Human Placental Estradiol 17β-Dehydrogenase: Sequence of a Histidine-Bearing Peptide in the Catalytic Region†

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Received July 22, 1985

ABSTRACT: The amino acid sequence of an octapeptide from the catalytic site of human placental estradiol 17β-dehydrogenase (EC 1.1.1.62) was established by affinity-labeling techniques. The enzyme was inactivated separately by 12β-hydroxy-4-estrene-3,17-dione 12-(bromo[2-^{14}C]acetate) and 3-methoxyestriol 16-(bromo[2-^{14}C]acetate) at pH 6.3. The inactivations, in both cases, followed pseudo-first-order kinetics with half-times for the 12β and 16α derivatives being 192 and 68 h, respectively. Both derivatives are known substrates that inactivate in a time-dependent, irreversible manner and that modify cysteine residues to form (carboxymethyl)cysteine and histidine residues to form either N^{τ}- or N^{π}-(carboxymethyl)histidine. The inactivated enzyme samples were separately reduced, carboxymethylated, and digested with trypsin. The tryptic digests were applied to Sephadex G-50 and the radioactive N^{τ}- and N^{π}-(carboxymethyl)histidine-bearing peptides identified. The peptides were further purified by cation-exchange chromatography and gel filtration. Final purification was achieved by HPLC prior to sequencing. It was determined that both steroid derivatives modified either of the two histidine residues in the peptide Thr-Asp-Ile-His-Thr-Phe-His-Arg. These histidines are different from a histidine that was previously shown to be alkylated by estrone 3-(bromoacetate) and that was presumed to proximate the A ring of the bound steroid. It is concluded that the two histidine residues identified in the present study proximate the D ring of the steroid as it binds at the active site and may participate in the hydrogen transfer effected by human placental estradiol 17β-dehydrogenase.

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. The topography of the active site has been studied by affinity labeling with various steroid bromoacetates. Steroids bearing the reagent group at the 3-, 12β- or 16α-position affinity alkylate histidine residues and yield N^{τ}-(carboxymethyl)histidine or N^{π}-(carboxymethyl)histidine on subsequent acid hydrolysis of the enzyme.

Previous work (Chin et al., 1982) has demonstrated that the 3-methoxyestriol 16-(bromoacetate) and 12β-hydroxy-4-estrene-3,17-dione 12-(bromoacetate) label different histidine residues to form N^{π}-(carboxymethyl)histidine. However, these data did not exclude the possibility of also labeling a single histidine at different positions in the imidazole ring by these two steroid derivatives. Other studies (Murdock et al., 1983) have shown that the 3- and 12β-steroid bromoacetates will modify the same histidine residue at the N^{π}-position of the histidine, which was presumed to be spatially oriented near the steroid A ring. We have demonstrated that there are at least two different histidine residues present at the active site and that one of these proximates the D ring of the steroid and, thus, may take part in the catalytic event. To support this proposal, we have isolated and sequenced a tryptic peptide of estradiol 17β-dehydrogenase containing a histidine residue that is modified by both 3-methoxyestriol 16-(bromoacetate) and 12β-hydroxy-4-estrene-3,17-dione 12-(bromoacetate).

Experimental Procedures

Reagent-grade salts, inorganic acids, reagent-grade organic solvents, bromoacetic acid, dicyclohexylcarbodiimide, dialysis tubing (Spectra/Por No. 2), and liquid scintillation counting fluor were obtained from Fisher. Steroids were from Steraloids. Nucleotides, Reactive Blue 2–agarose, Sephadexes G-10 and G-50, and human serum albumin were from Sigma. The AcA-34 gel filtration medium was from LKB. Preparative silica gel plates containing fluorescent indicator were obtained from Analtech. Trypsin–TPCK[1] was from Worthington. The AG 50W-X8 cation-exchange resin was from Bio-Rad. Buffers and reagents for the amino acid hydrolyses and analyses were from Pierce Chemical Co. The bromo[2-^{14}C]acetic acid was obtained from Amersham. Ammonium sulfate and urea (ultrapure grade) were from Schwarz/Mann. Sequencer-grade reagents and solvents for HPLC were obtained from Burdick and Jackson Laboratories.

Methods. The syntheses of the standard compounds N^{τ}-(carboxymethyl)histidine, N^{π}-(carboxymethyl)histidine, and N^{τ},N^{π}-bis(carboxymethyl)histidine were according to Crestfield et al. (1963). Amino acid analyses were performed according to the procedure of Spackman et al. (1959) with a Beckman Model 118C amino acid analyzer. Acid hydrolysis of protein was done in constant-boiling 6 N hydrochloric acid in evacuated, sealed tubes at 110 °C for 24 h. The effluent from the analyses was collected in 0.4-mL fractions and counted in 5 mL of fluor in a Model 3320 Packard Tri-Carb liquid scintillation spectrometer.

