Reactivation of Human Placental 17β,20α-Hydroxysteroid Dehydrogenase Affinity Alkylated by Estrone 3-(Bromoacetate): Topographic Studies with 16α-(Bromoacetoxy)estradiol 3-(Methyl ether)†

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ABSTRACT: Estradiol 17β-dehydrogenase and 20α-hydroxysteroid dehydrogenase, oxidoreductase activities copurified from the cytosol of human-term placenta as a homogeneous protein (native enzyme), were reactivated at equal rates to 100% activity following complete inactivation in the presence of cofactor (NADPH) with the affinity alkylator estrone 3-(bromoacetate). Reactivation was accomplished by base-catalyzed hydrolysis of steroidal ester–amino acid linkages in the enzyme active site. The rate of enzyme reactivation was pH dependent. In identical studies without NADPH, only 12% of the original enzyme activity was restored. Completely reactivated enzyme was repurified by dialysis. Enzyme in control mixtures (control enzyme) that contained estrone in place of alkylator was treated the same as the reactivated enzyme. Reactivated enzyme exhibited a 6.0-fold lower affinity for common substrates, a 1.8-fold lesser affinity for NAD^+ and NADH, and the same affinity for $NADP^+$ and NADPH compared to control enzyme. In incubations that included NADPH, the reactivated enzyme maintained full activity during a 20-h second exposure to estrone 3-(bromoacetate), but in identical incubations without NADPH, the reactivated enzyme was rapidly inactivated at the same rate as the control and native enzymes. The control and reactivated enzymes were inactivated at equal rates by 16α-(bromoacetoxy)estradiol 3-(methyl ether) in the presence or absence of cofactor ($NADP^+$) and exhibited similar Kitz and Wilson inhibition constants for this affinity alkylator. Estrone 3-(bromo[2′-^{14}C]acetate) incubated with native enzyme and NADPH produced radiolabeled 3-(carboxymethyl)histidine and *S*-(carboxymethyl)cysteine. 16α-(Bromo[2′-^{14}C]acetoxy)estradiol 3-(methyl ether) produced twice the amount of radiolabeled 1,3-bis(carboxymethyl)histidine and half the amounts of radiolabeled 3-(carboxymethyl)histidine and *S*-(carboxymethyl)cysteine in reactivated enzyme compared to control enzyme. The areas under the ninhydrin absorbance peaks of 3-(carboxymethyl)histidine and *S*-(carboxymethyl)cysteine from the radioalkylated reactivated, control, and native enzymes were compared. These results demonstrate that (1) the estradiol 17β-dehydrogenase and 20α-hydroxysteroid dehydrogenase activities reside at a single site on one protein, (2) the active site histidine alkylated by estrone 3-(bromoacetate) in the presence of NADPH, although not essential to catalytic activity, is involved in substrate binding, while cysteine alkylated in the absence of cofactor is essential to catalysis and resides in the cofactor binding site, (3) diphosphopyridine and triphosphopyridine nucleotides occupy the enzyme active site in slightly different orientations, and (4) 16α-(bromo[2′-^{14}C]acetoxy)estradiol 3-(methyl ether) nonspecifically alkylates 3-(carboxymethyl)histidine in reactivated enzyme to produce radiolabeled 1,3-bis(carboxymethyl)histidine and the two affinity alkylators modify common cysteinyl residues nonspecifically.

The enzyme 17β,20α-hydroxysteroid dehydrogenase, an oxidoreductase purified from the cytosol of human-term placental villous tissue, expresses both estradiol 17β-dehydrogenase and 20α-hydroxysteroid dehydrogenase activities (Purdy et al., 1964; Strickler & Tobias, 1980, 1982). Studies with affinity alkylators have suggested that the two activities reside at a single active site on one protein and have modeled how progestin and estrogen substrates become oriented within the same enzyme active site (Strickler et al., 1981; Thomas et al., 1983). We now report reactivation of the enzyme activities following complete inactivation by the affinity alkylator estrone 3-(bromoacetate).[1] Reactivation is accomplished by base-catalyzed hydrolysis of the ester linkage that binds the alkylating steroid to the amino acid residue(s) in the enzyme active site (Sweet, 1976; Sweet & Samant, 1980). Simultaneous reactivation of the 17β and 20α activities is additional evidence for bifunctional enzyme activity at a single site. Reactivated enzyme tests whether a second affinity-labeling steroid modifies the same amino acid residue(s) carboxymethylated by the originator alkylator. Comparison of the kinetic constants measured with reactivated enzyme vs. unmodified enzyme localizes active site amino acids to the substrate or cofactor binding region.

Experimental Procedures

Materials. Steroid hormones (chromatographically pure), pyridine nucleotides (NAD^+, NADH, $NADP^+$, NADPH), and 2-mercaptoethanol were purchased from Sigma Chemical Co. Reagent-grade salts, organic chemicals, ScintiVerse liquid scintillation counting fluid, analytical-grade solvents, Spectrapor dialysis tubing (no. 2), and Eastman thin-layer chromatography sheets were obtained from Fisher Scientific Co.

