Identification of Two Histidyl Residues in the Active Site of Human Placental Estradiol 17β-Dehydrogenase†

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ABSTRACT: To further characterize the active site of human placental estradiol 17β-dehydrogenase (EC 1.1.1.62), we have synthesized 12β-(bromoacetoxy)-4-estrene-3,17-dione and 16α-(bromoacetoxy)-1,3,5(10)-estratriene-3,17β-diol 3-methyl ether. Synthetic steps for the 12β derivative involved specific 12β-hydroxylation of 4-estrene-3,17-dione by *Collectotrichum gloeosporioides* and subsequent coupling with bromoacetic acid. Both of the affinity-labeling steroids are substrates and inactivate the enzyme in a time-dependent, irreversible manner, which is slowed by estradiol-17β. Samples of homogeneous enzyme (4 μM) were inactivated separately with 400 μM 12β-(bromo[2-^{14}C]acetoxy)-4-estrene-3,17-dione and 16α-(bromo[2-^{3}H]acetoxy)-1,3,5(10)-estratriene-3,17β-diol 3-methyl ether at pH 6.3. Aliquots of these samples, after acid hydrolysis, both yielded radioactive 1-(carboxymethyl)histidine, 3-(carboxymethyl)histidine, 1,3-bis(carboxymethyl)histidine, *S*-(carboxymethyl)cysteine, and small amounts of (carboxymethyl)lysine. The presence of estradiol-17β during the inactivation slowed histidyl alkylation (most significantly at the 3 position) but had little effect on alkylation of cysteine by both steroid bromoacetates. The two inactivated enzyme samples were combined, reduced and carboxymethylated, and hydrolyzed with trypsin. Three radioactive (carboxymethyl)histidyl-bearing peptide fractions were isolated by cation-exchange chromatography developed with a pyridine–acetate pH gradient. Each contained both ^{14}C and ^{3}H. The first of these contained only 1,3-bis(carboxymethyl)histidine as the labeled residue, the second only 3-(carboxymethyl)-histidine, and the third only 1-(carboxymethyl)histidine. Each of the three isolated fractions were treated with papain and chromatographed on a Sephadex G-15 column. In all three cases, two subpeptides were eluted, one bearing the [^{14}C]-carboxymethylated and the other bearing the [^{3}H]carboxymethylated histidyl residue. Therefore, a topography of the steroid-binding site is proposed in which two histidyl residues are present. One is affinity labeled by 12β-(bromo[2-^{14}C]-acetoxy)-4-estrene-3,17-dione and the other by 16α-(bromo-[2-^{3}H]acetoxy)-1,3,5(10)-estratriene-3,17β-diol 3-methyl ether, and neither residue is pinpointed at the site of the catalytic event.

Estradiol 17β-dehydrogenase (EC 1.1.1.62) has previously been purified to homogeneity (Chin & Warren, 1973) and crystallized (Chin et al., 1976) in this laboratory. Subsequent affinity labeling with 16α-(bromoacetoxy)estradiol 3-methyl ether revealed a histidyl residue presumed to be in the catalytic region of the active site (Chin & Warren, 1975). The study of Dreiding models reveals that if a substrate 12β-bromoacetoxy derivative should alkylate the same histidyl residue, that residue must be located at the site of the catalytic event, for it is only in the vicinity of the steroid 17α position that the rotamers of the reagent-bearing arms overlap.

Accordingly, we have synthesized 12β-(bromoacetoxy)-4-estrene-3,17-dione, shown that it is a substrate, and studied kinetic parameters. The enzyme was inactivated separately by the two affinity-labeling steroids. It was subsequently determined from the analyses of labeled histidyl-bearing peptides that 12β-(bromoacetoxy)-4-estrene-3,17-dione labels a histidyl residue that is different from the one labeled by 16α-(bromoacetoxy)estradiol 3-methyl ether.