The enzyme was prepared according to Jarabak (1969) through the heat-treatment step. The heat-treated enzyme was centrifuged at 36000*g* for 1.5 h, and the supernatant derived from eight placentas was stirred with 100 mL of Reactive Blue 2–agarose for 1 h. The slurry was filtered and the gel washed with at least 20 volumes of 0.01 M potassium

† This research was supported by Grant AM-15708 from the National Institutes of Health.
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[1] Abbreviations: trypsin–TPCK, trypsin–L-1-(tosylamido)-2-phenylethyl chloromethyl ketone; EDTA, ethylenediaminetetraacetic acid; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

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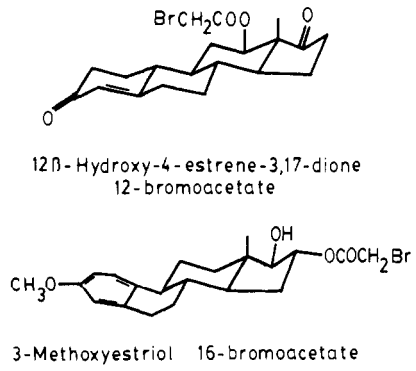

FIGURE 1: Affinity-labeling substrates for estradiol 17β-dehydrogenase.

phosphate buffer, pH 7.2, containing 5 mM EDTA and 20% glycerol (buffer A). The gel was washed until no protein could be detected in the filtrate. The enzyme was eluted 3 times in a batch with 100 mL of buffer containing 0.1 mM $NADP^+$ and 0.1 mM estrone. The eluates were pooled, and the enzyme was precipitated by 50% saturation with solid $(NH_4)_2SO_4$. The precipitate was resuspended in 20 mL of buffer A, applied to a column (2.6 × 100 cm) of AcA-34, eluted with buffer A. The eluted enzyme had a specific activity of 7.5 IU/mg and was shown to be a single band on SDS–PAGE. Enzyme activity was assayed according to Langer & Engel (1958). The enzyme preparation was stable at room temperature up to 3 months.

The syntheses of 12β-hydroxy-4-estrene-3,17-dione 12-(bromo[2-^{14}C]acetate) and 3-methoxyestriol 16-(bromo[2-^{14}C]acetate) (Figure 1) have been previously described (Chin et al., 1982; Chin & Warren, 1975). The specific activities of the purified 12β and 16α derivatives were 19.1 and 27.6 mCi/mmol.

Inactivation of Estradiol 17β-Dehydrogenase by 12β-Hydroxy-4-estrene-3,17-dione 12-(Bromo[2-^{14}C]acetate) and 3-Methoxyestriol 16-(Bromo[2-^{14}C]acetate). Thirteen milligrams of enzyme (0.19 μmol) was inactivated by 12β-hydroxy-4-estrene-3,17-dione 12-(bromo[2-^{14}C]acetate) (7.5 μmol) in a total volume of 32 mL of buffer A, pH 6.3, containing 10% ethanol. In a separate inactivation, the enzyme (40 mg, 0.58 μmol) was incubated in the same buffer system with 3-methoxyestriol 16-(bromo[2-^{14}C]acetate) (5.8 μmol) in a volume of 116 mL. Control samples containing no steroid maintained 100% activity through the experimental time. The rate of inactivation was monitored by assaying enzyme activity as described above. The reactions were stopped by the addition of a 100 molar excess of 2-mercaptoethanol. The reaction mixtures were dialyzed against 0.1 M NH_4HCO_3 buffer, pH 8.0, until the radioactivity in the dialyzate had returned to background values. An aliquot of the dialyzed, inactivated enzyme was removed from each sample for amino acid analysis, and the remaining solution was dialyzed against 5 volumes of 0.1 N NaOH for 2 h to facilitate the cleavage of the steroid ester bond. The removal of the steroid is necessary for efficient proteolytic digestion of the enzyme.

Peptide Isolation and Purification. The enzyme solutions were dialyzed against 10 volumes of 8 M urea in 1 M potassium phosphate buffer, pH 8.0, overnight with two changes. The resulting denatured enzyme solutions were flushed with N_2, and 2-mercaptoethanol was added to make a 10-fold molar excess with respect to the sulfhydryl groups of the enzyme. The samples were stirred 20 min at 25 °C, sealed under N_2. A 10-fold molar excess of unlabeled iodoacetic acid and a molar equivalent of NaOH were added, and the mixture was stirred an additional 15 min under N_2. The reaction was stopped by addition of excess 2-mercaptoethanol. The samples were dialyzed 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 addition, the samples were allowed to react overnight at 37 °C. The samples were lyophilized to reduce their volumes to less than 50 mL.