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[1] Abbreviations and trivial names: estrone 3-(bromoacetate), 3-(bromoacetoxy)estra-1,3,5(10)-trien-17-one; 16α-(bromoacetoxy)estradiol 3-(methyl ether), 16α-(bromoacetoxy)estra-1,3,5(10)-triene-3,17β-diol 3-(methyl ether); estrone 3-(iodoacetate), 3-(iodoacetoxy)estra-1,3,5(10)-trien-17-one; EDTA, ethylenediaminetetraacetic acid; SDS, sodium dodecyl sulfate.

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Coomassie brilliant blue G-250 was a product of Eastman Kodak Co. Bromo[2-^{14}C]acetate (sp act. 17.4 mCi/mmol) was obtained from Pathfinder Laboratories, St. Louis, MO. [6,7-^{3}H]Estrone (sp act. 55.9 Ci/mmol) was purchased from New England Nuclear. Silica gel (silica Woelm TSC) for dry-column chromatography was obtained from Universal Scientific Inc. Glass-distilled, deionized water was used for all aqueous solutions. Buffer solutions were as follows: (buffer A) 0.01 M potassium phosphate buffer, pH 7.0, containing 5 mM EDTA and 20% glycerol (v/v); (buffer B) 0.01 M potassium phosphate buffer, pH 6.5, containing 5 mM EDTA and 20% glycerol (v/v).

Synthesis of Estrone 3-(Bromoacetate) and Estrone 3-(Bromo[2'-^{14}C]acetate). Estrone 3-(bromoacetate) was synthesized and purified as previously reported (LaRochelle et al., 1984). The uncorrected melting point (measured in an electrothermal apparatus), ultraviolet absorbance maximum (obtained with a Varian Cary 219 spectrophotometer), and infrared spectrum peaks (obtained with a Beckman Acculab 4 spectrometer) of the product were identical to the values reported for estrone 3-(bromoacetate) synthesized by a similar method (Murdock & Warren, 1982).

The synthesis and purification of estrone 3-(bromo[2'-^{14}C]acetate) used bromo[2-^{14}C]acetate and yielded a product with 17.0 mCi/mmol specific activity. Silica gel thin-layer chromatography (developed with chloroform) of the purified product identified a single spot and peak of radioactivity with an R_f value (0.85) identical with that of unlabeled estrone 3-(bromoacetate).

Synthesis of 16α-(Bromoacetoxy)estradiol 3-(Methyl ether) and 16α-(Bromo[2'-^{14}C]acetoxy)estradiol 3-(Methyl ether). The unlabeled steroid was synthesized at 0.07 mole scale of the published procedure (Chin & Warren, 1975). The crude product was chromatographed on two preparative silica gel plates (0.25 mm) developed with chloroform–ethyl acetate (9:1). The product band (R_f 0.48) was scraped from the plate and extracted with chloroform. The dried extract was recrystallized from petroleum ether. The final product exhibited an uncorrected melting point, ultraviolet absorbance maximum, and infrared spectrum peaks identical with the reported values (Chin & Warren, 1975).

The synthesis and purification of the radiolabeled steroid with bromo[2-^{14}C]acetate gave a final product with a specific activity of 16.2 mCi/mmol. Silica gel thin-layer chromatography (chloroform–ethyl acetate, 9:1) of the purified product yielded a single spot and peak of radioactivity at R_f value of 0.48.

Assay of Purified 17β,20α-Hydroxysteroid Dehydrogenase. The enzyme (17β:20α specific activity ratio 100:1) was purified from human-term placenta by the method of Murdock et al. (1983). The final product was homogeneous as shown by SDS disc gel electrophoresis, had a monomeric M_r of 35 000 on SDS gels, and possessed specific activity for estradiol 17β-dehydrogenase of 7 units/mg. These properties are identical with those reported for crystalline estradiol 17β-dehydrogenase (EC 1.1.1.62) (Chin et al., 1976). Protein concentration was determined by the Coomassie blue technique (Bradford, 1976).

"Native enzyme" is the original product purified from human placenta. "Reactivated enzyme" is defined as native enzyme that was inactivated to 0% activity by an affinity alkylator, completely reactivated, and repurified by dialysis. "Control enzyme" refers to native enzyme in an identical control mixture that contained estrone in place of alkylator and was treated the same as the reactivated enzyme. "Second inactivation" describes the loss of reactivated enzyme activity during a second exposure to an affinity alkylator.

Enzyme assays for estradiol 17β-dehydrogenase and 20α-hydroxysteroid dehydrogenase activities were performed in duplicate at 22 °C on a Varian Cary 219 recording spectrophotometer under our published conditions (Tobias et al., 1982). The slope of the initial linear change in absorbance at 340 nm (due to oxidation or reduction of cofactor) as a function of time was used to calculate enzyme activity.

