REACTIVATION OF HUMAN PLACENTAL 17β,20α-HYDROXYSTEROID DEHYDROGENASE: AFFIRMATION OF AFFINITY LABELING PRINCIPLES

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ABSTRACT

Human placental 17β,20α-hydroxysteroid dehydrogenase was completely inactivated by the affinity alkylator, 3-bromoacetoxy-1,3,5(10)-estratrien-17-one (estrone 3-bromoacetate). The inactivated enzyme was then reactivated to 100% of the enzyme activity by base-catalyzed hydrolysis of the steroidalester-enzyme conjugate. After the reactivated enzyme was repurified by dialysis, re-inactivation studies were performed on it. The reactivated enzyme could not be re-inactivated by the original alkylator, estrone 3-bromoacetate. However, 16α-bromoacetoxyestradiol-17β 3-methyl ether caused a loss of reactivated enzyme activity at a rate comparable to that for the native enzyme. These observations demonstrate that a specific amino acid modification within the enzyme active site was produced by estrone 3-bromoacetate alkylation and suggest that the conformation of the active center was essentially unaltered. Thus, these successful reactivation studies of 17β,20α-hydroxysteroid dehydrogenase affirm the specificity of affinity labeling. This methodology also offers a new tool to investigate the steroid binding regions of macromolecular proteins.

INTRODUCTION

An affinity alkylator is defined as a compound which has a strong attraction to an active center of an enzyme and which carries reactive groups capable of forming a chemical bond with amino acid residues at the active center. Steroid hormones bearing reactive groups have been widely used to label amino acid residues within enzyme active sites (1-10). With the exception of Groman et al. (1) demonstrating the catalytic competence of affinity alkylated estradiol-17β dehydrogenase, the belief that affinity labels create specific, selective changes

within the enzyme structure is based on logical inference rather than on direct experimentation. Our laboratory has studied the reactivation of 17β,20α-hydroxysteroid dehydrogenase (HSD), the bifunctional oxidoreductase (5-12) purified to homogeneity from the cytosol of human term placenta, following affinity labeling with estrone 3-bromoacetate. In addition, we performed affinity alkylation studies on the reactivated enzyme to evaluate how affinity labeling by estrone 3-bromoacetate modifies the active site.

MATERIALS AND METHODS

Materials. Steroid hormones which were chromatographically pure, 2-mercaptoethanol, NADH, and NADPH were obtained from Sigma Chemical Co. Analytical grade solvents, Eastman thin layer chromatography sheets, organic chemicals, reagent grade salt, ScintiVerse liquid scintillation counting fluid, and Spectrapor dialysis tubing (No. 2) were purchased from Fisher Scientific Co. Silica gel (Silica Woelm TSC) was obtained from Universal Scientific Inc. [6,7-^{3}H]Estrone (specific activity 55.9 Ci/mmol) was acquired from New England Nuclear. 16α-Bromoacetoxyestradiol-17β 3-methyl ether was prepared in our laboratory according to a published synthesis (13). Aqueous solutions were prepared with glass-distilled deionized water. Buffer solutions contained 0.01 M potassium phosphate, 5 mM EDTA, and 20% glycerol (v/v): Buffer A, pH 7.0; Buffer B, pH 6.5.

Instruments. A Fisher Accumet Model 325 expanded scale pH meter was used to determine pH values to ± 0.02 pH units. Melting points were measured in an Electrothermal apparatus. Ultraviolet and visible absorbance spectra were determined with a Varian Cary 219 recording spectrophotometer and infrared absorption with a Beckman Acculab 4 spectrometer. Radioactivity was measured in a model LS7500 Beckman liquid scintillation spectrometer with 48% counting efficiency for tritium. A Texas Instruments SR-51-II calculator and a Digital Vax 11/780 computer were used to analyze data.

Purification and assay of 17β,20α-hydroxysteroid dehydrogenase. The human placental enzyme (native enzyme) isolated and purified by the method of Chin and Warren (14) was homogeneous on sodium dodecyl sulfate disc gel electrophoresis and possessed identical properties to those reported for crystalline estradiol-17β dehydrogenase (15). The Coomassie Blue technique (16) was used to determine protein concentration. Enzyme assays for 17β- and 20α-HSD activities were performed in duplicate at 22°C using our published conditions (9). The slope of the linear change in absorbance at 340 nm (due to cofactor oxidation or reduction) was recorded with respect to time and used to calculate enzyme activity.