The tryptic digests were applied to a Sephadex G-50 column (5 × 100 cm, V_0 = 500 mL) and eluted with the ammonium bicarbonate buffer. The effluent was collected in 3.5-mL fractions (18 mL/h). The radioactive profiles were determined by counting 5-μL aliquots of alternate fractions in 5 mL of scintillation fluor. Fractions were pooled for each radioactive peak, aliquots taken for amino acid analyses, and the pools lyophilized. The residues of those fractions containing radioactive N^{τ}-(carboxymethyl)histidine or N^{π}-(carboxymethyl)histidine were resuspended in a minimal volume of 0.05 M pyridine–acetate buffer, pH 2.3 (buffer I). The samples were applied to a column (2.6 × 35 cm) of AG 50W-X8 (400 mL) that had previously been washed with 0.1 L each of 1 N NaOH, H_2O, 3 N HCl, H_2O, and 2 M pyridine and equilibrated with buffer I at 41 °C for 3 h. The eluting pH gradient was started immediately with 300 mL of buffer I and 300 mL of the limit buffer of 2 M pyridine–acetate, pH 5.0. Fractions of 2.3 mL were collected at a rate of 20 mL/h. Radioactivity was determined by counting 10 μL of alternate fractions; pH measurements were made on every tenth fraction. Aliquots of each specific radioactive fraction were taken to dryness under reduced pressure at 40 °C. The residues of those fractions containing radioactive N^{τ}-(carboxymethyl)histidine or N^{π}-(carboxymethyl)histidine were dissolved in 0.2% formic acid and desalted on a Sephadex G-10 column (1.6 × 100 cm), which was eluted with 0.2% formic acid. The pooled radioactive fractions were lyophilized for final purification by HPLC.

The desalted fractions were resuspended in 500 μL of 0.05% trifluoroacetic acid. The samples were applied 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 either 0.25% or 1.0% limit buffer per min. The effluent was monitored at 214 nm, and 0.5-min fractions were collected. The radioactivity content of each fraction was determined. Those radioactive peptides eluting prior to the start of the gradient were subsequently chromatographed on an Altex/PTH Ultrasphere-octyl column eluted with the same gradient.

Amino Acid Sequencing. 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 a 95% repetitive yield. Each fraction was dried, dissolved in 50 μL of methanol, and converted to the phenylthiohydantoin derivative. A 5–20-μL aliquot was taken for radioactivity determination. The amino acid identification was performed on a Hewlett-Packard 1084B high-performance liquid chromatograph equipped with an Altex/PTH column. A gradient of sodium acetate (0.3 M) and methanol/acetonitrile (17:3) was used for elution.

RESULTS

Inactivation with 12β-Hydroxy-4-estrene-3,17-dione 12-(Bromoacetate) and 3-Methoxyestriol 16-(Bromoacetate). The inactivation of estradiol 17β-dehydrogenase at pH 6.3 by the two steroid derivatives in separate incubations followed

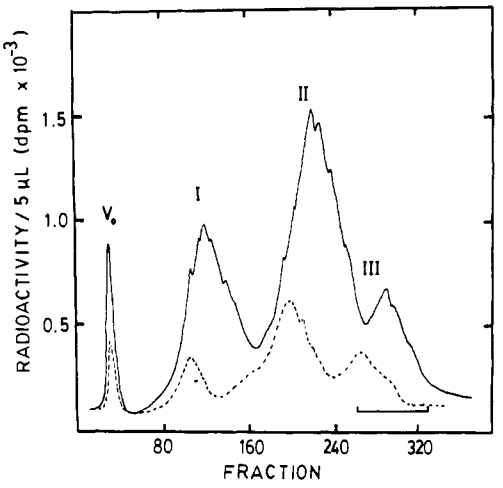

FIGURE 2: Elution profiles of tryptic digests on Sephadex G-50. Estradiol 17β-dehydrogenase, inactivated by either 12β-hydroxy-4-estrene-3,17-dione 12-(bromo[2-^{14}C]acetate) (--) or 3-methoxyestriol 16-(bromo[2-^{14}C]acetate) (—), was digested by trypsin–TPCK, applied to a column (5 × 100 cm, V_0 = 500 mL) at 25 °C, and eluted with 0.1 M NH_4HCO_3 buffer, pH 8.0. Fractions of 3.5 mL were collected. The fractions containing N^{τ}- or N^{π}-(carboxymethyl)histidine, peak III of each sample (indicated by bar), were pooled and lyophilized separately.