Inactivation and Reactivation of the Enzyme. Native enzyme (0.5 μM) was incubated at 22 °C in buffer B, pH 6.5, with estrone 3-(bromoacetate) (35.0 μM) in the presence or absence of NADPH (60.0 μM). An identical control mixture contained an equal concentration of estrone in place of the alkylator.

Following inactivation of the enzyme by estrone 3-(bromoacetate), 2-mercaptoethanol was added to the control and experimental incubation mixtures in every study. Each mixture was then dialyzed in Spectrapor dialysis tubing (no. 2) against 300 volumes/dialyzate change of buffer A, pH 7.0, at 4 °C and/or titrated to a specific pH value with equal volumes of 0.2 N NaOH, depending on the experiment performed. A Fisher Accumet Model 325 expanded-scale pH meter was used to determine pH values within ±0.02 pH unit.

In studies that measured reactivation of both estradiol 17β-dehydrogenase and 20α-hydroxysteroid dehydrogenase activities, the enzyme was completely inactivated in the presence of NADPH. The control and experimental mixtures were then dialyzed for 24 h with three dialyzate changes to remove estrone and alkylator–mercaptoethanol complexes. The protein concentration of both retentates were determined and equalized by appropriate dilution with buffer A, pH 7.0. The control and inactivated enzyme preparations were titrated to pH 9.3, incubated at 22 °C, and assayed for 17β and 20α activities at various time intervals over 48 h.

In the reactivation study where enzyme was inactivated in the absence of NADPH, the control and alkylated enzyme mixtures were titrated to pH 9.3, incubated at 22 °C, and assayed for 17β activity over 48 h.

In studies that determined the effect of pH on the rate of reactivation, the control and completely inactivated enzyme mixtures (both containing NADPH) were divided into 1.0-mL aliquots. Each control and experimental aliquot pair was titrated to a specific pH value (8.0, 8.5, 9.2, 9.3, 9.4, 9.5), incubated at 22 °C, and assayed for 17β activity until at least 60% of control activity was attained. Experiments were performed in duplicate.

To prepare reactivated enzyme for kinetic and second inactivation studies, the control and completely inactivated enzyme mixtures (both containing NADPH) were titrated to pH 9.3, incubated at 22 °C, and assayed for 17β activity until reactivation to 100% activity relative to the control was attained. [6,7-^{3}H]Estrone was added to each mixture, and the mixtures were dialyzed against a total of 3000 volumes of buffer A as described previously (LaRochelle et al., 1984). After dialysis, radioactivity in duplicate 0.05-mL aliquots of each retentate was measured in a Model LS7500 Beckman liquid scintillation spectrometer (efficiency of tritium counting 48%). The concentration of [^{3}H]estrone remaining was less than 0.01 μM (0.03% of the original concentration) in each experiment. The protein concentration of each retentate was measured (Bradford, 1976).

Kinetic Studies. Kinetic constants for estrone, 17β-estradiol, progesterone, NAD^+, NADH, $NADP^+$, and NADPH were determined in duplicate with both the control and reactivated enzyme preparations from at least five appropriate concen-

trations of each substrate or cofactor. Incubations at 22 °C in 0.05 M potassium phosphate buffer at the pH optimum for substrate oxidation (9.2) or reduction (6.5) contained 10% ethanol. The method of Lineweaver & Burk (1934) was used to calculate the kinetic constants.

Second Inactivation Studies. Reactivated enzyme, control enzyme, or native enzyme (0.5 μM) was incubated at 22 °C in 0.72 mL of buffer A, pH 7.0, with estrone 3-(bromoacetate) (23.3 μM added in 0.08 mL of ethanol). Experiments were performed in duplicate. Identical control mixtures contained both the same enzyme preparation and estrone in place of alkylator.

Reactivated enzyme, control enzyme, or native enzyme was incubated at 22 °C in buffer A, pH 7.0, with 16α-(bromoacetoxy)estradiol 3-(methyl ether) in the presence or absence of $NADP^+$. An equal concentration of estriol 3-(methyl ether) replaced the affinity alkylator in otherwise identical control mixtures.

Affinity Radioalkylation Studies. Reactivated enzyme [carboxymethylated by unlabeled estrone 3-(bromoacetate)] or control enzyme (each at 0.5 μM) was incubated at 22 °C in 3.6 mL of buffer A, pH 7.0, with 16α-(bromo[2′-^{14}C]-acetoxy)estradiol 3-(methyl ether) (195.0 μM, added in 0.9 mL of ethanol). Identical control mixtures contained estriol 3-(methyl ether) in place of the radioalkylator. Aliquots (0.05 mL) of each mixture were assayed at various time intervals for 17β activity. When the reactivated or control enzyme was 70% inactivated, 2-mercaptoethanol (0.45 mM) was added, and the incubation mixture (3.6 mL) was dialyzed against 0.001 M potassium phosphate buffer, pH 7.0, until the dialyzate contained radioactivity at background level. The retentate was lyophilized and then acid hydrolyzed in evacuated, sealed tubes with 2.0 mL of 6.0 N HCl at 110 °C for 22 h. Amino acid analysis was performed with a Beckman-Spinco Model 118C automatic amino acid analyzer as described previously (Thomas & Strickler, 1983). The elution profile of appropriate carboxymethylated amino acid standards was determined separately from the profiles of the radioalkylated enzyme samples.