Estrone 3-bromoacetate synthesis. A solution of 91.0 mg (0.34 mmol) estrone in 10.0 mL dry methylene chloride, 51.4 mg (0.37 mmol) of bromoacetic acid in 5.0 mL methylene chloride, and 77.2 mg (0.37 mmol) of dicyclohexylcarbiodiimide in 5.0 mL methylene chloride was stirred for 10 min at room temperature followed by an addition of 30 µL of pyridine. The reaction mixture was stirred for 1 h at 0°C and 1 h at room temperature. After a 30 µL addition of acetic acid and 20 min of stirring, the mixture was filtered to remove dicyclohexylurea. The solvent was removed with nitrogen and the residue was dissolved in 2.0 mL of chloroform. This sample was applied to a 1.0 cm x 20.0 cm silica gel dry-column and eluted with chloroform at 4°C. The infrared spectrum peaks, melting point, and ultraviolet absorbance maximum were in agreement with reported values obtained in a similar microsynthesis (17).

Inactivation with estrone 3-bromoacetate. Native enzyme (0.75 µM) was incubated at 22°C in Buffer B, pH 6.5, containing NADPH (60.0 µM), with estrone 3-bromoacetate (35.0 µM). An equivalent amount of estrone replaced the estrone 3-bromoacetate in control incubation mixtures. Periodically, aliquots (0.05 mL) of each mixture were assayed in duplicate for 17β-HSD activity. All concentrations reported are final concentrations.

Reactivation and repurification of enzyme for second inactivation studies. After 100% inactivation of the enzyme by estrone 3-bromoacetate, 2-mercaptoethanol (100.0 µM) was added to the reaction and control mixtures, and both were quickly titrated to pH 9.3 with equal volumes of 0.2 N NaOH solution. Aliquots of each mixture were assayed until 100% of the enzyme activity was attained relative to the control. A volume of [6,7-^{3}H]estrone was added to each mixture to yield a specific activity of 86.0 µCi/µmol, based on the assumption that alkylating steroid bound to the enzyme and any unbound alkylator-mercaptoethanol complexes were completely hydrolyzed to produce 35.0 µm estrone during base-catalyzed reactivation. Each mixture was dialyzed against 300 volumes of Buffer A, pH 7.0, at 4°C. The dialysate was changed twice daily over a 5 day period. After dialysis, the concentration of ^{3}H-estrone remaining in the retentates was calculated to be less than 0.01 µM. "Reactivated enzyme" is defined as enzyme which was 100% reactivated and repurified by dialysis. "Control enzyme" refers to enzyme in the control mixture which was treated identically to the reactivated enzyme. "Second inactivation" describes the loss of reactivated enzyme activity during a second exposure to an affinity alkylator.

Second inactivation studies. Reactivated, control, and native enzymes (0.50 µM) dissolved in Buffer A, pH 7.0 containing NADPH (60.0 µM), were incubated at 22°C with estrone 3-bromoacetate (23.33 µM). Identically prepared control incubations contained estrone in place of alkylator. Reactivated, control, and native enzymes (0.50 µM) were incubated at 22°C in Buffer A, pH 7.0, with 16α-bromoacetoxy-estradiol-17β 3-methyl ether (75.0 µM). An equivalent amount of estriol 3-methyl ether in place of the alkylator was used in control incubations. All incubation mixtures were assayed in duplicate for 17β-HSD activity.