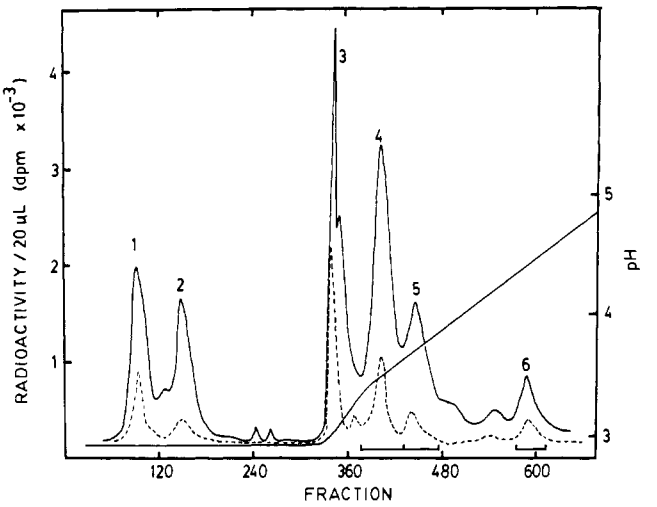

FIGURE 3: Cation-exchange chromatography of peak III eluted from Sephadex G-50. The peak III lyophilized samples, sample 12 (--) derived from 12β-hydroxy-4-estrene-3,17-dione 12-(bromo[2-^{14}C]-acetate) and sample 16 (—) from 3-methoxyestriol 16-(bromo[2-^{14}C]acetate) alkylation, were eluted from an AG 50W-X8 column (2.6 × 35 cm) by a gradient of 0.05 M pyridine–acetate, pH 2.3 (300 mL), and 2 M pyridine–acetate, pH 5.0 (300 mL), at 41 °C in fractions of 2.3 mL. The radioactivity determinations were corrected for pyridine quenching. The fractions in peptides 4–6 from each sample [containing N^{τ}- or N^{π}-(carboxymethyl)histidine] were pooled as indicated by the bar.

pseudo-first-order kinetics. The enzyme was 50% inactivated by the 12β-derivative ($t_{1/2}$ = 192 h, sample 12) and 43% by the 16α derivative ($t_{1/2}$ = 68 h, sample 16). Following dialysis of the inactivated enzyme, there remained 3.6 mg of sample 12 and 40 mg of sample 16 available for further peptide analysis. The amino acid analysis of each of the samples showed that the percent of radioactive label as carboxymethylated histidine [N^{τ}- and N^{π}-(carboxymethyl)histidine] was 28% in sample 12 and 17% in sample 16. The ratios of N^{τ}-(carboxymethyl)histidine to N^{π}-(carboxymethyl)histidine were 2 and 0.6, for the respective samples. In both samples, 69–75% of the radioactivity was associated with carboxymethylated cysteine. The peptide purification methodology was directed toward isolating those peptides containing the radioactive carboxymethylated histidine.

Gel Filtration of Tryptic Digest. Approximately 15% of the radioactivity applied to the Sephadex G-50 column was eluted at the void volume in each sample. The elution profiles of the two tryptic digests are shown in Figure 2. There were three main radioactive peaks (I–III) eluted beyond the void volume. The total recovery of radioactivity from the column was 83 and 100% of that applied for sample 12 and sample 16, respectively. The major quantity of radioactive (carboxymethyl)histidine was determined to be in the pooled fractions of peak III in both samples. The pooled fractions from peak I was mostly nonidentifiable radioactive material with less than 5% as N^{τ}- or N^{π}-(carboxymethyl)histidine. The radioactivity in peak II from both samples was primarily associated with (carboxymethyl)cysteine.

The amino acid analyses of the pooled fractions of sample 12-III revealed that 52% of the radioactivity was represented by carboxymethylated histidine residues with the ratio of the N^{τ} derivative to the N^{π} derivative being 1.2. The recovery from this step was 63 nmol of labeled peptide on the basis of the specific activity of the radioactive label. There was 672 nmol of peptide recovered in sample 16-III, and 44% of the radioactivity was associated with the carboxymethylated histidine. The ratio of N^{τ}-(carboxymethyl)histidine to N^{π}-(carboxymethyl)histidine was 0.45.

Peptide Purification by Cation-Exchange Chromatography. The resuspended residues of the Sephadex G-50 fractions 12-III and 16-III were chromatographed separately on AG 50W-X8, and the elution profiles of radioactivity are seen in Figure 3. The total recovery of radioactivity from the column was 93% in sample 16. The major portion of peaks 1–3 contained (carboxymethyl)cysteine with less than 5% of the label as either N^{τ}- or N^{π}-(carboxymethyl)histidine. The modified histidine residues were primarily found in peaks 4 and 6 of sample 12 and peaks 4–6 of sample 16.

In sample 12, peptide fraction III-4 (15% of applied radioactivity), the amount of modified histidine was 47% of the recovered radioactivity, with the ratio for the N^{τ} to N^{π} derivative of 0.62. The recovered histidine-bearing peptide totaled 10.3 nmol. Fraction III-6 from this sample was composed of 83% carboxymethylated histidine with the derivative ratio of 1.1, and it contained 7.2 nmol of labeled peptides (27% of radioactivity applied). Both of these fractions were desalted on Sephadex G-10 with a recovery of 66 (6.9 nmol) and 80% (14 nmol) for the pools 12-III-4 and 12-III-6, respectively.