Native enzyme (0.75 μM) was incubated at 22 °C in 3.6 mL of buffer B, pH 6.5, with estrone 3-(bromo[2′-^{14}C]acetate) (35.0 μM, added in 0.4 mL of ethanol) in the presence of NADPH (60.0 μM). Aliquots (0.05 mL) were assayed for 17β activity until complete inactivation was attained, 2-mercaptoethanol (100.0 μM) was added, and the mixture was titrated to pH 9.3. A control mixture containing estrone in place of the radioalkylator was treated identically. After reactivation to 100% of control activity was achieved, the radioalkylated enzyme mixture (2.8 mL) was dialyzed, acid hydrolyzed, and analyzed for amino acid composition by the procedure described above.

Radioactivity in the collected fractions (0.4 mL) was measured in a Model LS7500 Beckman liquid scintillation spectrometer (efficiency of carbon-14 counting 90%). The areas beneath ninhydrin absorbance peaks for amino acids of interest were calculated by manual integration.

Analysis of Data. Linear kinetic data were fitted by least mean squares on a Texas Instruments SR-51-II calculator. Curves representing inactivation and reactivation of enzyme activity were fitted by polynomial regression analysis on a Digital Vax 11/780 computer.

Results

Simultaneous Reactivation of Estradiol 17β-Dehydrogenase and 20α-Hydroxysteroid Dehydrogenase Activities after Inactivation in the Presence of NADPH. The enzyme was completely inactivated by estrone 3-(bromoacetate) (steroid:enzyme molar ratio 46.7:1) in the presence of NADPH (cofactor:steroid molar ratio 1.7:1) as shown in Figure 1A. The reaction was quenched with a 2.9 molar excess of 2-mercaptoethanol, and the control and inactivated enzyme mixtures were dialyzed against buffer A, pH 7.0, for 24 h. The inactivated enzyme "spontaneously" regained 15% activity during dialysis. After the retentates were titrated to pH 9.3, and 17β and 20α activities in the experimental mixture underwent reactivation at equal rates to 100% activity relative to the control (Figure 1A). No enhancement of either enzyme activity beyond 100% was observed over 48 h. The absence of "superactivation", as reported by Boussioux et al. (1973) for human placental estradiol 17β-dehydrogenase using estrone 3-(iodoacetate), was discussed previously (LaRochelle et al., 1984).

