Human Placental Estradiol 17β-Dehydrogenase: Evidence for Inverted Substrate Orientation ("Wrong-Way" Binding) at the Active Site†

Gary L. Murdock,* James C. Warren, and Frederick Sweet

Department of Obstetrics and Gynecology and Department of Biological Chemistry, Washington University School of Medicine, St. Louis, Missouri 63110

Received November 5, 1987; Revised Manuscript Received February 18, 1988

ABSTRACT: Human placental estradiol 17β-dehydrogenase (EC 1.1.1.62) was affinity labeled with 17α-estradiol 17-(bromo[2-^{14}C]acetate) (10 μM) or 17β-estradiol 17-(bromo[2-^{14}C]acetate) (10 μM). The steroid bromoacetates competitively inhibit the enzyme (against 17β-estradiol) with K_i values of 90 μM (17α bromoacetate) and 134 μM (17β bromoacetate). Inactivation of the enzyme followed pseudo-first-order kinetics with a $t_{1/2}$ = 110 min (17α bromoacetate) and $t_{1/2}$ = 220 min (17β bromoacetate). Amino acid analysis of the affinity radioalkylated enzyme samples from the two bromoacetates revealed that N^{π}-(carboxy[^{14}C]methyl)histidine was the modified amino acid labeled in each case. Digestion with trypsin produced peptides that were isolated by reverse-phase high-performance liquid chromatography and found to contain N^{π}-(carboxy[^{14}C]methyl)histidine. Both the 17α bromoacetate and also the 17β bromoacetate modified the same histidine in the peptide Phe-Tyr-Gln-Tyr-Leu-Ala-His(π-CM)-Ser-Lys. Previously, the same histidine had been exclusively labeled by estrone 3-(bromoacetate) and shown not to be directly involved in catalytic hydrogen transfer at the D-ring of estradiol. Therefore, this histidine was presumed to proximate the A-ring of the bound steroid substrate. The present results suggest that the 17α bromoacetate and 17β bromoacetate D-ring analogues of estradiol react with the same active site histidine residue as estrone 3-(bromoacetate), the A-ring analogue of estrone. Moreover, as each of the estradiol 17-(bromoacetates) undergoes the reversible binding step at the enzyme active site, its D-ring is in a reversed binding position relative to that of the natural substrate 17β-estradiol as it undergoes catalytic hydrogen transfer at the same active site.

Estradiol 17β-dehydrogenase has been isolated and crystallized in this laboratory (Chin & Warren, 1973; Chin et al., 1976). Previous studies aimed at determining the structure of the enzyme active site by affinity labeling techniques employed various bromoacetate analogues of natural substrates (Murdock & Warren, 1982; Chin et al., 1982). The bromoacetate analogues were proven substrates and, therefore, must undergo the reversible binding step at the active site in an orientation similar to the natural substrate. Analogues with a bromoacetate reagent group at the 3-, 12β-, or 16α-position modified the histidine residue(s), which was (were) identified by amino acid analysis of the affinity-alkylated enzyme, as either N^{τ}- or N^{π}-(carboxymethyl)histidine. Furthermore, this work revealed that the peptides presumed to be derived from the active site contain at least three histidine residues (Murdock et al., 1983, 1986). Finally, affinity labeling studies suggested that one of the histidines most likely proximates the A-ring of a bound substrate, because it was exclusively modified by estrone 3-bromoacetate and was found to be unnecessary for catalytic hydrogen transfer at the steroidal D-ring (Murdock et al., 1983; Pons et al., 1977).

We have synthesized 17α-estradiol 17-(bromoacetate) and 17β-estradiol 17-(bromoacetate) for the present study. These two steroids cannot be substrates for estradiol 17β-dehydrogenase because the hydroxyl group at the 17-position on the D-ring has been replaced by the bromoacetate moiety. Nevertheless, we assumed that placing an alkylating bromoacetate side chain at the 17-position would facilitate reaction with the enzyme active site residues which participate in the catalytic event. The assumption was based on the premise that the two estradiol analogues would bind in the same orientation as the natural substrates at the enzyme active site.

Experimental Procedures

Reagents. Reagent-grade salts, inorganic acids, reagent-grade organic solvents, bromoacetic acid, dicyclohexylcarbodiimide, dialysis tubing (Spectra/Por No. 2), thin-layer chromatography sheets (Eastman silica gel GF, No. 13181), and liquid scintillation counting fluor were obtained from Fisher Scientific. Steroids, nucleotides, *p*-toluenesulfonic acid, and guanidine hydrochloride were from Sigma Chemical Co. Preparative silica gel TLC[1] plates containing fluorescent indicator were from Analtech. Trypsin–TPCK was obtained from Worthington. Buffers and reagents for amino acid hydrolyses and analyses were from Pierce Chemical Co. Bromo[2-^{14}C]acetic acid (17 mCi/mmol) was from Pathfinder Laboratories (St. Louis, MO). Sequencer-grade reagents and solvents for HPLC and amino acid sequencing were from Burdick and Jackson Laboratories.

Amino Acid Analyses. Acid hydrolysis of protein was conducted with constant-boiling hydrochloric acid in evacuated, sealed tubes at 110 °C for 24 h. Amino acid analyses of the hydrolysates were performed with a Beckman Model 118C

† This research was supported by Grants AM-15708 and HD 12533 from the National Institutes of Health. A preliminary account of this work was presented at the 190th National Meeting of the American Chemical Society, Chicago, September 1985.

* Address correspondence to this author at the Department of Obstetrics and Gynecology.

[1] Abbreviations: trypsin–TPCK, trypsin–L-1-(tosylamido)-2-phenylethyl chloroethyl ketone; His(π-CM), N^{π}-(carboxymethyl)histidine; TLC, thin-layer chromatography; HPLC, high-performance liquid chromatography.