Experimental Procedures

Reagent-grade salts and inorganic acids were from Mallinckrodt or Fisher. Reagent-grade organic solvents, bromoacetic acid, cyanogen bromide, Scintiverse, and Eastman thin-layer chromatography sheets (No. 6060) were from Fisher. Anhydrous organic solvents were freshly prepared according to Fieser & Fieser (1967–1979). All steroids were purchased from Steraloids, Inc. Nucleotides, hydroxylamine hydrochloride, Sepharose 4B, 2-mercaptoethanol, ammonium sulfate treated with EDTA,[1] Sephadex G-10 (120 mesh), G-15 (120 mesh), and G-50 (40 mesh), papain, standard amino acids, and *S*-(carboxymethyl)cysteine were from Sigma. Preparative silica gel plates containing fluorescent indicator were acquired from Analtech, Inc. Trypsin–TPCK was from Worthington Biochemical Co. The AG 50W-X8 cation-exchange resin was from Bio-Rad. Buffers and reagents for the amino acid hydrolyses and analyses were purchased from Pierce Chemical Co. Bromo[2-^{14}C]acetic acid (13 μCi/μmol) and bromo[2-^{3}H]acetic acid (23 μCi/μmol) were obtained from Amersham Corp. Dialysis tubing (No. 2) was from Spectrum Medical Industries.

Methods. The synthesis of 1-(carboxymethyl)histidine, 3-(carboxymethyl)histidine, and 1,3-bis(carboxymethyl)-histidine was according to Crestfield et al. (1963). Melting points were determined on a Meltemp apparatus and are reported without correction. Ultraviolet absorption spectra were recorded on a Cary 14 spectrophotometer. Infrared spectra were measured with a Beckman IR-18A spectrophotometer. Elemental analysis was performed by Galbraith Laboratories, Inc.

Amino acid analysis was performed according to the procedure of Spackman et al. (1958) with a Beckman Model

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[1] Abbreviations: estratriol 3-methyl ether, 1,3,5(10)-estratriene-3,16α,17β-triol 3-methyl ether; estradiol-17β, 1,3,5(10)-estratriene-3,17β-diol; 16α-(bromoacetoxy)estradiol 3-methyl ether, 16α-(bromoacetoxy)-1,3,5(10)-estratriene-3,17β-diol 3-methyl ether; EDTA, ethylenediaminetetraacetic acid; trypsin–TPCK, L-1-(tosylamido)-2-phenylethyl chloromethyl ketone–trypsin.

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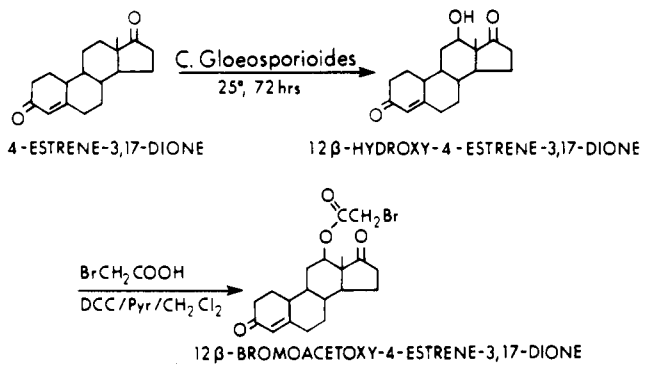

FIGURE 1: Synthesis of 12β-(bromoacetoxy)-4-estrene-3,17-dione as described in the text.

118C amino acid analyzer. Acid hydrolysis of protein was done in constant-boiling 6 N hydrochloric acid or 4 M methanesulfonic acid in evacuated, sealed tubes at 110 °C for 24 h. The effluent was collected in 0.4-mL fractions and counted in 10 mL of Scintiverse in a Model 3320 Packard Tri-Carb liquid scintillation spectrometer.

The enzyme was prepared according to Jarabak (1969) through the heat treatment step. It was further purified by affinity chromatography and crystallized by electrophoretic diffusion as previously reported (Chin & Warren, 1973; Chin et al., 1976). The final enzyme had a specific activity of 7.1 units/mg and was homogeneous on disc gel electrophoresis at pH 7.0 and 8.3. Activity was assayed according to Langer & Engel (1958).