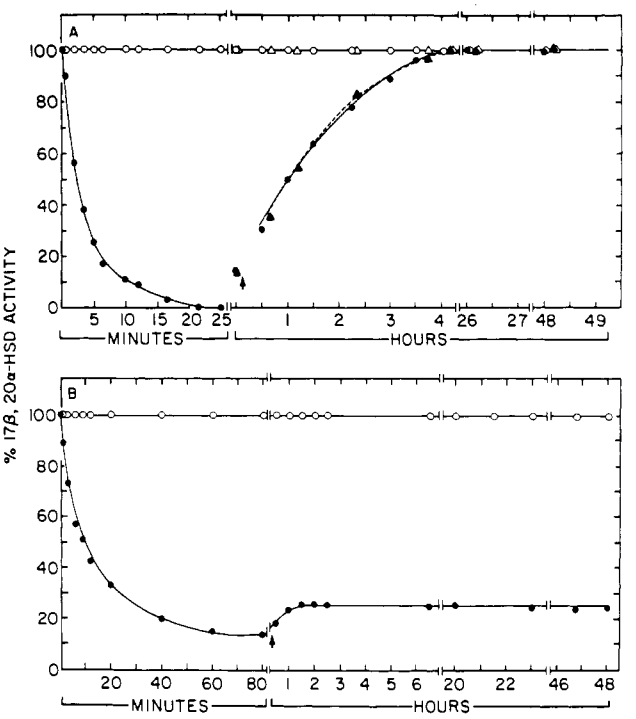

FIGURE 1: Inactivation of 17β,20α-hydroxysteroid dehydrogenase by estrone 3-(bromoacetate) in the presence and absence of cofactor followed by base-catalyzed reactivation. Enzyme (0.75 μM) dissolved in 9.0 mL of buffer B, pH 6.5 (with and without NADPH), was incubated at 22 °C with estrone 3-(bromoacetate) (35.0 μM added in 1.0 mL of ethanol). Identical control incubations that contained estrone in place of alkylator were assayed for either 17β (○) or 20α (△) activity when appropriate. (A) Incubations contained NADPH (60.0 μM). At various time intervals, 0.05 mL was assayed for 17β activity until complete inactivation in the experimental mixture (●) was observed. 2-Mercaptoethanol (100.0 μM) was added, incubations were dialyzed for 24 h, and the pH of the retentates was raised to 9.3 (↑). Periodically over 48 h, either 0.05 mL was assayed for 17β activity (●) with estrone (0.10 mM) as substrate, or 0.29 mL was assayed for 20α activity (△, broken line) with progesterone (0.18 mM) as substrate. (B) Identical incubations of 2.0-mL total volume did not contain cofactor. At various time intervals, 0.05 mL of control and experimental (●) mixtures were assayed for 17β activity until no further inactivation was seen. 2-Mercaptoethanol (100.0 μM) was added, and each incubation was titrated to pH 9.3. Aliquots (0.05 mL) were assayed for 17β activity over 48 h. The percentage of enzyme activity is plotted on a linear scale along the ordinates, and time is represented by the linear scale along the abscissas. The values are the means of at least duplicate experiments.

Dialysis was performed so that 20α activity could be measured with progesterone, a low-affinity substrate of the enzyme relative to estrone and the alkylator [K_m = 10 μM using NADH (Murdock & Warren, 1982)]. No detection of enzyme activity in assay cuvettes that contained 10% ethanol

Table I: Kinetic Constants of Substrates for Control and Reactivated 17β,20α-Hydroxysteroid Dehydrogenase

steroid (variable)	cofactor (constant)	control enzyme K_m (μM)	control enzyme V_{max} (μmol min^{-1} μg^{-1})	reactivated enzyme K_m (μM)	reactivated enzyme V_{max} (μmol min^{-1} mg^{-1})
estrone	NADH	3.3	2.1	28.5	1.8
17β-estradiol	NAD^+	4.9	2.3	31.0	1.8
progesterone	NADH	323.0	0.03	1108.0	0.05

Table II: Kinetic Constants of Cofactors for Control and Reactivated 17β,20α-Hydroxysteroid Dehydrogenase

cofactor (variable)	steroid (constant)	control enzyme K_m (μM)	control enzyme V_{max} (μmol min^{-1} mg^{-1})	reactivated enzyme K_m (μM)	reactivated enzyme V_{max} (μmol min^{-1} mg^{-1})
$NADP^+$	17β-estradiol	4.2	2.9	4.4	3.1
NADPH	estrone	2.8	1.9	2.5	1.9
NAD^+	17β-estradiol	12.5	2.0	24.9	2.1
NADH	estrone	7.7	2.5	11.8	1.7

without progesterone indicates that the dialysis effectively removed free estrogens.

Reactivation of Enzyme Activity after Inactivation in the Absence of NAPDH. By use of control and experimental incubation mixtures that contained steroid and enzyme concentrations equal to those described for Figure 1A but that excluded NADPH, the enzyme was inactivated to 14% of control activity as shown in Figure 1B. This "plateau" results because the spontaneous hydrolysis of estrone 3-(bromoacetate) over 80 min produces a concentration of estrone sufficient to prevent further inactivation by competition with the remaining alkylator for access to the enzyme active site (Sweet & Samant, 1980). After addition of 2-mercaptoethanol and titration of the mixtures to pH 9.3, reactivation to 26% activity relative to the control was attained, and the enzyme activity remained at this level for 48 h (Figure 1B).

pH Dependency of the Rate of Enzyme Reactivation. Experiments were performed that correlated the half-times of enzyme reactivation and the pH values to which the enzyme mixtures were adjusted following complete inactivation by estrone 3-(bromoacetate) (steroid:enzyme molar ratio 46.7:1) in the presence of NADPH (cofactor:steroid molar ratio 1.7:1). There is a linear relationship ($r^2 = 0.999$) between the rate of reactivation and pH (data not shown).

Comparison of Kinetic Constants Observed for Reactivated and Control Enzyme. Michaelis constants of the common substrates measured with the reactivated enzyme were an average of 6-fold greater than the K_m values of the substrates obtained with the control enzyme (Table I). The maximum turnover rates (V_{max}) obtained with reactivated enzyme were similar to the control values. The kinetic constants of the substrates measured with the control enzyme preparation compared favorably to our previously reported values obtained with the native enzyme (Tobias et al., 1982).

Table II shows that the affinities and maximum turnover rates of reactivated and control enzymes for $NADP^+$ and NADPH were very similar. The affinities of the reactivated enzyme for NAD^+ and NADH were 2.0-fold and 1.5-fold lower, respectively, relative to those of the control enzyme, although the maximum turnover rates compared more favorably. The kinetic constants obtained with the control enzyme are similar to those reported for these cofactors and estradiol 17β-dehydrogenase (Karavolas et al., 1970).