RESULTS

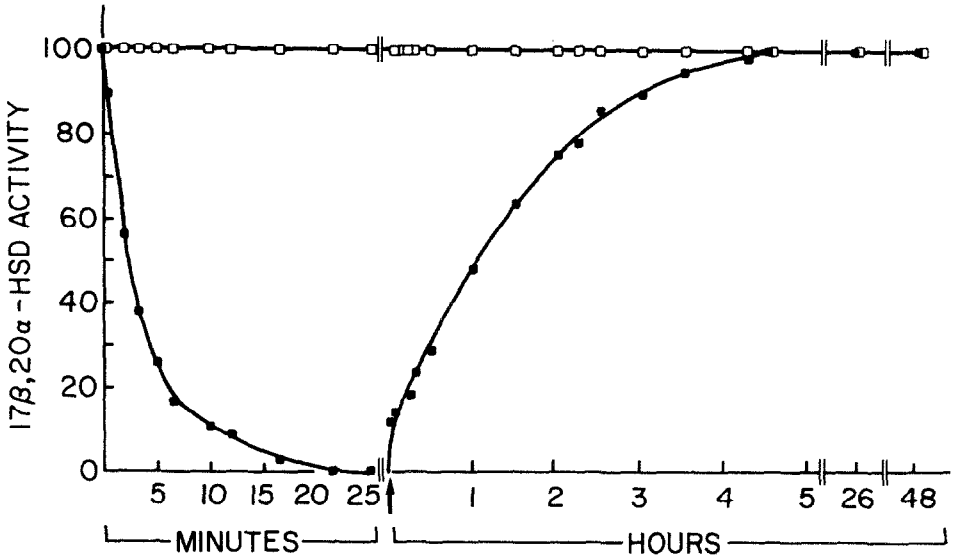

Figure 1. Inactivation of 17β,20α-hydroxysteroid dehydrogenase (HSD) by estrone 3-bromoacetate followed by base-catalyzed reactivation. Enzyme (0.75 μM) dissolved in 9.0 mL of Buffer B, pH 6.5 containing NADPH (60.0 μM), was incubated at 22°C with estrone 3-bromoacetate (35.0 μM added in 1.0 mL ethanol). An identical control incubation (□) contained estrone in place of alkylator. At various time intervals, 0.05 mL was assayed for 17β-HSD activity until complete inactivation in the experimental mixture (■) was observed. 2-Mercaptoethanol (100.0 μM) was added and the pH of the retentates was raised to 9.3 (↑). Periodically over 48 h, 0.05 mL was assayed for 17β-HSD activity (■). The percentage of enzyme activity is plotted on a linear scale along the ordinate, and time is represented by the linear scale along the abscissa. The values are the means of at least triplicate experiments.

Reactivation of 17β,20α-hydroxysteroid dehydrogenase activity after inactivation by estrone 3-bromoacetate. Native enzyme was completely inactivated ($t_{1/2}$ = 2.5 min) by estrone 3-bromoacetate (steroid:enzyme molar ratio 46.7:1) in the presence of NADPH (cofactor:steroid molar ratio 1.7:1) (Fig. 1). After quenching the

reaction with a 2.9 molar excess of 2-mercaptoethanol and titrating to pH 9.3, the inactivated enzyme underwent 100% reactivation of 17β-HSD activity relative to the control over 48 h. Consistent with several observations that 17β- and 20α-HSD activities occur at one active site (5-12), simultaneous rates of reactivation to 100% of both activities were attained (data not shown).

Absence of second inactivation of reactivated enzyme by estrone 3-bromoacetate. Reactivated enzyme was not inactivated by a second exposure to estrone 3-bromoacetate (steroid:enzyme molar ratio 46.7:1) in the presence of NADPH (cofactor:steroid molar ratio 2.6:1) over 20 h. The control and native enzymes were rapidly inactivated ($t_{1/2}$ = 2.0 min) by the alkylator in identical incubations (Fig. 2).

Second inactivation of reactivated enzyme by 16α-bromoacetoxy-estradiol-17β 3-methyl ether. Reactivated, control, and native enzymes were inactivated by 16α-bromoacetoxyestradiol-17β 3-methyl ether (steroid:enzyme molar ratio 150:1) at similar rates (Fig. 3).