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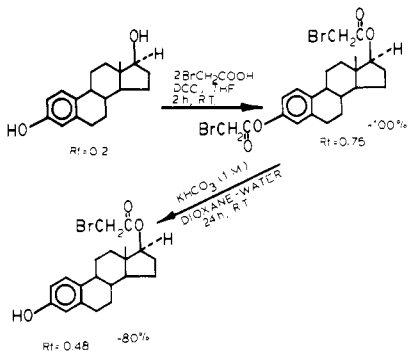

FIGURE 1: Scheme for syntheses of 17β- and 17α-estradiol 17-(bromoacetate).

amino acid analyzer according to the procedure of Spackman et al. (1958). The effluent was collected in 0.4-mL fractions. Five milliliters of counting fluor was added to each fraction, and the ^{14}C radioactivity was measured in a Packard Tri-Carb Model 3320 liquid scintillation spectrometer. Synthesis of the reference compounds N^{τ}- and N^{π}-(carboxymethyl)histidine was by the method of Crestfield et al. (1963).

Synthesis of 17β- (or 17α-) Estradiol 3,17-Bis(bromoacetate). To a stirred solution of 545 mg (2 mmol) of 17β- (or 17α-) estradiol in 10 mL of tetrahydrofuran at 0 °C were added 611 mg (4.4 mmol) of bromoacetic acid in 5 mL of tetrahydrofuran and 908 mg (4.4 mmol) of dicyclohexylcarbodiimide in 5 mL of tetrahydrofuran. Pyridine (50 μL) was added, and the stirring was continued at 0 °C for 10 min and at room temperature for 1 h. Thin-layer chromatograms (developed in benzene–ethyl acetate, 9:1, visualized by I_2 vapors) of the reaction mixture showed four components with R_f values of 0.2 (17β-estradiol), 0.35, 0.45, and 0.7 (major component). The addition of 400 mg of dicyclohexylcarbodiimide to the reaction mixture with stirring for 1 h at room temperature completely converted the remaining starting material and the two intermediates to the major component (R_f 0.7). Water (4 drops) was added, and after being stirred for 10 min, the mixture was filtered. The solid residue was rinsed with two 5-mL portions of tetrahydrofuran. The combined filtrates were concentrated under reduced pressure, leaving a viscous residue. The residue was mixed with 10 mL of methanol and evaporated to dryness. The solid residue was dissolved in 15 mL of boiling methanol and kept at 4 °C overnight. The colorless, crystalline 17β-estradiol 3,17-bis(bromoacetate) (930 mg) was collected by filtration. The crystals (mp 90–92 °C) produced a nuclear magnetic resonance (NMR) spectrum with proton signals centered at 4.77 ppm (dd, H-17α, $J_{17\alpha,16\alpha/16\beta}$ = 6.0 Hz), 4.04 ppm (s, C-3, $BrCH_2COO$–), 3.85 ppm (split singlet, C-17β, $BrCH_2COO$–), 2.87 ppm (m, H-6α + H-6β), and 0.87 ppm (s, C-18, CH_3–). All of the steroid samples were analyzed in solutions of $CDCl_3$ with tetramethylsilane as an internal standard in a 300-MHz, Varian 300XL spectrometer and are reported in parts per million (ppm). The NMR and infrared data of the steroid bromoacetates were all consistent with the structures represented in Figure 1 .

Synthesis of 17β- (or 17α-) Estradiol 17-(Bromoacetate). A solution of 514 mg (1 mmol) of 17β-estradiol 3,17-bis(bromoacetate) in 20 mL of dioxane was mixed at room temperature with 120 mg (1.2 mmol) of $KHCO_3$ in 2 mL of H_2O. The hydrolysis reaction was monitored by TLC. Within 20 min a new component (R_f 0.35) evolved from the starting material (R_f 0.7), and after 18 h, all of the starting material was converted to product. Glacial acetic acid (2 drops) was added to the mixture, and the solvent was removed under reduced pressure at 35 °C. The residue was mixed with 5 mL of ethanol, again evaporated, and dissolved in 10 mL of ethanol. Water was added dropwise until the solution remained cloudy. The solution was stored overnight at 4 °C. A colorless, crystalline precipitate was collected, giving 360 mg (90%) of product with a trace of contaminants (TLC). Recrystallization of this material from ethanol–H_2O gave a pure product (R_f 0.35) with mp 182 °C. The infrared spectrum (KBr pellet) showed absorptions at 3410 cm^{-1} (OH) and at 1730 cm^{-1} (aliphatic ester). The NMR signals of this compound were centered at 4.77 ppm (dd, H-17α, $J_{17\alpha,16\alpha/16\beta}$ = 6 Hz), 3.85 ppm (split singlet, C-17β, $BrCH_2COO$–), 2.82 ppm (m, H-6α + H-6β), and 0.86 ppm (s, C-18, CH_3–). Comparison of this spectrum with that of the known compound 17β-estradiol 17-acetate showed corresponding signals for H-17α and H-6α + H-6β and for the C-18 methyl group (0.83 ppm), with the acetate methyl signal at 2.06 ppm. These data and the elemental analysis of the compound were consistent with the structure of 17β-estradiol 17-(bromoacetate) (Figure 1). This compound was stable (TLC) in ethanol solutions at room temperature for over one week.

17α-Estradiol 17-(bromoacetate) was prepared by the above method, starting with 545 mg of 17α-estradiol. The overall yield of 17α-estradiol 17-(bromoacetate) was 85%. The R_f values on TLC and the infrared spectrum were not significantly different from those of the corresponding 17β derivatives. The melting point range of the final crystalline product was 136–138 °C. The NMR signals of this compound were centered at 4.93 ppm (dd, H-17β, $J_{17\beta,16\alpha/16\beta}$ = 4.0 Hz), 3.85 ppm (split singlet, C-17α, $BrCH_2COO$–), 2.83 ppm (m, H-6α + H-6β), and 0.80 ppm (s, C-18, CH_3–).