The radioactive fractions having the same mobilities on the cation-exchange resin were isolated from sample 16. Fraction 16-III-4 (18% of appplied radioactivity) contained 121 nmol of labeled peptides, and 52% of this was associated with (carboxymethyl)histidine. A lesser quantity (75 nmol) was isolated as fraction III-5 (11% total sample), which contained 66% of the label as modified histidine. Fraction III-6 had 48 nmol (7%) of radioactive peptide, and 62% of the radioactivity was as (carboxymethyl)histidine. In each of the three fractions, the ratio of N^{τ}-(carboxymethyl)histidine to N^{π}-(carboxymethyl)histidine was 0.27 (III-4), 0.24 (III-5), and 0.55 (III-6). Subsequent to desalting, the amount of isolated labeled peptides was 117 (III-4), 64 (III-5), and 43 nmol (III-6).

HPLC of Histidine-Bearing Peptides. The desalted peptides were chromatographed 2 times by HPLC as the final purification steps. The recovery of radioactivity from each run was approximately 45%. The profiles for the first HPLC separation of fraction 12-III-4 and 16-III-4 are seen in Figures 4A and 5A, respectively. One major peak of radioactivity (III-4a) was isolated for each sample. The radioactivity was

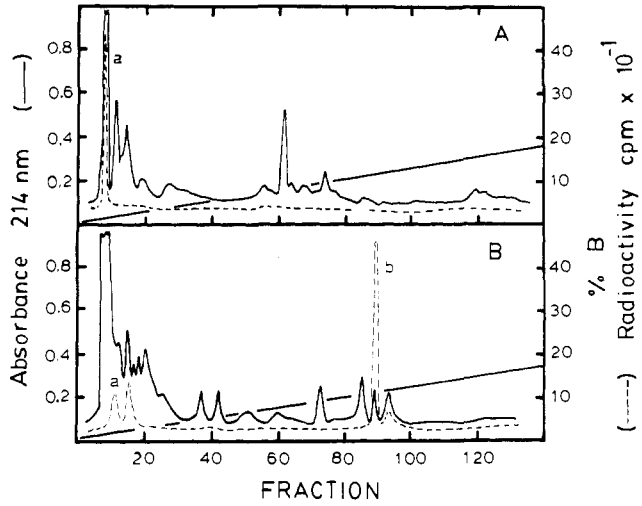

FIGURE 4: HPLC elution profiles of peptides alkylated by 12β-hydroxy-4-estrene-3,17-dione 12-(bromo[2-^{14}C]acetate). Two radioactive peaks eluted from cation-exchange resin, peptides III-4 (A) and III-6 (B), were desalted and applied to a C_{18} HPLC column (0.4 × 25 cm). The peptides were eluted at a flow rate of 1.0 mL/min with a gradient of buffer A (0.05% trifluoroacetic acid) and 0.25%/min change of buffer B (0.05% trifluoroacetic acid/acetonitrile). The effluent was monitored at 214 nm, and fractions of 0.5 mL were collected. Aliquots of 10 μL were taken for radioactivity counting at an efficiency of 0.86.

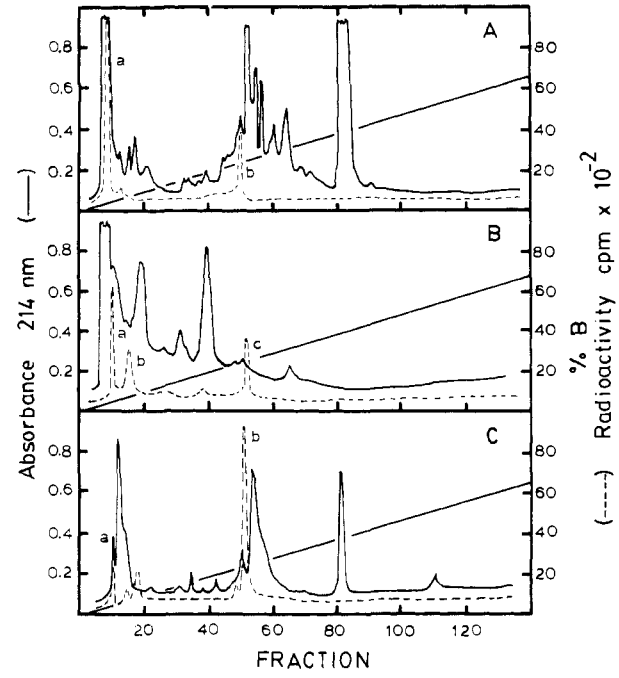

FIGURE 5: HPLC elution profiles of peptides alkylated by 3-methoxyestriol 16-(bromo[2-^{14}C]acetate). Three radioactive peaks eluted from cation-exchange chromatography were desalted and applied to a C_{18} column (0.4 × 25 cm). Peptides III-4 (A), III-5 (B), and III-6 (C) were separately chromatographed and eluted at a flow rate of 1.0 mL/min with a gradient of buffer A (0.05% trifluoroacetic acid) and 1.0%/min change of buffer B (0.05% trifluoroacetic acid/acetonitrile). The effluent was monitored at 214 nm, and fractions of 0.5 mL were collected. Aliquots of 5 μL were taken for radioactivity determinations and counted with an efficiency of 0.86.

