Synthesis of 4-Bromoacetamidoestrone Methyl Ether and Study of the Steroid Binding Site of Human Placental Estradiol 17β-Dehydrogenase*

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To further characterize the active site of human placental oestradiol 17β-dehydrogenase (EC 1.1.1.62), we have synthesized 4-bromoacetamidoestrone methyl ether. Starting with estrone, synthetic steps involved nitrination, isolation of the 4-nitro derivative with subsequent methylation, selective reduction of the nitro group, and coupling of the resulting 4-amino group with bromoacetic acid. The affinity-labeling steroid is a substrate for the enzyme with an apparent $K_m$ of $1.18 \times 10^{-3}$ M and apparent $V_{max}$ value of 0.79 nmol/min/μg of enzyme. It inactivates the enzyme in a time-dependent, irreversible manner which follows pseudo-first order kinetics. Further, inactivation conducted with varying steroid concentrations displays saturation kinetics (with an I-nitro derivative as it undergoes the reversible binding step. Therefore, affinity-labeling methodology should allow evaluation of the comparative topography of the steroid-binding sites of various proteins.

Accordingly, we purified oestradiol 17β-dehydrogenase (EC 1.1.1.62) from human placenta to homogeneity by a simple scheme of affinity chromatography (6) and subsequently crystallized it (7). We began affinity-labeling studies on the human placental enzyme with 16α-bromocetoxyestriadiol 3-methyl ether and found a histidyl residue present in the catalytic region of the active site (8), as is the case for 20β-hydroxysteroid dehydrogenase.

Thinking that the major differences in amino acid residue content of these two enzymes might be expected to occur in the A ring region, we have now synthesized 4-bromoacetamidoestrone methyl ether and employed it to affinity-label the human placental enzyme. The results indicate that lysyl and cysteinyl residues proximate this region of the steroid as it binds at the active site and that the presence of cofactor (NADP+) at the active site makes the cysteinyl residue unavailable either by interposing itself between the steroid and this residue or by changing the conformation of the active site.

**EXPERIMENTAL PROCEDURES**

The "Experimental Procedures" and part of the "Results" are described in the miniprint supplement.1

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1 The "Experimental Procedures" and part of the "Results" (Figs. 1 to 6 and Tables I and II) and Refs. 9 to 20 are presented in a miniprint format immediately following this paper. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, Md. 20014. Request Document No. 77M-994, cite author(s) and include a check or money order for $1.50 per set of photocopies.

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We have previously used affinity-labeling steroids to study the steroid-binding site of 20β-hydroxysteroid dehydrogenase

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Inactivation of Estradiol 17β-Dehydrogenase by Tritiated 4-Bromoacetamidoestrone Methyl Ether—The amino acid residues to be affinity labeled at the active site of estradiol 17β-dehydrogenase by 4-bromoacetamidoestrone methyl ether were investigated by inactivation of the enzyme with the radioactive steroid derivative. The control and inactivation preincubations were made by first mixing 3.5 mg of estradiol 17β-dehydrogenase (50 nmol) in 54.0 ml of Buffer A with 6.0 ml of ethanol (10% ethanol v/v) and keeping it at room temperature for 18 h. The sample was then divided into three parts. In Sample A, to 2.7 ml of the enzyme solution (23 nmol), 0.3 ml of ethanol was added making the ethanol content 20%. This was used as control. In Sample B, to 27 ml of the same enzyme solution (23 nmol), 1.45 mg (3.45 pmol) of 4-bromo[2'-3H]acetamidoestrone methyl ether in 3 ml of ethanol was added. In Sample C, to 97 ml of the same enzyme solution (23 nmol), 5.27 mg (6.9 pmol) of NADP+ and 1.45 mg (3.45 pmol) of 4-bromo[2'-3H]acetamidoestrone methyl ether in 3 ml of ethanol were added. Enzyme activity was assayed every 1 to 2 h from zero time.

The control (Sample A) retained its original activity throughout the course of the experiment. For the sample undergoing inactivation with 4-bromo[2'-3H]acetamidoestrone methyl ether, 8-ml portions of the reaction mixture were removed at the time when 25, 48, and 80% inactivation had occurred. Further reaction was immediately stopped in each of these portions with 0.1 ml of 1.4 M 2-mercaptoethanol. They were then separately dialyzed against distilled water with frequent changes of the dialysate. When the dialysate was found to have radioactivity identical with background, dialysis was terminated, and the contents of the bags were lyophilized. The dry enzyme was dissolved in 10 ml of 0.5 N NaOH. Parts of the alkaline enzyme solution were used to study the stoichiometry of inactivation (see miniprint supplement), and the remaining portions were used to identify the affinity-labeled amino acid residues.

Identification of Amino Acid Residues at Steroid-binding Site of Estradiol 17β-Dehydrogenase—The remaining alkaline protein solutions left after determining stoichiometry were neutralized to pH 7 by 6 N HCl, dialyzed against water for 24 h, and lyophilized. The dry enzyme samples were then hydrolyzed separately with 6 N HCl at 110° for 24 h. The hydrolysates were evaporated to dryness, and residues were dissolved in 0.5 ml of 0.2 M sodium citrate buffer, pH 5.2. Aliquots of 10 to 25 μl were used to quantitate radioactivity, and 0.25 ml of sample was put on an amino acid analyzer. The effluent fractions were collected every minute (1.6 ml) and counted on a Packard Tri-Carb liquid scintillation spectrometer. Over 90% of the radioactivity applied to the column was recovered in each case. For all the samples inactivated by 4-bromo[2'-3H]acetamidoestrone methyl ether in the absence of cofactor, 30% of the recovered radioactivity appeared in a peak corresponding to S-carboxymethylcysteine and 70% in a peak corresponding to ε-carboxymethyllysine (Fig. 7, III). For the sample inactivated by 4-bromo[2'-3H]acetamidoestrone methyl ether and NADP+, only the radioactive peak corresponding to ε-carboxymethyllysine appeared (Fig. 7, IV). Radioactivity recovered from the column runs of the 10-h and 24-h samples (14% inactivation) was 75 and 72%, respectively. The radioactive hydrolysates were also run together with a standard mixture of nonradioactive S-carboxymethylcysteine and ε-carboxymethyllysine. The ninhydrin peaks of the amino acids coincided with their corresponding radioactive peaks (Fig. 7, V).