Synthesis of 17β- (or 17α-) Estradiol 17-(Bromo[2-^{14}C]acetate). A 250-μL aliquot containing 8.2 mg (0.118 mmol) of bromoacetic acid was transferred from a stock solution to an ampule containing 2 mCi of bromo[2-^{14}C]acetic acid (17 mCi/mmol). The resulting solution was quantitatively transferred to a solution of 16.07 mg (0.059 mmol) of 17β-estradiol in 0.5 mL of tetrahydrofuran and 36.5 mg (0.177 mmol) of dicyclohexylcarbodiimide in 0.2 mL of tetrahydrofuran at 0 °C. The solution was stirred for 10 min. Pyridine (2 μL) was added to the reaction mixture, and stirring was continued at room temperature for 1 h. An aliquot of the reaction mixture was analyzed by TLC (benzene–ethyl acetate, 9:1), and the radioactivity in the product spot (R_f 0.7), intermediate spots (R_f 0.35 and 0.45), and the origin was measured by liquid scintillation counting. The product contained 70% of the total radioactivity. The reaction mixture was applied to a preparative silica gel G TLC plate which was developed with benzene–ethyl acetate (9:1). The zone on the TLC plate which contained the product was removed, extracted with methanol, and filtered. The concentrated filtrate gave 16.5 mg (0.032 mmol, 54% yield) of 17β-estradiol 3,17-bis(bromo[2-^{14}C]acetate). This material was dissolved in 2 mL of dioxane and mixed with 0.25 mL of water containing 3.5 mg (0.035 mmol) of $KHCO_3$. The reaction mixture was stirred at room temperature overnight. TLC analysis showed that 17β-estradiol 17-(bromo[2-^{14}C]acetate) (R_f 0.35) was the major component. Preparative chromatography of the reaction mixture and isolation of the product provided 8.5 mg of pure 17β-estradiol 17-(bromo[2-^{14}C]acetate) (8 mCi/mmol) with physical properties identical with those of the authentic, nonradioactive compound. The 17α bromoacetate was prepared under similar conditions, beginning with 2 mCi of bromo[2-^{14}C]acetic acid (33 mCi/mmol). The overall yield for 17α-estradiol 17-(bromo[2-^{14}C]acetate) (17.7

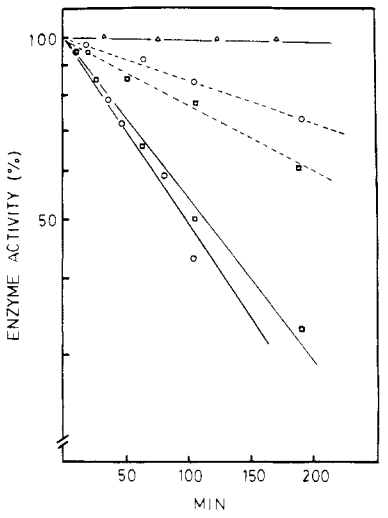

FIGURE 2: Inactivation of estradiol 17β-dehydrogenase (1 μM) by 17α-estradiol 17-(bromoacetate) [10 μM (○)] or 17β-estradiol 17-(bromoacetate) [10 μM (□)]. Those incubations containing an excess of 17β-estradiol (100 μM) are represented by the corresponding symbols connected with (--). All incubations were performed in the presence of 40 μM NADH under the conditions described under Experimental Procedures. The control (△), containing only cofactor, remained unchanged throughout the experiment.

mCi/mmol) was 60%. The radioactive derivatives were stored in ethanol at 0 °C until the start of the inactivation experiment.

K_i Determinations and Inactivation of Estradiol 17β-Dehydrogenase with 17α- (and 17β-) Estradiol 17-(Bromoacetates). The 17α- and 17β-estradiol 17-(bromoacetates) were used as inhibitors in the assay of estradiol 17β-dehydrogenase. The assay conditions were similar to those employed for the inactivation of the enzyme. The enzyme (0.15 μM) was assayed in 0.1 M K_xPO_4 buffer, pH 6.3, containing 20% ethanol and 200 μM NAD^+ in a total volume of 3.0 mL. This concentration of ethanol was used throughout the inactivation studies in order to maintain the solubility of the steroids. The reaction was started by the addition of enzyme. The substrate (17β-estradiol) concentration was varied from 10 to 250 μM, and the concentration of either inhibitor, 17α- (or 17β-) estradiol 17-(bromoacetate), was 10–75 μM. The initial velocities were plotted according to Lineweaver–Burk, and the K_i values were determined from the plots.

The enzyme used for inactivation studies was purified to homogeneity from human placenta as previously reported (Murdock et al., 1986). The specific activity of pooled preparations was 7.5 IU/mg as assayed according to Langer and Engel (1958). The initial pilot inactivations were carried out with inactivating steroid (10 μM) and cofactor (40 μM) alone or in the presence of 17β-estradiol (100 μM). The incubation mixture contained 10% ethanol in 0.1 M K_xPO_4 buffer and 10% glycerol at pH 6.3 and room temperature (Figure 2). For the purpose of peptide analyses, site-directed inactivations of the enzyme with the two steroids were carried out in the same buffer containing 20 μM NADH and 20% ethanol. The first inactivation was performed by incubating 25 mg of enzyme (1 μM) with 17β-estradiol 17-(bromo[2-^{14}C]acetate) (10 μM) in a total volume of 360 mL. In a separate experiment, 15 mg of enzyme (1 μM) was inactivated by 17α-estradiol 17-(bromo[2-^{14}C]acetate) (10 μM) in a volume of 220 mL. The reactions were stopped by the addition of a 50-fold molar excess of 2-mercaptoethanol to the mixture. Control incubations containing only enzyme in the incubation buffer were assayed to monitor activity throughout the inactivation time. The samples were dialyzed 3 times against 0.1 M NH_4HCO_3, pH 8.0, at which time the radioactivity in the dialyzate was at background levels. The samples were frozen and lyophilized.