DISCUSSION

Reactivation of human placental estradiol-17β dehydrogenase was first reported by Boussioux et al. (18) who observed spontaneous recovery of activity when enzyme inactivated by estrone 3-iodoacetate was incubated for 48 h at 37°C in 0.03 M phosphate buffer, 20% glycerol, pH 7.2. Indeed, these workers observed recovery of 300% activity relative to their control enzyme solutions. However, they were unable to inactivate or alkylate the repurified "superactive" enzyme with 16α-iodoacetoxyestrone or with other affinity labels. This suggests that their reactivation and repurification procedures induced a major conformational change of the enzyme active center. Despite

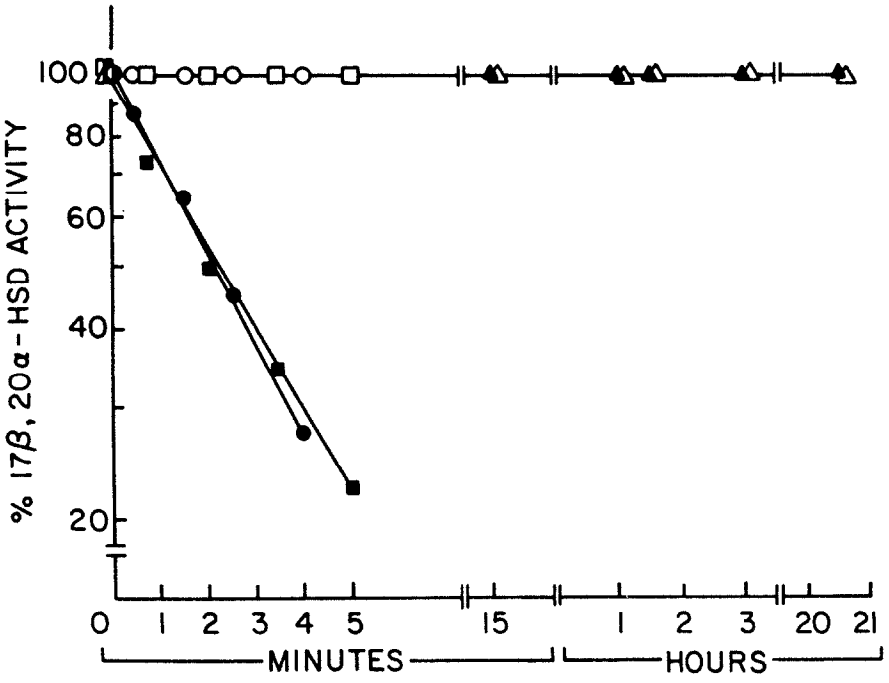

Figure 2. Effect of second exposure of reactivated 17β,20α-hydroxysteroid dehydrogenase (HSD) to estrone 3-bromoacetate. Reactivated (▲), control (■), and native (●) enzymes (0.50 μM) dissolved in 0.72 mL of Buffer A, pH 7.0 containing NADPH (60.0 μM), were incubated at 22°C with estrone 3-bromoacetate (23.33 μM added in 0.08 mL of ethanol). Identically prepared control incubations contained estrone in place of alkylator (Δ, reactivated; □, control; ○, native enzyme). At various time intervals, 0.05 mL of each incubation was assayed for 17β-HSD activity. The percentage of enzyme activity is plotted on a logarithmic scale along the ordinate, and time is represented by the linear scale along the abscissa. The values are the means of duplicate experiments.

using the incubation conditions of Boussioux et al. followed by observation for 120 h, we never saw "superactivation".

Sweet restored activity to the bacterial enzyme, 20β-hydroxysteroid dehydrogenase, which had been completely inactivated by 6β-bromoacetoxyprogesterone (19). By titrating the inactivated reaction mixture to pH 9.0 with sodium hydroxide, he showed that