Second Inactivation Studies Using Estrone 3-(Bromoacetate). The reactivated enzyme was not inactivated again by a second exposure to estrone 3-(bromoacetate) in the presence of NADPH over a 20-h period, but the control enzyme and native enzyme were rapidly inhibited at equal rates in identical incubations containing cofactor (LaRochelle et al., 1984). When NADPH was not present in the incubations, estrone 3-(bromoacetate) (steroid:enzyme molar ratio 46.7:1) inactivated the reactivated, control, and native enzyme preparations at equal rates (time to 50% inactivation ranged from 10.0 to 10.7 min, data not shown).

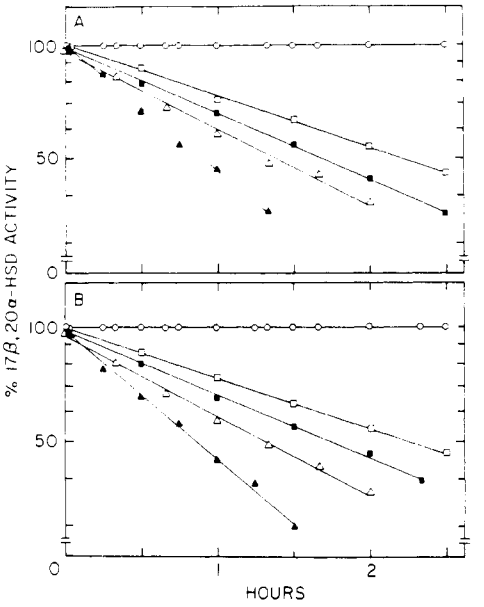

FIGURE 2: Second inactivation of reactivated 17β,20α-hydroxysteroid dehydrogenase by 16α-(bromoacetoxy)estradiol 3-(methyl ether). Various concentrations of 16α-(bromoacetoxy)estradiol 3-(methyl ether) [195.0 (▲), 130.0 (△), 105.0 (■), and 78.0 μm (□); each added in 0.16 mL of ethanol] were incubated in 0.64 mL of buffer A, pH 7.0, at 22 °C with (A) reactivated enzyme (0.5 μM) or (B) control enzyme (0.5 μM). Indentical control incubations (○) contained estriol 3-(methyl ether) in place of the alkylator and the appropriate enzyme preparation. At various time intervals, 0.05 mL of each incubation was assayed for 17β 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.

Second Inactivation Studies with 16α-(Bromoacetoxy)estradiol 3-(Methyl ether). The rates of inactivation of reactivated enzyme (Figure 2A) and control enzyme (Figure 2B) by 16α-(bromoacetoxy)estradiol 3-(methyl ether) were approximately equal at each alkylator concentration (steroid:enzyme molar ratios 156:1 to 390:1). In addition, when incubations contained evolutive cofactor ($NADP^+$, 10.0 μM), this affinity alkylator (195.0 μM) inactivated the reactivated, control, and native enzyme preparations (each at 0.5 μM) at similar rates (data not shown).

On the basis of the model of irreversible covalent inhibition (Kitz & Wilson, 1962), k_{app} values were determined from the ln (percent activity) vs. time plots for inactivation of the reactivated and control enzymes in the absence of cofactor, and the double-reciprocal plots, $1/k_{app}$ vs. $1/[I]$, were constructed. Both plots were linear, and the abscissa and ordinate intercepts yielded values for the inhibition constants (K_I) and the rate constants for covalent binding (k_3), respectively. The kinetic constants for the reactivated enzyme ($K_I = 1.64 \times 10^{-3}$ M; $k_3 = 2.02 \times 10^{-3}$ s^{-1}) were similar to those obtained for the control enzyme ($K_I = 1.97 \times 10^{-3}$ M; $k_3 = 2.36 \times 10^{-3}$ s^{-1}).

Affinity Radioalkylation Studies. Table III compares the relative quantities of [^{14}C]carboxymethylated amino acid residues produced in the reactivated and control enzymes by

Table III: Relative Quantities of Amino Acids Radioalkylated in Control, Reactivated, and Native 17β,20α-Hydroxysteroid Dehydrogenase

	16-BAE[a]		3-BAE[b]
amino acid[c]	control enzyme	reactivated enzyme	native enzyme
1,3-DCM-His	25.8 ± 0.7[d]	50.0 ± 2.3	5.7 ± 0.3
S-CM-Cys	49.8 ± 3.4	29.1 ± 2.2	43.5 ± 1.4
3-CM-His	9.4 ± 1.1	4.2 ± 0.5	47.4 ± 1.3
1-CM-His	4.2 ± 0.4	2.7 ± 0.3	0.0
ε-CM-Lys	3.7 ± 0.6	2.2 ± 0.7	1.6 ± 0.1

[a] Control or reactivated enzyme was incubated with 16α-(bromo[2′-^{14}C]acetoxy)estradiol 3-(methyl ether) (16-BAE) as described in the text. [b] Native enzyme was incubated with estrone 3-(bromo[2′-^{14}C]-acetate) (3-BAE) and NADPH as described in the text. [c] CM, carboxymethyl; DCM, bis(carboxymethyl). [d] Mean percent of total dpm ± standard error of the mean for duplicate experiments.