DISCUSSION

In a previous work (8), affinity-labeling studies of estradiol 17β-dehydrogenase with 16-α-bromoacetoxyestradiol 3-methyl ether indicated that a histidyl residue proximates position 16 of the bound steroid at the active site (8). In the present investigation, 4-bromoacetamidoestrone methyl ether was used for affinity labeling and lysyl and cysteinyl residues were found to proximate position 4 of the bound steroid. The bromoacetamido derivative (BrCH2CONH−) was selected because the resistance of its peptide bond to hydrolysis precludes the possibility of formation of a dicarboxymethyl derivative by transacylation (5).

In the present study, kinetic evidence supports the conclusion that the substrate analog, 4-bromoacetamidoestrone methyl ether, alkylates residues within the active site of estradiol 17β-dehydrogenase. First, both 4-bromoacetamidoestrone methyl ether and estradiol are substrates and therefore must compete for the active site. Second, the rate of inactivation of estradiol 17β-dehydrogenase by 4-bromoacetamidoestrone methyl ether displays saturation kinetics, so that a
double-reciprocal plot of the pseudo-first order rate constant ($k_{\text{obs}}$) against 4-bromoacetamidoestron methyl ether concentration ($1/[I]$) shows Michaelis-Menton kinetics as described in Equations a and b. Third, the rate of inactivation is slowed by the presence of estradiol 17β and NADP$^+$. It was reported by Pons et al. (21, 22) that 3 cysteinyl and 1 histidyl residues are located at or near the steroid-binding site when estradiol 17β-dehydrogenase is inactivated by iodoacetoxyl-3-estrone and that the histidyl residue is not essential for enzyme activity. With the use of 15 different haloacetoxy and haloacetamido derivatives of estradiol or estrone at nine different positions on the A, B, C, and D rings of the steroid nucleus, Pons et al. (23) indicated that all of these compounds affinity-labeled the same histidyl residue and 3 cysteinyl residues. The histidyl residue is alkylated less as the alkylating side chain is shifted from C-3 to C-16. On the other hand, the reactivity of the cysteinyl residue located at the active site shows increasing alkylation when the alkylating side chain is similarly shifted. They also reported that a cysteinyl residue was labeled by 3-chloroacetoxypyridine adenine nucleotide (24). They concluded that a histidyl residue was located in the vicinity of the A ring of the bound steroid while 1 cysteinyl residue was located in the catalytic region close to the D ring, 1 was probably located in the cofactor binding site and 1 was probably out of the active site. They presumed that in the absence of cofactor, the alkylating steroid may bind randomly at several positions of the active site and also in the cofactor binding site, so different derivatives may alkylate the same amino acid residue.

We presume that the histidyl residue labeled by 1-bromoacetamidoestradiol 3-methyl ether (8) is not the one that was alkylated by 3-iodoacetoxyestrone, and that quite possibly two different histidyl residues are in the peptide chain which they produced by chymotrypsin cleavage. When the enzyme was inactivated by 4-bromo[2'-'H]acetamidoestrone methyl ether, a lysyl residue (70%) and a cysteinyl residue (30%) (the difference of radioactivity distribution among these 2 residues may be due to intrinsic difference in reactivity, environment, or proximity to the reagent group as the steroid binds) were alkylated and thus are presumably located near position 4 of the bound steroid. We observed no carboxymethylhistidine on amino acid analysis of the hydrolysate of the inactivated enzyme. Moreover, in the presence of NADP$^+$ only the lysyl residue was carboxymethylated. These results indicate that the accessibility of the alkylating reagent group on the steroid to the cysteinyl residue is greatly diminished when the cofactor region is occupied by NADP$^+$. In terms of cysteine alkylation (and the effect of the presence of cofactor on same), the results obtained parallel those the French workers observed with 3-iodoacetoxyestrone (21). On the other hand, that derivative affinity labeled a histidyl residue, while 4-bromoacetamidoestrone methyl ether clearly affinity labels a lysyl residue. We are aware that destruction or exchange of alkylated residues (particularly with use of tritium) may occur during acid hydrolysis even when it is preceded by extensive evacuation of the tube. Therefore, we alkylated standard histidine with iodo[2'-'H]acetate and noticed that 30% of the radioactivity in carboxymethylhistidine so generated was lost during acid hydrolysis. Nevertheless, losses of this magnitude would not have prevented recognition of affinity-labeled histidine if it were present, and we conclude that the histidyl residue affinity labeled by 3-iodoacetoxyestrone (21) is too distant from position 4 to undergo significant alkylation.

Previous studies of 20β-hydroxysteroid dehydrogenase revealed that a cysteinyl residue was affinity labeled by 6-bromoprogesterone (1) and by 6-bromoacetoxyprogesterone (2). Therefore, both of these steroid dehydrogenases appear to have histidyl residues in the catalytic region of the active site and cysteinyl residues in the region which proximates the lower AB ring area of the bound steroid.

REFERENCES

Refs. 9 to 20 will be found on p. 815.
Synthesis of 4-bromoacetamidoestrone methyl ether and study of the steroid binding site of human placental estradiol 17beta-dehydrogenase.
Y M Bhatnagar, C C Chin and J C Warren


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