Syntheses of Affinity-Labeling Steroids. The synthesis of 12β-(bromoacetoxy)-4-estrene-3,17-dione was by specific 12β-hydroxylation of 4-estrene-3,17-dione and subsequent bromoacetylation (Figure 1). The 12β-hydroxylation was carried out with *Collectrotrichum gloeosporioides* as described by Pan et al. (1967). Surface growth cultures were maintained at 25 °C in a medium consisting of 200 mL of cherry decot (the liquid portion of 1 kg of cherries boiled in 1 L of water for 1 h), 50 g of agar, and 800 mL of water. Growth of the organism in Petri dish cultures was allowed to proceed for 7–10 days prior to use. Inocular from the surface cultures were then transferred to 500-mL flasks, each containing 200 mL of medium consisting of 40 mL of cherry decot and 160 mL of water. After 48–72 h of incubation with continuous agitation (rotary or linear) at 25 °C, each of the 200-mL cultures was transferred to a 3-L flask containing 1.5 L of growth medium. Growth was allowed to proceed for an additional 24 h with agitation at 25 °C as previously noted. Then 1 g of 4-estrene-3,17-dione, dissolved in 10 mL of *N,N*-dimethylformamide, was added to each flask. Incubation proceeded with continuous agitation for 10 days. The cells were collected on a fritted suction funnel and washed twice with 100-mL portions of hot water, which were added to the filtrate. The filtrate was extracted with an equal volume of chloroform, and the solvent was removed under reduced pressure. The residue was taken up in chloroform and chromatographed on 500-μm preparative silica gel plates developed with chloroform–methanol (95:5). The R_f values for 12β-hydroxy-4-estrene-3,17-dione and 4-estrene-3,17-dione are 0.72 and 0.92, respectively. The steroid was eluted with chloroform and the solvent evaporated. The product was crystallized from hot ethanol, yielding 240 mg (23%): mp 183–185 °C; $[\alpha]_D$ +121° (chloroform); IR ν_{max}^{KBr} 3448 (OH), 1745 (C=O), 1672 (C=O), and 1610 cm^{-1} (C=C). These data agree with those of Pan et al. (1967).

Bromoacetylation was accomplished by sequential addition of 1.2 mmol of bromoacetic acid, 2.0 mmol of dicyclohexylcarbodiimide, and 1.2 mmol of dry pyridine to a stirred solution of 1.0 mmol of 12β-hydroxy-4-estrene-3,17-dione in 40 mL of dry methylene chloride cooled to 0 °C. The reaction mixture was stirred for 1 h at 0 °C and at room temperature for 1 h. The mixture was filtered and the filtrate concentrated and purified on 500-μm preparative silica gel plates developed with chloroform–acetone (1:1). The 12β-(bromoacetoxy)-4-estrene-3,17-dione (R_f 0.8) was eluted with acetone and recrystallized from ethanol, yielding 128 mg (32%): mp 195–198 °C dec; IR ν_{max}^{KBr} 1745 (C=O), 1672 (C=O), and 1610 cm^{-1} (C=C). The characteristic absorbance of the hydroxyl group (3448 cm^{-1}) that was observed with the starting material (12β-hydroxy-4-estrene-3,17-dione) had disappeared. Anal. Calcd for $C_{20}H_{25}O_4Br$: C, 58.78; H, 6.11; Br, 19.55. Found: C, 59.65; H, 6.49; Br, 18.64.

The 12β-(bromo[2-^{14}C]acetoxy)-4-estrene-3,17-dione was synthesized in a similar manner by reacting 130 μmol of 12β-hydroxy-4-estrene-3,17-dione, 130 μmol of bromo[2-^{14}C]acetic acid (13 μCi/μmol), 260 μmol of dicyclohexylcarbodiimide, and 240 μmol of dry pyridine. After purification and crystallization, the specific activity of the radioactive product was determined to be 13 μCi/μmol with a yield of 14.9 mg (28%).

The syntheses of 16α-(bromoacetoxy)estradiol 3-methyl ether and its radioactive derivatives were accomplished by bromoacetylation of estratriol 3-methyl ether with bromoacetic acid, bromo[2-^{3}H]acetic acid, or bromo[2-^{14}C]acetic acid as described in an earlier report (Chin & Warren, 1975). The specific activities of the ^{3}H and ^{14}C derivatives were 23 and 13 μCi/μmol, respectively.

Kinetic Experiments. The assay using 12β-(bromoacetoxy)-4-estrene-3,17-dione was conducted at pH 6.3 as described by Jarabak (1969). Assays were carried out at 25 °C, and the enzyme activity was determined from the initial linear decrease in absorbance at 340 nm. The K_m of the derivative was determined by varying the amount of substrate for each assay and plotting the reciprocals of velocity and substrate concentration according to the method of Lineweaver & Burk (1934). It has previously been determined that 16α-(bromoacetoxy)estradiol 3-methyl ether is a substrate for estradiol 17β-dehydrogenase (Chin & Warren, 1975).