not significantly retained on the column and was eluted with other contaminating peptides. The amino acid analysis showed the radioactivity (90%) to be N^{τ} and N^{π}-(carboxymethyl)-histidine (ratio = 0.60). High-voltage paper electrophoresis at pH 6.3 of this fraction showed a single radioactive spot, but subsequent purification steps did not yield a purified peptide suitable for sequencing. A minor peak (III-4b) of radioactivity from sample 16 (Figure 5A) eluted at 11% of the gradient also contained both N^{τ}- and N^{π}-(carboxymethyl)histidine (ratio = 0.47).

The elution profile of fraction III-5 from sample 16 (60 nmol applied) is shown in Figure 5B. Three radioactive peaks were isolated: III-5a (10 nmol recovered) eluted at the gradient start; III-5b (6 nmol) eluted at 10–12% gradient; III-5c (13 nmol) eluted at 20–24% gradient. The amino acid analysis of III-5c showed it to contain both N^{τ}- and N^{π}-(carboxymethyl)histidine at a ratio of 0.60. A second application of III-5c to HPLC with a pH 7.5 gradient did not yield a sufficient amount of purified peptide to allow sequencing. The major radioactive peptide eluted at 28% of this basic gradient.

The HPLC of fraction 12-III-6 (15 nmol applied) yielded one major radioactive peptide (12-III-6b, 4 nmol recovered) eluting at 11% gradient (Figure 4B). Smaller amounts of radioactivity were found at the beginning of the gradient. Peptide 12-III-6b was lyophilized for sequencing. Sample 16-III-6 also had one predominant radioactive peak (Figure 5C), which was 97% carboxymethylated histidine. The ratio of the N^{τ} and N^{π} derivatives was 0.81. The isolated fraction (8 nmol) was purified by a second HPLC run under the same conditions. The purified peptide, 16-III-6b (4 nmol) eluting at 11% gradient, was sequenced.

Amino Acid Sequence of Modified Peptides. Two sequence determinations of approximately 2 nmol each established the sequence of peptide 16-III-6b as Thr-Asp-Ile-CMHis(His)-Thr-Phe-CMHis(His)-Arg. In peptide 12-III-6b, only the first six residues of this same peptide could be identified. Each peptide was run for 10 cycles with aliquots taken from each cycle for radioactivity measurements. In sample 12, 60% of the radioactivity was found in cycle 7, with the remainder in cycle 4. Sample 16 contained 80% of the radioactivity in cycle 7. There also was found free histidine in cycles 4 and 7. However, it could not be established whether the (carboxymethyl)histidine was modified at either the N^{τ} or N^{π} position of the imidazole ring. The amino acid composition of the peptide from both samples was identical.

Discussion

Several pyridine nucleotide dependent dehydrogenases utilize the imidazole ring of one or more histidine residues in the catalytic process (Keleti, 1970). In addition, crystallographic studies of several 2-hydroxy acid dehydrogenases have indicated the involvement of a histidine residue in catalysis (Parker & Holbrook, 1977; Birktoft et al., 1982). We have presumed this to be the case with human placental estradiol 17β-dehydrogenase. The studies from this laboratory have determined several structural aspects of the catalytic site of the human enzyme, including the isolation of the peptide containing the presumed catalytically active histidine residue.

The enzyme has been inactivated by estrone 3-(bromoacetate), 12β-hydroxy-4-estrene-3,17-dione 12-(bromoacetate), and 3-methoxyestriol 16-(bromoacetate) (Murdock & Warren, 1982; Chin et al., 1982). All three steroid derivatives are substrates for estradiol 17β-dehydrogenase and, therefore, must bind at the active site. Each steroid modifies one or more histidine residues, and in each case the alkylation is reduced in the presence of excess 17β-estradiol. While we have emphasized in these previous studies the fact that the majority of residues modified by these steroid derivatives were cysteine residues, other investigators (Pons et al., 1977) have shown that most of these cysteine residues are nonessential for catalytic activity or are located in the cofactor binding region of the enzyme. Therefore, our more recent studies have been directed toward evaluation of the involvement of histidine