Purification and Sequencing of Radiolabeled Tryptic Peptides. The lyophilized samples were separately resuspended in a minimal amount of 0.1 M NH_4HCO_3 buffer, pH 8.0, and made 0.05 N in NaOH. They were left overnight at room temperature to ensure hydrolysis of the steroid ester. Guanidine hydrochloride (6 M final concentration) was added to the samples, the pH was adjusted to 8.0 with 1 N NaOH, and the samples were stirred 30 min. A sufficient quantity of 2-mercaptoethanol was added to the denatured protein to make a 10-fold molar excess with respect to sulfhydryl groups. The samples were flushed with N_2 and stirred for 20 min. A 10-fold molar excess (relative to 2-mercaptoethanol) of unlabeled iodoacetic acid and a molar equivalent of NaOH were added, and the mixture was stirred an additional 15 min, under N_2, at room temperature. The reaction was stopped by the addition of an excess of 2-mercaptoethanol. The samples were dialzyed 4 times against 10 volumes of 0.1 M NH_4HCO_3, pH 8.0. The dialyzed, carboxymethylated enzyme was treated 3 times at 4-h intervals with trypsin–TPCK (4% w/w). After the last treatment, the samples were allowed to react overnight at 37 °C and then frozen and lyophilized. The lyophilized residue was washed twice with H_2O to remove the bicarbonate salt.

The radioactive peptides were isolated by HPLC. The desalted, lyophilized samples were resuspended in 500 μL of 0.05% trifluoroacetic acid for application to a Waters C-18 Bondapak column on a Varian Model 5020 high-performance liquid chromatograph. Peptides were eluted with a gradient of 0.05% trifluoroacetic acid/H_2O with a limit buffer of 0.05% trifluoroacetic acid/acetonitrile at a rate of 0.25% limit buffer per minute. The effluent was monitored at 214 nm, and fractions were collected every 0.5 min (1.0 mL/min). The radioactivity content was determined in each fraction. The radioactive peptides eluting prior to the start of the gradient were rechromatographed on an Altex/PTH Ultrasphere octyl column eluted with the same gradient. The remaining isolated radioactive peptides from the first chromatography were rechromatographed on the C-18 column, after the starting buffer was adjusted to pH 7.5 with diluted triethylamine. The recovered radioactive peptides were lyophilized, and the residues were washed twice with H_2O.

The sequences of the isolated peptides were determined in an Applied Biosystems Model 470A gas-phase sequencer. Each determination was run with a precycled program with Polybrene (Abbott Laboratories) and had an 85–95% repetitive yield. Each lyophilized fraction was dissolved in 50 μL of methanol and converted to the phenylthiohydantoin derivative. A 5–20-μL aliquot was taken for radioactivity measurements. The amino acid identification was performed on 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).

RESULTS

Kinetics of Enzyme Inactivation with 17α- (or 17β-) Estradiol 17-(Bromoacetate). The maximum solubility of the bromoacetate derivatives was 75 μM in the assay mixtures which contained 20% ethanol at pH 6.3. The calculated K_i values were 90 μM and 134 μM for 17α-estradiol 17-(bromoacetate) and 17β-estradiol 17-(bromoacetate), respectively, from duplicate determinations at three inhibitor concentrations. Both of the derivatives were competive inhibitors. Under these assay conditions, the K_m value of 17β-estradiol was calculated

Table I: Amino Acid Sequence Analysis of Tryptic Peptides from Estradiol 17β-Dehydrogenase Modified by either 17α-Estradiol 17-(Bromo[2-^{14}C]acetate) or 17β-Estradiol 17-(Bromo[2-^{14}C]acetate), As Described under Experimental Procedures[a]

	sequence cycle								
HPLC peptide	1	2	3	4	5 (1)	6 (2)	7 (3)	8 (4)	9 (5)
17β-2	Phe	Tyr	Gln	Tyr	Leu	Ala	His(π-CM)[b]	Ser	Lys
17β-3	Phe	Tyr	Gln	Tyr	Leu	Ala	His(π-CM)[b]	ND[c]	ND
17α-1b					Leu	Ala	His(π-CM)[b]	Ser	Lys
17α-2	Phe	Tyr	Gln	Tyr	Leu	Ala	His(π-CM)[b]	ND	ND
17α-4					Leu	Ala	His(π-CM)[b]	ND	ND
17α-5					Leu	Ala	His(π-CM)[b]	Ser	Lys

[a]Some of the samples were pseudotryptic peptides (cycle number in parentheses). [b]Radioactivity was found in the sequence cycle. [c]ND, not determined due to peptide washout.

to be 83 μM. The inactivation of estradiol 17β-dehydrogenase at pH 6.3 by the two steroid derivatives in separate incubations followed pseudo-first-order kinetics throughout the time course of the experiment. The pilot incubations demonstrated that natural substrate protects the enzyme from inactivation by either steroid bromoacetate (Figure 2). The rate of inactivation was decreased 3–4-fold in the presence of 17β-estradiol. In the large batch incubations, the enzyme was 65% inactivated by the 17α derivative ($t_{1/2}$ = 110 min, sample 17α) and 60% by the 17β derivative ($t_{1/2}$ = 220 min, sample 17β) under similar conditions of reagent and enzyme concentrations. Amino acid analyses of aliquots of the dialyzed incubation mixture of each of the samples showed that the radioactivity occurring in N^{π}-(carboxymethyl)histidine was 90 and 92% of the total incorporated in sample 17α and sample 17β, respectively. Small quantities of radioactive N^{τ}-(carboxymethyl)histidine and *S*-(carboxymethyl)cysteine could be detected in the samples. The peptide purification methodology was directed toward isolating the peptides which contained radioactive N^{π}-(carboxymethyl)histidine.