16α-(bromo[2′-^{14}C]acetoxy)estradiol 3-(methyl ether). The table also shows which amino acids are alkylated in the native enzyme by estrone 3-(bromo[2′-^{14}C]acetate) in the presence of NADPH. These carboxymethylated amino acid residues were present, but not radiolabeled, in the reactivated enzyme before it was second alkylated by 16α-(bromo[2′-^{14}C]acetoxy)estradiol 3-(methyl ether).

The percent of radiolabeled 1,3-bis(carboxymethyl)histidine measured in the reactivated enzyme was 1.9-fold greater than in the control enzyme. The possibility that 16α-(bromo[2′-^{14}C]acetoxy)estradiol 3-(methyl ether) radioalkylated the unlabeled 3-(carboxymethyl)histidine in the reactivated enzyme to produce the additional 1,3-bis(carboxymethyl)histidine was addressed. The normalized area under the ninhydrin absorbance peak of 3-(carboxymethyl)histidine in radioalkylated reactivated enzyme was 76% less than the area under this peak in the native enzyme radioalkylated by estrone 3-(bromo[2′-^{14}C]acetate) (Figure 3C). In addition, the percent of radiolabeled *S*-(carboxymethyl)cysteine in the reactivated enzyme was 1.7-fold less than that in the control enzyme. Comparison of the normalized areas under the ninhydrin absorbance peaks of *S*-(carboxymethyl)cysteine in the radioalkylated reactivated, control, and native enzymes demonstrates whether the two affinity alkylators modify common cysteinyl residues. The area under this peak in the reactivated enzyme was 58% of the sum of the corresponding areas in the control enzyme radioalkylated by 16α-(bromoacetoxy[2′-^{14}C]acetoxy)estradiol 3-(methyl ether) and native enzyme radioalkylated by estrone 3-(bromo[2′-^{14}C]acetate) (Figure 3D).

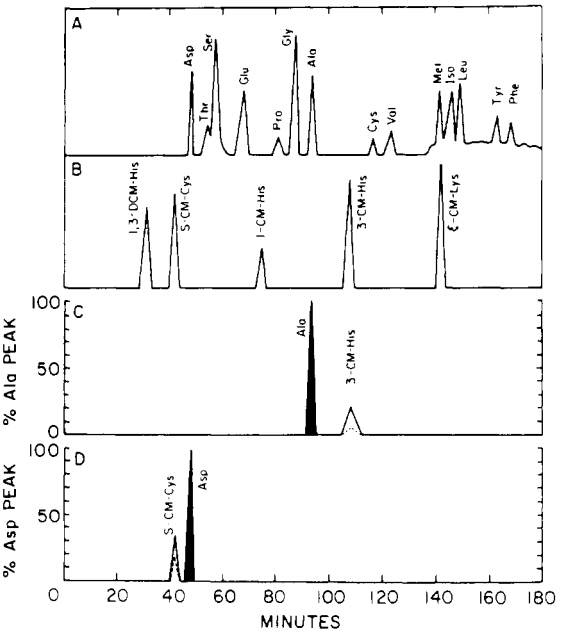

FIGURE 3: Ninhydrin elution profiles of amino acid analyses. (A) Ninhydrin profile of unmodified amino acid residues in the acid hydrolysate of 17β,20α-hydroxysteroid dehydrogenase. (B) Ninhydrin profile of carboxymethylated (CM) amino acid standards [DCM, bis(carboxymethyl)]. (C) Comparison of the areas under the ninhydrin absorbance peaks for 3-(carboxymethyl)histidine in acid hydrolysates of native enzyme radioalkylated by estrone 3-(bromo[2′-^{14}C]acetate) in the presence of NADPH (solid line) and reactivated enzyme radioalkylated by 16α-(bromo[2′-^{14}C]acetoxy)estradiol 3-(methyl ether) (broken line). Peak height represents peak area expressed as a percentage of the alanine peak area (shaded peak). (D) Comparison of the areas under the ninhydrin absorbance peaks for *S*-(carboxymethyl)cysteine: (1) the solid line represents the sum of these peak areas in acid hydrolysates of both native enzyme radioalkylated by estrone 3-(bromo[2′-^{14}C]acetate) in the presence of NADPH and control enzyme radioalkylated by 16α-(bromo[2′-^{14}C]acetoxy)estradiol 3-(methyl ether); (ii) the broken line represents this peak area in reactivated enzyme radioalkylated by 16α-(bromo[2′-^{14}C]acetoxy)-estradiol 3-(methyl ether); (iii) peak height represents peak area expressed as a percentage of the aspartate peak area (shaded peak). Peak areas are the mean values from duplicate experiments.