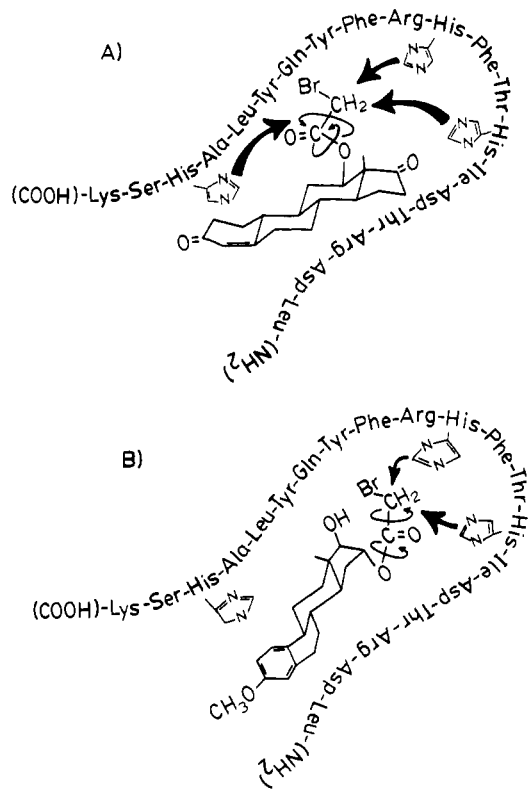

FIGURE 6: Schematic representation of the modification of histidine by 12β-hydroxy-4-estrene-3,17-dione 12-(bromoacetate) (A) and 3-methoxyestriol 16-(bromoacetate) (B). The proposed binding site of estradiol 17β-dehydrogenase contains three histidine residues. One histidine, which may participate in the catalytic hydrogen transfer, is predominantly alkylated by both steroid derivatives. The 12β derivative is also known to modify the histidine proximating the A ring of the bound steroid.

residues in the catalytic hydrogen transfer.

The enzyme is inactivated by the 3-derivative at a significantly faster rate than with either 12β-hydroxy-4-estrene-3,17-dione 12-(bromoacetate) or 3-methoxyestriol 16-(bromoacetate). The stoichiometry of alkylation by the latter derivative approximates 2 mol of steroid covalently bound per mole of enzyme inactivated (Chin & Warren, 1975). The estrone 3-(bromoacetate) almost exclusively alkylates at the N^{π} position of a histidine found in the peptide Leu-Ala-His-Ser-Lys (Murdock et al., 1983). In contrast, the 12β and 16α derivatives will modify histidine at either the N^{τ} or N^{π} position. One of the histidines modified by 12β-hydroxy-4-estrene-3,17-dione 12-(bromoacetate) is found in the same peptide as that alkylated by estrone 3-(bromoacetate).

In the present study, we have isolated an octapeptide, Thr-Asp-Ile-His-Thr-Phe-His-Arg (peptide III-6), that was modified by 12β-hydroxy-4-estrene-3,17-dione 12-(bromoacetate) and 3-methoxyestriol 16-(bromoacetate). The study of Dreiding models reveals that either of these derivatives should alkylate a histidine in proximity of C-17 of the bound steroid. Our data show that either histidine within this peptide can be alkylated by the two different steroid derivatives, but the histidine in position 7 of the peptide was predominantly attacked as determined by radioactivity measurements. This fact suggests that this histidine is in closer proximity to C-17 and may be the active residue. It could not be ascertained whether either of the histidines was preferentially alkylated at the N^{τ} or N^{π} position.

Two other major peptides were isolated by cation-exchange chromatography, which contained carboxymethylated histidine residues, III-4 and III-5 (Figure 3). These could not be successfully sequenced. These may be fragments or other artifacts due either to trypsin digestion or the purification methodology. We know from previous studies that the 12β derivative does attack the histidine in the pentapeptide Leu-Ala-His-Ser-Lys. Because of this fact, and the fact that it elutes at the beginning of the HPLC gradient (Figure 4A), we have speculated that peptide III-4 may be the smaller pentapeptide. If we assume that this peptide proximates the A ring of the steroid bound in a position conducive to catalysis, there are at least two possibilities for that histidine to be alkylated by 12β-hydroxy-4-estrene-3,17-dione 12-(bromoacetate) and 3-methoxyestriol 16-(bromoacetate). In the case of the 12β derivative, the bromoacetate side chain could rotate to such a position as to allow for alkylation. Second, either of the derivatives may bind in a "wrong-way" position, such that the A and D rings were reversed in the steroid binding site. Further experiments are being conducted to determine this possibility.