Isolation of Radioactive His(π-CM)-Bearing Peptides. Aliquots of each lyophilized, trypsin-digested sample were chromatographed twice by HPLC for purification of the peptides. Sample 17β (containing 130 nmol of radioactive label) was applied to the HPLC for the first run (Figure 3). Forty-eight percent of the applied radioactivity was recovered (all subsequent recoveries are based on the percent radioactive peptide recovered from the quantity applied to the column). The radioactivity was distributed among three collected peptide fractions: fraction 1, 21.6 nmol; fraction 2, 18.8 nmol; fraction 3, 22.8 nmol. All three pools contained only radioactive N^{π}-(carboxymethyl)histidine. After two subsequent chromatographic runs, fraction 1 could not be resolved into a distinct radioactive peak (except for consistent, small quantities which had retention times corresponding to peaks 2 and 3). Purified peptides for amino acid sequence analysis were not obtained for fraction 1. Fractions 2 and 3 were rechromatographed at pH 7.5. The second HPLC resulted in a reduced retention time for the major radioactive peak, relative to the first chromatography. The second HPLC of fraction 2 yielded 10 nmol (17β-2, 53% recovery). The second HPLC of fraction 3 provided 12.4 nmol (17β-3, 54%). Aliquots of the isolated peptides were used for amino acid sequence analysis.

The tryptic digest profile of sample 17α was more complex than that of sample 17β. Figure 4 shows only the radioactive peaks obtained from the first HPLC analysis of sample 17α (190 nmol applied). Five major fractions were collected for rechromatography: fraction 1, 23.9 nmol; fraction 2, 13 nmol; fraction 3, 12 nmol; fraction 4, 16.8 nmol; fraction 5, 20.6 nmol. The amino acid analyses of all fractions showed N^{π}-(carboxymethyl)histidine as the sole radioactive amino acid residue. Total recovery of radioactivity from HPLC in the five peaks was 45%. Each peak was rechromatographed at

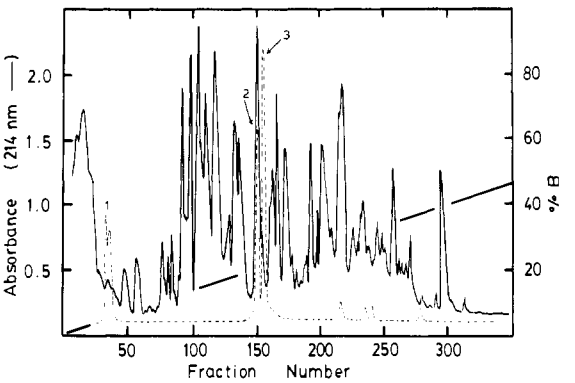

FIGURE 3: HPLC elution profile of tryptic digest of estradiol 17β-dehydrogenase inactivated by 17β-estradiol 17-(bromo[2-^{14}C]acetate) (10 μM). The desalted, lyophilized digest was dissolved in 0.05% trifluoroacetic acid/H_2O (buffer A) and applied to a Waters C-18 Bondapak column. The peptides were eluted with a 0.25%/min gradient of 0.05% trifluoroacetic acid/acetonitrile (buffer B) at a flow rate of 1.0 mL/min. Fractions of 0.5 mL were collected and aliquots taken for determination of radioactivity (--). Radioactive peptides 1–3 were collected for subsequent rechromatography.

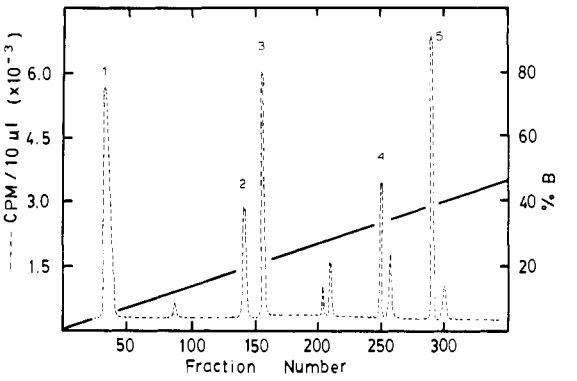

FIGURE 4: HPLC elution profile of radioactive tryptic peptides from estradiol 17β-dehydrogenase inactivated by 17α-estradiol 17-(bromo[2-^{14}C]acetate) (10 μM). The digest was dissolved in 0.05% trifluoroacetic acid/H_2O (buffer A) and applied to a Waters C-18 Bondapak column. Column elution with a 0.25%/min gradient of 0.05% trifluoroacetic acid/acetonitrile (buffer B) was at a flow rate of 1.0 mL/min. Aliquots of 50 μL were taken from each fraction (0.5 mL) for measurement radioactivity. Peaks 1–5 were collected for further purification as described under Experimental Procedures.

pH 7.5 (50–53% recovery). Fraction 1 produced two isolated radioactive peaks; 17α-1a (5 nmol) and 17α-1b (8 nmol). From the experience gained with the HPLC of sample 17β, fractions 2 and 3 from sample 17α were combined. Rechromatography yielded only one major radioactive peak, 17α-2 (13 nmol). A single peak was isolated from each of fraction 4 (17α-4, 6 nmol) and fraction 5 (17α-5, 7 nmol).

Amino Acid Sequence of the His(π-CM) Peptides. Aliquots of 1–3 nmol of each peptide were applied in duplicate runs for amino acid sequence analysis. The results of these analyses

are shown in Table I. Most of the radioactivity was found in one cycle, although there was consistent tailing in subsequent cycles. Generally, the yield was satisfactory with an unambiguous identification of N^{π}-(carboxymethyl)histidine from the sequenator. Only in peptides 17α-1b and 17α-4 could the PTH derivatives not be readily identified, although radioactivity was detected in the cycle, as indicated. Furthermore, amino acid analyses with larger samples of these peptides consistently demonstrated that N^{π}-(carboxymethyl)histidine was the only radioactive component. For peptides 17β-3, 17α-2, and 17α-4, sequences could not be determined in cycles subsequent to N^{π}-(carboxymethyl)histidine due to washing out of the peptide. The sequences of peptide 17β-2 showed that a second, minor, peptide was in the sample. The apparent association of these peptides through the purification procedure most likely was the reason for variations in the HPLC retention times of fractions 17β-2 and 17β-3.