Inactivation of Estradiol 17β-Dehydrogenase by 12β-(Bromoacetoxy)-4-estrene-3,17-dione and 16α-(Bromoacetoxy)estradiol 3-Methyl Ether. All enzyme samples were mixed with 10% ethanol in buffer A (10 mM potassium phosphate, 1 mM EDTA, and 20% glycerol, pH 7.0) at 25 °C for 24 h before the inactivation experiments were started. This was due to the slight increase in enzyme activity upon the addition of ethanol. The inactivations were commenced by the addition of 100-fold molar excess of either 12β-(bromo[2-^{14}C]acetoxy)-4-estrene-3,17-dione, 16α-(bromo[2-^{14}C]acetoxy)estradiol 3-methyl ether, or 16α-(bromo[2-^{3}H]acetoxy)estradiol 3-methyl ether to the preincubated enzyme solution. Estradiol-17β was present in some incubations at a concentration 4 times that of the affinity-labeling steroid. The rate of inactivation was monitored by assaying enzyme activity as described above. The reactions were performed at both pH 7.0 and pH 6.3. (The enzyme is stable from pH 6.2 to pH 9.8.) The pH was adjusted by adding 0.5 M KH_2PO_4. Portions of the reaction mixtures were removed at various times and the inactivations stopped by the addition of a molar excess of 2-mercaptoethanol. Control samples containing no steroid were also maintained. Samples were dialyzed against 100 volumes of water (three changes) for 1 day, 0.05 N NaOH for 1 day, and water again until the radioactivity outside the

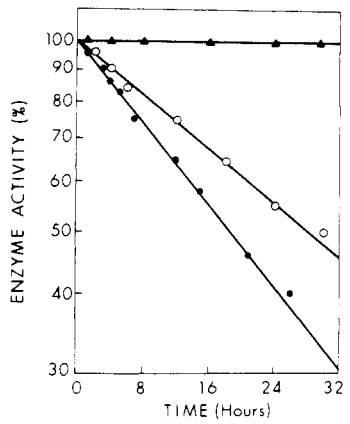

FIGURE 2: Inactivation of 1.0 μM estradiol 17β-dehydrogenase in buffer A, containing 20% glycerol, pH 7, 20 °C, by 100 μM 12β-(bromoacetoxy)-4-estrene-3,17-dione (●), 100 μM 12β-(bromoacetoxy)-4-estrene-3,17-dione and 100 μM estradiol-17β (○), and either a control of enzyme only or with 100 μM bromoacetate (▲). Each point is a mean of three determinations.

bag was reduced to background. The dialyzed samples were lyophilized.

Tryptic Hydrolysis of Inactivated Enzyme. Lyophilized samples for hydrolyses with trypsin–TPCK were resuspended in a minimal amount of 0.1 M NH_4HCO_3 buffer, pH 8.0, and dialyzed against 100 volumes of 1 M phosphate–8 M urea buffer, pH 8.0. The samples were treated for 3 h with 1 mM unlabeled iodoacetic acid at 25 °C under nitrogen. Each of the reduced and carboxymethylated samples was dialyzed against 100 volumes of 0.1 M NH_4HCO_3 buffer, pH 8.0, then lyophilized, and kept desiccated below 0 °C. A 2.3-mg aliquot of lyophilized powder of enzyme inactivated 45% by 12β-(bromo[2-^{14}C]acetoxy)-4-estrene-3,17-dione (1.2×10^7 dpm) was mixed with 1.9 mg of powder from enzyme 48% inactivated by 16α-(bromo[2-^{3}H]acetoxy)estradiol 3-methyl ether. The residues were dissolved in 2.0 mL of 0.1 M NH_4HCO_3 buffer, pH 8.0, 0.2 mg of trypsin–TPCK was added, and the solution was allowed to react for 17 h at 37 °C. The sample was applied to a Sephadex G-50 (40 mesh) column (5 × 100 cm, V_0 = 600 mL) and eluted with the ammonium bicarbonate buffer. The effluent was monitored at 254 nm, and aliquots were counted for the determination of radioactivity. The effluent eluted between 800 and 1400 mL (containing 80% of total radioactivity) was pooled and lyophilized. The residue was resuspended in 5 mL of 0.05 M pyridine–acetate buffer, pH 2.5, applied to an AG 50W-X8 column (2.5 × 100 cm), and eluted with a linear gradient from 0.2 M pyridine–acetate, pH 3.0, to a limit buffer of 2 M pyridine–acetate, pH 4.5. Fractions of 2 mL were collected at a rate of 20 mL/h. Aliquots were taken for determination of radioactivity. The major radioactive peaks were collected separately and rechromatographed in the same system. Aliquots were taken from each peak for amino acid analysis.