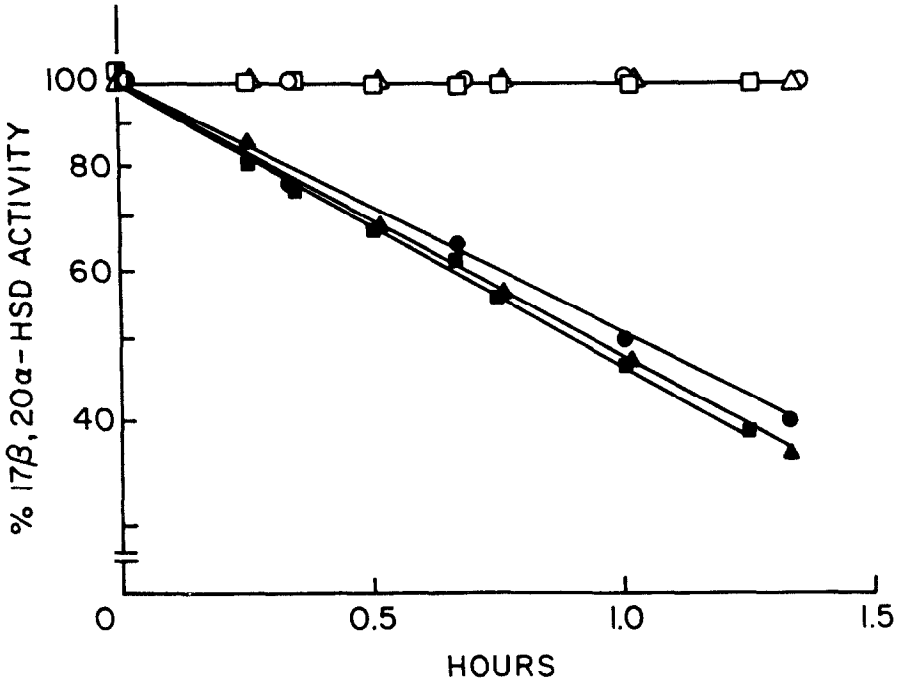

Figure 3. Second inactivation of reactivated 17β,20α-hydroxysteroid dehydrogenase (HSD) by 16α-bromoacetoxyestradiol-17β 3-methyl ether. Reactivated (▲), control (■), and native (●) enzymes (0.50 μM) dissolved in 0.64 mL of Buffer A, pH 7.0, were incubated at 22°C with 16α-bromoacetoxyestradiol-17β 3-methyl ether (75.0 μM added in 0.16 mL of ethanol). Identically prepared control incubations contained estriol 3-methyl ether in place of the alkylator (Δ, reactivated; □, control; ○, native enzyme). At various time intervals, 0.05 mL of each incubation was assayed for 17β-HSD activity. The percentage of enzyme activity is plotted on a logarithmic scale along the ordinate, and time is represented by the linear scale along the abscissa. The values are the means of duplicate experiments.

base-catalyzed hydrolysis of the ester linkage between the steroid nucleus and the alkylating side arm liberated the steroid from the active site. This was similar to the observation of Boussioux et al. (18) that ^{3}H-estrone was released at the same rate as that of enzyme reactivation following inactivation by 3-iodoacetoxy[6,7-^{3}H] estrone.

We developed an improved method for reactivating 17β,20α-hydroxysteroid dehydrogenase. Estrone 3-bromoacetate was chosen over

the iodinated alkylator because it is less susceptible to hydrolysis during inactivation at pH 6.5. After quenching the unreacted alkylator with the nucleophilic scavenger 2-mercaptoethanol, the pH of the enzyme mixture was rapidly titrated to pH 9.3, similar to Sweet's method (19). Whereas Sweet was able to recover only 80% of the control enzyme activity, our preparation was restored to 100% of the activity of the control enzyme. Finally, to repurify the reactivated enzyme, dialysis against a pH 7.0 buffer removed steroid, 2-mercaptoethanol, and cofactor. This system was reproducible during eight reactivation experiments.

Reactivated enzyme could not be inactivated a second time by the same alkylator, estrone 3-bromoacetate. However, 16α-bromoacetoxy-estradiol-17β 3-methyl ether caused loss of enzyme activity at a rate very similar to that observed when native enzyme reacts with this D-ring substituted alkylator. Thus, although there had been modification of an amino acid within the active site, these studies suggest that the conformation of the site was essentially unaltered.

The results of this investigation confirm that inactivation of human placental 17β,20α-hydroxysteroid dehydrogenase by estrone 3-bromoacetate results because the alkylating steroid aligns within the enzyme active site and then covalently binds through an ester bridge to an amino acid residue. The alkylation is so specific that reactivated enzyme cannot be inactivated a second time by the same alkylator. Furthermore, this shows that the alignment of steroid within the active site is very precise, offering little opportunity for steroid to move about, and thereby alkylate amino acids non-specifically . Finally,

reactivation of the affinity alkylated enzyme offers a new tool to explore the steroid binding regions of macromolecular proteins.

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