The amino acid sequences of peptides from both sample 17α and sample 17β showed that only a single histidine residue had been modified. This histidine was found in the nonapeptide Phe-Tyr-Gln-Tyr-Leu-Ala-His(π-CM)-Ser-Lys. The pseudotryptic peptide Leu-Ala-His(π-CM)-Ser-Lys, which was found in 17α-1b, 17α-4, and 17α-5, is a tryptic fragment from the nonapeptide. No identifiable sequence could be determined for 17α-1a, although radioactivity was found in the third cycle.

Discussion

The imidazole ring of histidine has been shown to participate in the catalytic hydrogen transfer at the active site of several dehydrogenases (Parker & Holbrook, 1977; Birktoft et al., 1982). Recent studies in our laboratory identified three histidine residues in the steroid binding region of the active site of estradiol 17β-dehydrogenase. The two tryptic peptides containing the three histidine residues are adjacent to one another in the primary amino acid sequence of human placental estradiol 17β-dehydrogenase (Warren et al., 1984). Therefore, we surmised that the imidazole ring of one of these histidines is most likely involved in catalytic hydrogen transfer. Estrone 3-(bromoacetate) was found to affinity label a histidine residue, presumed to proximate the A-ring of the steroid as it binds to the active site of estradiol 17β-dehydrogenase (Murdock et al., 1983). This histidine residue was identified in the pseudotryptic peptide Leu-Ala-His(π-CM)-Ser-Lys, but it is not involved in catalytic hydrogen transfer. This conclusion followed from the finding that hydrolytic release of the estrone moiety from the affinity-labeled enzyme caused reactivation of the enzyme (i.e., resumption of catalytic hydrogen transfer), even though the histidine remained modified as N^{π}-(carboxymethyl)histidine (Pons et al., 1977). However, two other histidines (now called "geminal" histidines, Figure 5) were identified in a single tryptic octapeptide (Thr-Asp-Ile-His-Thr-Phe-His-Arg) after both of the histidines were modified in affinity labeling experiments with either 12β-hydroxy-4-estrene-3,17-dione 12-(bromoacetate) or 3-methoxyestriol 16-(bromoacetate) (Murdock et al., 1986). Dreiding molecular models showed that rotation of the 12-(bromoacetate) and 16-(bromoacetate) groups allowed each of the side chains to react with some of the same amino acids in a common region of space. The two histidines were presumed to proximate the D-ring of estrone (or estradiol) as it binds to the steroid region of the enzyme active site. Hydrolytic release of the corresponding two steroids from the affinity-labeled enzyme did not produce reactivation of enzyme activity in contrast to the earlier results with estrone 3-(bromoacetate) (Pons et al., 1977). Thus, one or perhaps both histidines may be involved in catalytic hydrogen transfer. Inano and Tamaoki

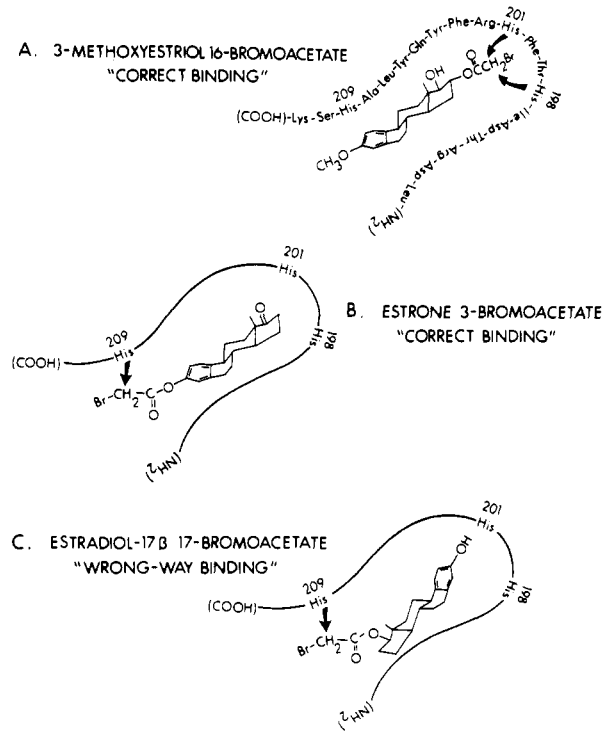

FIGURE 5: Schematic representation for binding of affinity labeling substrate analogues at the catalytic site of estradiol 17β-dehydrogenase. Amino acid residue numbers are tentatively assigned. The orientation of substrate which is conducive to catalytic hydrogen transfer is shown for 3-methoxyestriol 16-(bromoacetate) (A) and estrone 3-(bromoacetate) (B). The wrong-way binding of 17β- (or 17α-) estradiol 17-(bromoacetate) (C) promotes the alkylation of His-209, the same histidine attacked by estrone 3-(bromoacetate). The term geminal histidines is used to collectively describe His-198 and His-201.

affinity labeled a single arginine residue with 16-oxoestrone, but they did not report the amino acid sequence of the peptide that they isolated (Inano & Tamaoki, 1983). Nevertheless, we have found an arginine residue adjacent to one of the geminal histidines in the amino acid sequence of the octapeptide (Figure 5). Because the D-ring of 16-oxoestrone reacts with the arginine residue, we are inclined to the view that the segment Arg-His^{201}-Phe-Thr-His^{198} forms the catalytic hydrogen-transfer "zone" at the enzyme active site.