Papain Digestion and Isolation of Peptides. The peaks eluted from the cation-exchange column containing both ^{14}C- and ^{3}H-labeled carboxymethylated histidyl residues (fractions II, IV, and V) were collected as separate samples. The solvent was removed from aliquots of each sample under reduced pressure and the residue resuspended in a minimal volume of 0.1 M acetate buffer, pH 5.5, and digested with 5 μg of papain for 17 h at 37 °C. The digests were applied to a column of Sephadex G-15 (120 mesh) (1.5 × 100 cm) and eluted with 0.05 M pyridine–acetate buffer, pH 2.5. Fractions of 2.0 mL were collected. When no further radioactivity could be detected in the effluent, the column was eluted with 1.0 L of 0.1 M NH_4HCO_3 buffer, pH 8.0, and 1.0 L of water.

Table I: Effect of pH on Affinity Labeling of Estradiol 17β-Dehydrogenase[a]

affinity-labeling steroid	CM amino acid[b] 1,3-DCM-His	1-CM-His	3-CM-His	*S*-CM-Cys	CM-Lys
enzyme (58%)[c] + 12β-BAE, pH 7.0	870[d]	1420	790	40 500	2020
enzyme (34%) + 12β-BAE, pH 6.3	1700	5730	6070	12 740	2110
enzyme (38%) + 16α-BAE_2, pH 7.0	800	1450	720	31 800	1190
enzyme (24%) + 16α-BAE_2, pH 6.3	1990	3250	3670	11 260	3730

[a] Enzyme (5 μM) was incubated with either 12β-(bromo[2-^{14}C]acetoxy)-4-estrene-3,17-dione (12β-BAE, 500 μM) or 16α-(bromo[2-^{14}C]acetoxy)estradiol 3-methyl ether (16α-BAE_2, 500 μM) at pH 7.0 or 6.3 as described in the text. [b] CM, carboxymethyl; DCM, bis(carboxymethyl). [c] Percent of enzyme inactivated by the steroid. [d] Radioactivity is expressed in dpm.

Table II: Effect of Estradiol-17β on Affinity Labeling by 16α-(Bromoacetoxy)estradiol 3-Methyl Ether at pH 6.3[a]

inactivated sample	CM amino acid[b] 1,3-DCM-His	1-CM-His	3-CM-His	*S*-CM-Cys	CM-Lys
enzyme (29%)[c] + 16α-BAE_2	2410[d]	8400	7850	38 110	2560
enzyme (19%) + 16α-BAE_2 + E_2	1700	4640	2460	19 480	1130
enzyme (55%) + 16α-BAE_2	5010	11870	10830	32 200	6810
enzyme (39%) + 16α-BAE_2 + E_2	1990	6310	3900	31 080	3060

[a] Enzyme (5 μM) was incubated with 16α-(bromo[2-^{14}C]-acetoxy)estradiol 3-methyl ether (16α-BAE_2, 500 μM) with and without estradiol-17β (E_2, 2 mM) at pH 6.3 as described in the text. [b] CM, carboxymethyl; DCM, bis(carboxymethyl). [c] Percent of enzyme inactivated by the steroid. [d] Radioactivity is expressed in dpm.

Results

Inactivation with 12β-(Bromoacetoxy)-4-estrene-3,17-dione and 16α-(Bromoacetoxy)estradiol 3-Methyl Ether. The kinetics of inactivation of estradiol 17β-dehydrogenase (1 μM) at pH 7.0 by 12β-(bromoacetoxy)-4-estrene-3,17-dione (100 μM) with or without equimolar estradiol-17β are shown in Figure 2. The $t_{1/2}$ = 18 h for the affinity-labeling steroid alone but is slowed in the presence of estradiol-17β ($t_{1/2}$ = 30 h). The control remained stable throughout this time. The K_m for 12β-(bromoacetoxy)-4-estrene-3,17-dione was determined to be 133 μM with a V_{max} = 0.2 nmol min^{-1} (μg of enzyme)$^{-1}$. As it is a substrate, it must bind at the active site. The rate of inactivation by 16α-(bromoacetoxy)estradiol 3-methyl ether has previously been shown to be slowed by the presence of excess estradiol-17β (Chin & Warren, 1975).

