor NADP at concentrations equal to NADPH provided less than 10% recovery of the 3β,20α-HSD bound to the Cibacron blue–agarose even with progesterone present. The difference in affinities of 3β,20α-HSD for NADP and for NADPH results in the corresponding degrees of enzyme recovery from the blue matrix. These results are analogous to those in which the positive charge on the nicotinamide portion of the oxidized dinucleotide NAD was correlated with its reduced binding relative to NADH by more than 2 orders of magnitude (Sweet & Samant, 1981). The effectiveness of affinity chromatography depends upon manipulating the position of the equilibrium of a protein between a solution and a solid phase consisting of a ligand (having high affinity for the protein) attached to a stationary matrix. The present results provide a classic example of affinity chromatography because the most effective conditions for removing 3β,20α-HSD from the stationary matrix require that the enzyme's natural substrate and cofactor *both* be present in the buffer during recovery of the enzyme from the stationary matrix.

The relationship between the 3β and 20α reductase activities of 3β,20α-HSD was studied in a series of affinity alkylation experiments with 19-nortestosterone 17-bromoacetate (NTBA). NTBA was first synthesized in this laboratory for reproductive biology experiments, and it was found to terminate pregnancy in rats and also in primates (Clark et al., 1974). Incubations of 3β,20α-HSD with NTBA caused simultaneous losses of both 3β and 20α activities by a first-order kinetic process (Figure 4). The presence of progesterone or 17β-hydroxy-5α-androstan-3-one during similar incubations protected 3β,20α-HSD against inactivation by NTBA. If either of these two substrates were present at a concentration equal to their respective K_m values, then both of the first-order rate constants for 3β and 20α inactivation were reduced to half of the rate constant for the reaction of NTBA alone with 3β,20α-HSD (Figure 4). These results lead us to conclude that progesterone, 17β-hydroxy-5α-androstan-3-one, and NTBA all compete for the same active site of 3β,20α-HSD. Therefore, 3β and 20α reductase activities of the single-stranded polypeptide 3β,20α-HSD constitute a system of dual activity at the same active site. Models which can account for this type of dual activity have been presented by us elsewhere (Sweet & Samant, 1980a).

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