The present studies were intended to test our hypothesis that the octapeptide (Thr-Asp-Ile-His-Thr-Phe-His-Arg) in estradiol 17β-dehydrogenase contains the histidine residue(s) involved in catalytic hydrogen transfer. We assumed that a bromoacetate substituent strategically located on the D-ring of 17α-estradiol or 17β-estradiol would be oriented at the steroid binding region of the enzyme active site in a manner similar to the natural substrate during catalysis. The presence of a bromoacetate group at the 17-position of estradiol makes enzyme-catalyzed dehydrogenation of the C-17α hydrogen in the steroid impossible. Still, it seemed reasonable to assume that either 17α-estradiol 17-(bromoacetate) or 17β-estradiol 17-(bromoacetate) or both steroids would be in close enough proximity to modify one of the catalytically important geminal histidines by affinity alkylation. Dreiding molecular models show that during their rotation the 17β bromoacetate and 17α bromoacetate groups share a region of space within which they can modify the same amino acid.

The results reveal that both 17α-estradiol 17-(bromoacetate) and 17β-estradiol 17-(bromoacetate) affinity label estradiol 17β-dehydrogenase. 17α-Estradiol 17-(bromoacetate) affinity labels the enzyme faster than does the 17β isomer. These data can be interpreted to suggest that the imidazole ring of the

modified histidine residue is located closer to the α-side than to the β-side of the two steroids as they bind at the active site just prior to the alkylation reaction. Thus the 17α bromoacetate group may be able to approach the histidine closer and react with it faster than the 17β bromoacetate. The pseudo-first-order kinetic $t_{1/2}$ values of enzyme inactivation for both steroidal 17-(bromoacetates) are large (i.e., relatively slow inactivation) because very low concentrations of the steroids were used for affinity labeling the enzyme. The concentrations of the two steroids (10 μM), which were lower than the corresponding K_i values for reversible binding with the enzyme, were chosen to promote specificity in labeling the amino acids at the enzyme active site by preventing nonspecific bimolecular reactions (Sweet & Murdock, 1987).

Both the C-17α and C-17β bromoacetate derivatives alkylate the N^{π}-position of the same histidine, as does estrone 3-(bromoacetate) (Murdock et al., 1983). Amino acid sequence analyses of the modified peptides demonstrate that all three steroidal bromoacetates alkylate the same histidine residue in the tryptic peptide Phe-Tyr-Gln-Tyr-Leu-Ala-His-Ser-Lys. The finding that 17α-estradiol 17-(bromoacetate), 17β-estradiol 17-(bromoacetate), and estrone 3-(bromoacetate) react with the same histidine at the active site of human placental estradiol 17β-dehydrogenase strongly suggests that the two estradiol 17-(bromoacetates) bind at the catalytic site in a "wrong-way" position relative to the orientation required for catalytic hydrogen transfer at the 17-position of a correctly oriented substrate (Figure 5).

We should not exclude the possibility that a small fraction of the molecules of 17α-estradiol 17-(bromoacetate) [or 17β-estradiol 17-(bromoacetate)] binds at the enzyme active site in a "right-way" orientation during the affinity labeling experiments. This would produce N^{π}-(carboxymethyl)histidine in a correspondingly small amount of peptide fragment, different from the isolated peptide described under Results. Right-way binding would involve reaction of the 17-(bromoacetoxy) group with one of the two geminal histidines (Figure 5), which we call the "putative catalytic histidine". But we did not detect the hypothetical radiolabeled peptide during the present series of experiments. Either the fraction of 17-(bromoacetate) molecules which reacts with the enzyme active site in a right-way binding manner is undetectably small, or the putative catalytic histidine is not favorably situated to react with the 17-(bromoacetate) group during right-way binding. Currently, a series of experiments is under way with new steroid analogues to further clarify this possibility.

Indications of a wrong-way binding possibility for steroids at enzyme active sites have been previously encountered. Sweet and Samant (1980) demonstrated that microbial 3α,20β-hydroxysteroid dehydrogenase has both 3α and 20β activity at the same active site and were the first to propose that inverted substrate binding can account for dual enzyme activity. Similarly, dual substrate activity was discovered in 3β,20α-hydroxysteroid oxidoreductase from bovine fetal blood (Sharaf & Sweet, 1982), and Strickler and Tobias reported that human placental estradiol 17β-dehydrogenase can accommodate a variety of substrates which must bind somewhat differently to allow for 17β and 20α activity at a single active site (Strickler & Tobias, 1980).

In studies of the Δ^5-3-ketosteroid isomerase, Pollack and co-workers (Bevins et al., 1980; Kayser et al., 1983; Bounds & Pollack, 1987) demonstrated an inverted binding mode for substrate derivatives having a reactive oxirane group at opposite ends of the molecule. The irreversible inhibitors, one with a D-ring 17β-oxirane and the other with an A-ring 3β-oxirane, modified the same Asp-38 amino acid at the steroid binding site. These workers used the term "mode A" in describing the catalytically favorable substrate binding orientation and the terms "backwards binding" and "mode D" to describe the complex in which the steroid D-ring is located at the region of the active site that is normally occupied by the A-ring during enzyme catalysis (Kayser et al., 1983). Our results from the present study strongly suggest that a similar type of backwards binding or wrong-way binding occurs with human placental estradiol 17β-dehydrogenase.

Results from the present studies have not tested the hypothesis that one or both of the geminal histidines in the sequenced octapeptide are involved in catalytic hydrogen transfer at the active site of estradiol 17β-dehydrogenase. We are continuing the study of wrong-way binding in addition to the nature of catalytic hydrogen transfer on the basis of our hypothesis that hydrogen bonding between hydroxyl groups on estradiol or other substrates and the geminal histidines (Figure 5) at the active site of placental estradiol 17β-dehydrogenase influence both the force of binding and the binding orientation of steroidal substrates.

ACKNOWLEDGMENTS

We thank Dr. Greg Grant of the Washington University Chemistry Facility for his advice and technical assistance with the amino acid sequencing of the peptides. The NMR studies were performed by Dr. Leonard Rosik using the equipment of the Washington University High Resolution NMR Facility funded in part by NIH Shared Instrument Grant 1-S10-RR02004.