Amino Acid Analyses of Estradiol 17β-Dehydrogenase Inactivated at pH 6.3 and 7.0. The relative quantity of histidine alkylation was significantly increased at pH 6.3 compared to that found at pH 7.0 for both alkylating steroids (Table I). When estradiol 17β-dehydrogenase (5 μM) was inactivated by either 12β-(bromo[2-^{14}C]acetoxy)-4-estrene-3,17-dione or 16α-(bromo[2-^{14}C]acetoxy)estradiol 3-methyl ether (500 μM) at pH 6.3, there was an increase in the absolute counts in (carboxymethyl)histidyl residues even though the percent of enzyme inactivated was less than in samples

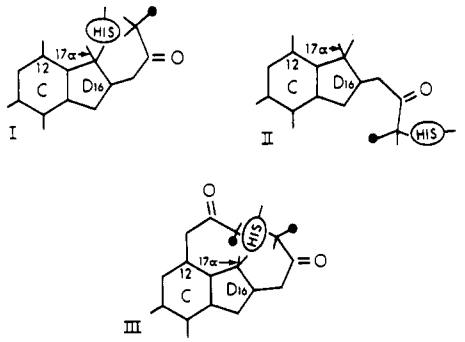

FIGURE 3: The two bromoacetoxy groups of 12β-(bromoacetoxy)-4-estrene-3,17-dione and 16α-(bromoacetoxy)estradiol 3-methyl ether overlap the catalytic region of estradiol 17β-dehydrogenase and would both affinity label a single histidyl residue, which proximates the steroid 17α hydrogen. For clarity, the 18-methyl group has been left out.

reacted at pH 7.0. Also at pH 6.3, the presence of excess estradiol-17β, which previously was shown to slow the rate of inactivation, decreases the relative quantity of histidine that is alkylated by 16α-(bromo[2-^{14}C]acetoxy)estradiol 3-methyl ether (Table II).

The stoichiometry of the inactivation was determined for 12β-(bromo[2-^{14}C]acetoxy)-4-estrene-3,17-dione on samples of 11, 22, and 58% inactivated enzyme. At pH 7.0, 2 mol of steroid was covalently bound per mol of enzyme inactivated. When repeated at pH 6.3, again 2.0, 2.1 and 2.1 mol are incorporated for each mol of enzyme inactivated at 14, 34, and 48%, respectively. Similar stoichiometry was reported for the 16α derivative (Chin & Warren, 1975). For both steroids, the stoichiometry of millimoles of histidine alkylated per millimole of enzyme inactivated was 0.1 at pH 7.0 and 0.6 at pH 6.3.

Isolation of Histidyl-Bearing Peptides from Tryptic Digests. As mentioned earlier, a histidyl residue proximating the 17α position of the bound steroid should be accessible to the reactive bromoacetoxy arms of both 12β-(bromoacetoxy)-4-estrene-3,17-dione and 16α-(bromoacetoxy)estradiol 3-methyl ether (Figure 3). If one steroid is labeled with ^{14}C and the other with ^{3}H, then the peptide fraction containing a single histidyl residue near the steroid 17α hydrogen should be labeled with both ^{14}C and ^{3}H after affinity labeling.

The experiment to determine this was conducted by incubating 4 μM enzyme in buffer A separately with 400 μM 12β-(bromo[2-^{14}C]acetoxy)-4-estrene-3,17-dione and 16α-(bromo[2-^{3}H]acetoxy)estradiol 3-methyl ether at pH 6.3. The final volumes were 50 and 35 mL, respectively. Approximately 80% of the radioactivity was recovered from Sephadex G-50 chromatography of the tryptic digests of the inactivated enzyme by pooling the effluent between 800 and 1400 mL. The ratio of ^{14}C/^{3}H was 4.8, which was the same as that of the original sample. By discarding the eluate between 600 and 800 mL, we removed most of the unhydrolyzed protein and unlabeled material (defined by absorbance). The reference tetrapeptide, Trp-Met-Asp-Phe, which has a molecular weight of 633, eluted at 1600 mL. Since the enzyme contains 20 lysyl and 44 argininyl residues and has a molecular weight of 68 000 (Engel & Groman, 1974), the average molecular weight of peptides expected from trypsin digestion is 1000.