Results from the present experiments enhance our theory that one or more histidines may be involved in the catalytic event of estradiol 17β-dehydrogenase. This octapeptide and the previously identified pentapeptide have been shown to be in close proximity to each other in the primary structure of the native enzyme.[2] We have schematically represented the position of these amino acid sequences relative to the bound steroid (Figure 6). That this peptide contains the catalytically active residues is also suggested by the study of Inano & Tamaoki (1983). They have presented indirect evidence that two arginine residues are specifically labeled by 16-oxoestrone. Although their data do indicate the presence of an arginine in the catalytic site, their claim that an arginine residue is essential for catalytic activity was not directly established. The modification of a residue resulting in enzyme inactivation does not necessarily indicate the function of that residue in catalysis or binding. Our study does establish the presence of two histidines in the region of the steroid binding site where catalysis occurs. The specific determination that histidine is involved in the catalytic process of human placental estradiol 17β-dehydrogenase will hopefully be resolved by the X-ray crystallographic studies now being pursued in our laboratory.

ACKNOWLEDGMENTS

We thank Dr. Greg Grant of the Washington University Protein Chemistry Facility for his advice and technical expertise with the peptide sequencing.

Registry No. His, 71-00-1; estradiol 17β-dehydrogenase, 9028-61-9; 12β-hydroxy-4-estrene-3,17-dione 12-bromoacetate, 64890-82-0; 3-methoxyestriol 16-bromoacetate, 57456-71-0.

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Elucidation of the Chemical Nature of the Steady-State Intermediates in the Mechanism of Carboxypeptidase A†

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Received August 12, 1985

ABSTRACT: Cryospectrokinetic studies of zinc and cobalt carboxypeptidase A disclosed two intermediates in the hydrolysis of both peptides and depsipeptides and furnished all the rate and equilibrium constants for the reaction scheme $E + S \rightleftharpoons ES_1 \rightleftharpoons ES_2 \rightarrow E + P$ [Auld, D. S., Galdes, A., Geoghegan, K. F., Holmquist, B., Martinelli, R. A., & Vallee, B. L. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 5041-5045]. Since the ES_2 intermediate is the predominate enzyme species present at steady state, its chemical nature is deducible from subzero chemical quench studies done after steady state is established. Extrapolation of the product concentration to zero time, $[P_0]$, measures the concentration of the enzyme species in which bond cleavage has occurred. For peptides, the $[P_0]$ values are zero, indicating that no product is generated prior to turnover and therefore the ES_2 intermediate involves a complex between enzyme and intact peptide substrate. For depsipeptides, $[P_0]$ values are 1 mol of product per mole of enzyme over the entire temperature range -20 to -50 °C, indicating cleavage of the ester bond occurs prior to the rate-limiting step so that ES_2 is more properly denoted by EP_1P_2, where P_1 and P_2 are the substrates for the reverse reaction. The rate-limiting step for depsipeptides thus involves release of the products which may occur directly or through a mandatory conformational change followed by rapid product release.

Carboxypeptidase A is one of the best characterized metalloenzymes and is often considered a prototype of the large family of zinc proteases (Vallee & Galdes, 1984). Until recently, however, its mechanism was largely unknown, although it was the subject of numerous speculations [reviewed in Vallee et al. (1983)]. This uncertainty was a result of its high catalytic efficiency which precluded identification of intermediates formed during substrate hydrolysis. We have recently described a multifaceted strategy designed to obviate the problem and have applied it successfully to establish the pre-steady-state kinetics of the enzyme, as well as to spectrally characterize the intermediates that accumulate at steady state. We have used subzero temperatures to extend the lifetime of the intermediates in nonperturbing cryosolvents and rapid-scanning stopped-flow procedures to monitor their rapid formation and interconversion. The intermediates can be visualized directly through the absorption spectra of the cobalt-substituted enzyme or the fluorescence generated by radiationless energy transfer between enzyme tryptophans and dansylated substrates. The cryokinetic studies disclose two intermediates in the hydrolysis of both peptide and depsipeptide substrates and furnish all the rate and equilibrium constants for the reaction scheme $E + S \rightleftharpoons ES_1 \rightleftharpoons ES_2 \rightarrow E + P$. The results show that, contrary to popularly held views (Cleland, 1977; Rees & Lipscomb, 1981), peptide and depsipeptide substrates give rise to different steady-state metallointermediates.

The present work extends our studies by identifying the chemical nature of the steady-state peptide and ester intermediates. This identification was achieved by rapidly quenching reaction mixtures in which the intermediates had accumulated at subzero temperatures and then analyzing the mixtures by high-performance liquid chromatography (HPLC) techniques. The results show that hydrolysis of the scissile amide bond in peptides occurs during, or after, the rate-limiting step, while hydrolysis of the ester bond in depsipeptides precedes the rate-limiting step.

Materials and Methods

Bovine carboxypeptidase A (Cox) was purchased as a crystalline suspension from Sigma Chemical Co. and purified further by recrystallization and affinity chromatography (Galdes et al., 1983).

† This work was supported in part by Grant-in-Aid GM-24967 from the National Institutes of Health and Human Services to Harvard Medical School.

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0006-2960/86/0425-0646$01.50/0