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^{31}P NMR Probes of Sipunculan Erythrocytes Containing the O_2-Carrying Protein Hemerythrin†

Pierre-Marie L. Robitaille
Department of Chemistry, Iowa State University, Ames, Iowa 50011
Donald M. Kurtz, Jr.*
Department of Chemistry, University of Georgia, Athens, Georgia 30602
Received August 24, 1987; Revised Manuscript Received January 5, 1988

ABSTRACT: Reported are the first examinations by ^{31}P NMR of erythrocytes containing the non-heme iron O_2-carrying protein hemerythrin (Hr). Intact coelomic erythrocytes from the sipunculids *Phascolopsis gouldii* and *Themiste zostericola* were shown by ^{31}P NMR to contain *O*-phosphorylethanolamine and 2-aminoethylphosphonate as the major soluble phosphorus metabolites. This combination of major metabolites appears to be unique to sipunculan erythrocytes. Nucleoside triphosphates and mannose 1-phosphate were present in lower concentrations. The concentration of *O*-phosphorylethanolamine within *P. gouldii* erythrocytes was established to be >20 mM. *T. zostericola* erythrocytes contained relatively high levels of 2-aminoethylphosphonate (on the order of 0.1 M) and lower levels of *O*-phosphorylethanolamine compared with those of *P. gouldii*. For *P. gouldii* and *T. zostericola* the intracellular pHs were determined to be 7.2 ± 0.1 and 7.1 ± 0.1, respectively, in air-equilibrated erythrocytes, and 6.5 ± 0.1 in anaerobic *P. gouldii* erythrocytes. *O*-Phosphorylethanolamine was found to bind weakly to *P. gouldii* metHr ($K_f \sim 7\ M^{-1}$). This interaction is best characterized by either negative cooperativity or nonspecific binding. *O*-Phosphorylethanolamine strongly inhibits azide binding to the iron site of *P. gouldii* metHr at pH 7.2. The rate of azide binding decreases by ~85-fold in the presence of 0.33 M *O*-phosphorylethanolamine. However, neither *O*-phosphorylethanolamine nor 2-aminoethylphosphonate at 0.33 M was found to have any significant effect on O_2 affinity of *P. gouldii* deoxyHr. Alternative functions for the two metabolites are suggested.

Hemoglobin has served as a focal point for numerous studies concerning requirements for reversible binding of O_2, as well as the structure, metabolism, and enzymology of erythrocytes. The non-heme iron O_2-carrying protein hemerythrin (Hr),[1] found in a few phyla of marine invertebrates, presents physiological and biochemical contrasts to the more widespread heme oxygen carriers (Klotz & Kurtz, 1984; Mangum, 1985, Terwilliger, 1985). Although a large body of information is available concerning the structure and reactivity of Hr itself (Wilkins & Harrington, 1983; Kurtz, 1986; Wilkins & Wilkins, 1987), relatively little is known about other constituents of Hr-containing erythrocytes.

Hr from erythrocytes of sipunculids and brachiopods usually consists of octamers ($M_r \sim 108\,000$) of essentially identical subunits. Each subunit contains a binuclear iron site that reversibly binds one molecule of O_2:

$$\underset{\text{deoxy}}{[Fe^{II},Fe^{II}]} + O_2 \rightleftharpoons \underset{\text{oxy}}{[Fe^{III},Fe^{III}O_2^{2-}]}$$

OxyHr slowly autoxidizes to $[Fe^{III},Fe^{III}]$metHr, which forms a particularly stable adduct with azide, metHrN_3. In this artificial adduct, azide is coordinated to one iron atom of the binuclear site (Stenkamp et al., 1984; Sieker et al., 1982), as is the peroxide in oxyHr (Stenkamp et al., 1985; Shiemke et al., 1984).

O_2 binding to sipunculan Hrs does not exhibit any significant cooperativity; however, brachiopodan Hrs do exhibit cooperativity in O_2 binding with a maximum Hill coefficient, n_{max} = 1.6-2.0 (Richardson et al., 1987). Intact coelomic cells containing Hr are reported to have a lower O_2 affinity than does isolated and purified deoxyHr (Weber & Fange, 1980; Mangum & Kondon, 1975). However, no physiological effector of deoxyHr has been identified. Ca^{2+}, Mg^{2+}, Cl^-, D- and L-lactate, ATP, and H^+ are all reported to have no appreciable effect on the O_2 affinities of purified sipunculan deoxyHrs (Petrou et al., 1981; Terwilliger et al., 1985; Mangum & Burnett, 1987; Richardson et al., 1987). Perchlorate is known to be an artificial heterotropic allosteric effector of sipunculan Hrs; its binding sites have been located 12-15 Å distant from the iron atoms (Stenkamp et al., 1978, 1983). Perchlorate has been shown to lower the affinities of anions for the iron site of metHr (Garbett et al., 1971a) and has recently been reported to lower the affinity of O_2 for the iron site of deoxyHr (Richardson et al., 1987).

† This research was supported by Grant GM 37851 from the National Institutes of Health (to D.M.K.).

[1] Abbreviations: Hr, hemerythrin; PEA, *O*-phosphorylethanolamine ($O_3^{2-}POCH_2CH_2NH_3^+$); 2-AEP, 2-aminoethylphosphonate ($O_3^{2-}PCH_2CH_2NH_3^+$); PEP, phosphoenolpyruvate; 2,3-DPG, 2,3-diphosphoglycerate; NTP, nucleoside triphosphate; NDP, nucleoside diphosphate; UDPG, uridine diphosphoglucose; NAD/NADH, nicotinamide adenine dinucleotide in its oxidized and reduced forms; PIPES, 1,4-piperazinediethanesulfonate; Bis-Tris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)-propane-1,3-diol; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonate.

0006-2960/88/0427-4458$01.50/0