The recovered sample was chromatographed on AG 50W-X8 as seen in Figure 4. Six radioactive peaks were eluted and identified as to their radioactive residue (peaks I–VI). All contained both ^{14}C label and ^{3}H label. Rechromatography of each separately in the same system revealed a single peak in each case. The amino acid analyses demonstrate that, in terms of radiolabeled residues, the peaks I, II, III, IV, and V

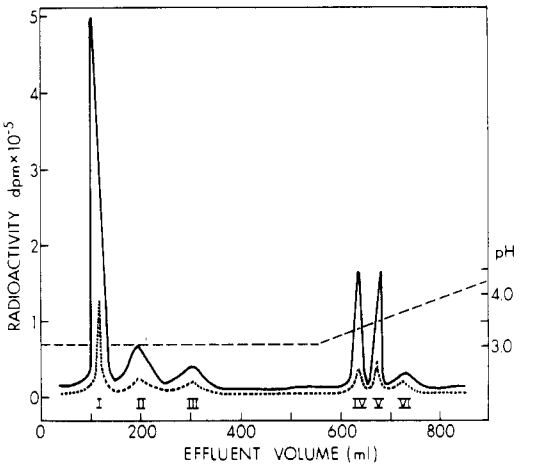

FIGURE 4: Tryptic digests of enzyme inactivated by 12β-(bromo[2-^{14}C]acetoxy)-4-estrene-3,17-dione or 16α-(bromo[2-^{3}H]acetoxy)estradiol 3-methyl ether were eluted from Sephadex G-50 (40 mesh), lyophilized, applied to an AG 50W-X8 column (2.5 × 100 cm), and eluted with a linear gradient of 500 mL of 0.2 M pyridine–acetate buffer, pH 3.0, to a limit buffer of 2 M pyridine–acetate, pH 4.5 (500 mL). Fractions of 2 mL were collected and aliquots counted to determine the ^{14}C (—) and ^{3}H (---) content.

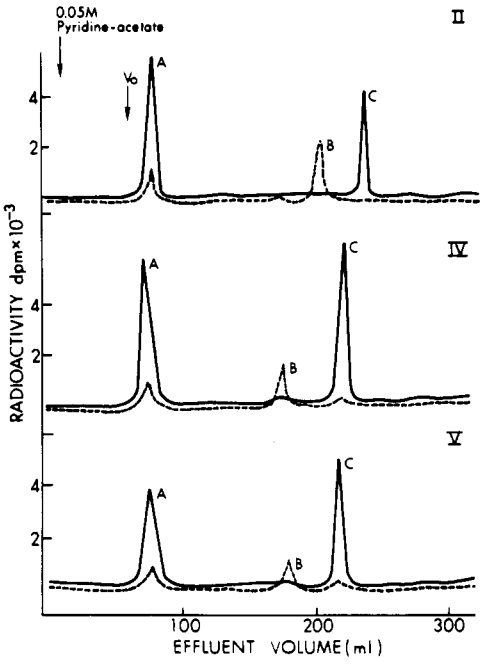

FIGURE 5: Profiles of papain digests on a Sephadex G-15 (120 mesh) column (1.5 × 100 cm) when eluted as described in the text. The samples were previously eluted from AG 50W-X8, treated with papain, lyophilized, and applied to the column. Fractions of 2 mL were collected and aliquots counted to determine the ^{14}C (—) and ^{3}H (---) content.

contained *exclusively* S-(carboxymethyl)cysteine, 1,3-bis-(carboxymethyl)histidine, ε,ε-bis(carboxymethyl)lysine, 3-(carboxymethyl)histidine, and 1-(carboxymethyl)histidine, respectively. Fraction VI disappeared on rechromatography, distributed among several small peaks. The ratio of ^{14}C/^{3}H in each of the histidyl-bearing fractions was as follows: II, 4.4; IV, 8.1; V, 5.7.

Hydrolysis of (Carboxymethyl)histidyl-Bearing Peptides (II, IV, and V) with Papain. The chromatographic profile from Sephadex G-15 for each of these three peptide fractions, hydrolyzed separately, is shown in Figure 5. In all three cases, the following applies. Three major fractions were developed. Fraction A retains the same mobility as that of the original sample (as shown by running unhydrolyzed controls). A

second digestion of fraction A with papain produced more of fractions B and C. Fraction B contained only ^{3}H and fraction C only ^{14}C. Following the second digestion, no more than 20% of the radioactivity remained in fraction A. Amino acid analyses of fractions B and C derived from the ion-exchange fraction IV revealed only ^{3}H-labeled 3-(carboxymethyl)histidne and ^{14}C-labeled 3-(carboxymethyl)histidine, respectively.

Discussion

It has been shown that both cysteinyl and histidyl residues are essential for the catalytic activity of several pyridine nucleotide dependent dehydrogenases (Keleti, 1970). Biellman et al. (1976) affinity labeled three cysteinyl residues in human placental estradiol 17β-dehydrogenase with 3-(chloroacetyl)pyridine adenine dinucleotide and presumed some or all of these to be at the active site. Indeed every bromoacetoxy steroid used with this enzyme has alkylated cysteinyl residues. Unfortunately, the rapidity of the inherent reaction rate of such compounds with model cysteinyl derivatives at pH 7.0 is 50–100 times faster than those seen with the model compounds of histidine, lysine, methionine, and tryptophan. Further, three to four of the six cysteinyl residues in the enzyme can be modified by non-site-directed sulfhydryl reagents without loss of activity, and all residues are "protected" by a cofactor (Pons et al., 1977). This makes localization of cysteinyl residues in this enzyme by affinity-labeling techniques difficult, if not impossible. Therefore, in this study, we concentrated on the histidyl residues by conducting the affinity-labeling studies at pH 6.3, where considerably less cysteinyl alkylation is noted and the histidyl residues are better detected.

This and previous work (Chin & Warren, 1975) shows that both 12β-(bromoacetoxy)-4-estrene-3,17-dione and 16α-(bromoacetoxy)estradiol 3-methyl ether are substrates for the enzyme and that both affinity label histidyl residues. Affinity labeling with both steroids at pH 6.3 yields 1,3-bis-(carboxymethyl)histidine, 1-(carboxymethyl)histidine, and 3-(carboxymethyl)histidine. Although generation of all three is diminished by estradiol-17β, the effect is clearly most impressive in the case of 3-(carboxymethyl)histidine. The data presented show that the histidyl residue carboxymethylated in the 3 position by 12β-(bromo[2-^{14}C]acetoxy)-4-estrene-3,17-dione is different from, and therefore must be at a different region of the active site than, the one carboxymethylated by 16α-(bromo[2-^{3}H]acetoxy)estradiol 3-methyl ether. Similar nonidentity of position in the protein is also the case in terms of 1-(carboxymethyl)histidine and bis(carboxymethyl)histidine found on amino acid analyses after affinity labeling by these two steroids. It is also very likely that some of the histidyl alkylation is nonspecific in consideration of the small inhibition by the excess estradiol-17β.

Thus, none of these residues appears to proximate the region of the steroid (as it undergoes reversible binding at the active site) where the catalytic event occurs. Further, the 3-(carboxymethyl)histidyl-bearing peptide generated by affinity labeling with 12β-(bromo[2-^{14}C]acetoxy)-4-estrene-3,17-dione has mobility on electrophoresis at pH 6.5, 3.5, and 1.9 identical with that of the 3-(carboxymethyl)histidyl-bearing peptide generated on affinity labeling with 3-(bromo[2-^{14}C]acetoxy)-1,3,5(10)-estratrien-17-one (unpublished results). Thus, it appears that the histidyl residue alkylated by both of these steroids lies in the upper A–B-ring region of the steroid, some distance from the site of the catalytic event.

Structural, crystallographic studies of the 2-hydroxy acid dehydrogenases have indicated that in those that do not require zinc, the transition state at the site of catalysis is induced by hydrogen bonding of the reduced substrate –OH hydrogen with the unprotonated imidazole ring of a specifically located histidyl residue while the oxidized substrate carbonyl group forms a hydrogen bond with the same but protonated imidazole. The histidine at the site of the catalytic event is His-195 in lactate dehydrogenase (Parker & Holbrook, 1977), His-176 in glyceraldehyde-3-phosphate dehydrogenase (Buehner et al., 1974), and His-181 in malate dehydrogenase (Banaszak & Bradshaw, 1975). Because all of these structurally defined dehydrogenases have a histidyl residue at the site of the catalytic event, we presumed this mechanism would be operative in the steroid dehydrogenases. Therefore, we were surprised when the 12β and 16α derivatives failed to label the same histidyl residue.

One can only conclude either (a) that the predicted mechanism is not operative or (b) that some obstacle limits access of the 12β-bromoacetoxy group to the upper anterior (β face) D-ring region of the steroid. We favor the latter possibility, considering the steric hindrance of the angular 18-methyl group, the preferential orientation of the bromoacetoxy side chain induced by the steroid moiety, or the physical interposition of hydrophobic amino acid residues at the binding site may direct the 12β-bromoacetoxy side chain toward the upper A–B-ring region of the steroid and limit its access to a histidyl residue that does, indeed, reside at the site of catalysis. Accordingly, as we have crystallized this enzyme (Chin et al., 1976), we are currently engaged in generation of crystals satisfactory for X-ray crystallographic studies to settle this point.

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