Discussion

The bifunctional activity of 17β,20α-hydroxysteroid dehydrogenase has been supported by studies wherein progestin (Strickler et al., 1981; Tobias et al., 1982; Thomas & Stricker, 1983), estrogen (Thomas et al., 1983), and cofactor analogue (Tobias & Strickler, 1981) affinity alkylators effected the simultaneous inactivation of the two oxidoreductase activities. In the present investigation, the simultaneous reactivation of the estradiol 17β-dehydrogenase and 20α-hydroxysteroid dehydrogenase activities to 100%, following complete inactivation by estrone 3-(bromoacetate), provides additional evidence that both enzyme activities reside at a single active site on one protein. The pH dependency of the rate of enzyme reactivation demonstrates that the mechanism of reactivation is base catalyzed hydrolysis of the (carboxymethyl)oxy ester linkage that binds the alkylating steroid to an amino acid residue in the enzyme active site, as proposed by Sweet (1976).

Estrone 3-(bromo[2′-^{14}C]acetate) in the presence of NADPH radiolabels histidine and cysteine in native 17β,20α-hydroxysteroid dehydrogenase. Murdock & Warren (1982) obtained a similar result in studies with human placental estradiol 17β-dehydrogenase. Since complete enzyme reactivation is observed despite carboxymethylation of these amino acid residues, neither of them is essential to the catalytic activity of the enzyme. Groman et al. (1975) showed that histidine alkylated by estrone 3-(bromoacetate) in the active site of human placental estradiol 17β-dehydrogenase was not essential by demonstrating "catalytic competence" of the affinity-alkylated enzyme. However, the relationship of the identified cysteinyl residue to catalytic activity has not been previously demonstrated.

When estrone 3-(bromoacetate) inactivates the enzyme in the absence of NADPH, only 12% of the activity is restored by base-catalyzed hydrolysis of steroidal ester–amino acid linkages. Previous studies (Pons et al., 1973; Murdock & Warren, 1982) demonstrated that substantially more cysteine relative to histidine is alkylated under these inactivation conditions. Incubation of the completely reactivated enzyme with estrone 3-(bromoacetate) and NADPH a second time results in no change in enzyme activity (LaRochelle et al., 1984), while in identical incubations without NADPH the reactivated enzyme is inactivated at the same rate as the control and native enzyme preparations. The cysteinyl residues in the reactivated enzyme active site that were unmodified due to protection by NADPH are alkylated by second exposure

to the affinity-labeling steroid when cofactor is not present. Therefore, at least one of the cysteinyl residues modified by estrone 3-(bromoacetate) in the absence of NADPH is essential to the correct alignment of cofactor so that catalytic activity can be expressed. A similar conclusion was proposed by Pons et al. (1977) in studies with human placental estradiol 17β-dehydrogenase using a variety of affinity alkylators.

Substrate kinetic constants of reactivated enzyme measure a 6-fold lower affinity for the common substrates relative to the control enzyme. The reactivated and control enzymes exhibit no differences in kinetic constants for $NADP^+$ or NADPH, while the reactivated enzyme has an average of 1.8-fold lesser affinity for NAD^+ and NADH than the control. Therefore, the active site histidyl residue that proximates the bound A ring of estrone 3-(bromoacetate) (Groman et al., 1975) must be unmodified for proper substrate alignment. Carboxymethylation of this histidine affects the binding of NAD^+/NADH to some extent but does not hinder the binding of $NADP^+$/NADPH. Thus, the diphosphopyridine and triphosphopyridine nucleotides occupy the active site in slightly different orientations.

Reactivated enzyme exposed to 16α-(bromoacetoxy)estradiol 3-(methyl ether) in the presence or absence of evolutive cofactor ($NADP^+$) is inactivated at a rate similar to that observed with control enzyme. Thus, the orientation of the bound alkylating steroid in the modified enzyme is essentially unaltered. Further, the similar inhibition kinetic constants (K_I, k_3) obtained with reactivated and control enzymes demonstrate that the presence of a 3-(carboxymethyl)histidine near the bound steroid A ring does not alter the efficiency of affinity alkylation by this D ring substituted steroid.

The patterns of amino acids in control and reactivated enzymes radioalkylated by 16α-(bromoacetoxy)estradiol 3-(methyl ether) are different. The amount of radiolabeled *S*-(carboxymethyl)cysteine produced in reactivated enzyme by 16α-(bromoacetoxy)estradiol 3-(methyl ether) is reduced compared to the amount observed after inactivation of the control enzyme. If common cysteinyl residues were not labeled by the 3- and 16-substituted estrogen analogues, the area under the ninhydrin absorbance peak of *S*-(carboxymethyl)cysteine in reactivated enzyme would equal the sum of these peaks from control enzyme alkylated by 16α-(bromoacetoxy)estradiol 3-(methyl ether) and native enzyme alkylated by estrone 3-(bromoacetate). Since reactivated enzyme contain 42% less *S*-(carboxymethyl)cysteine than the combined amount in control and native enzymes, the two estrogen alkylators must access one or more common cysteinyl residues. The common residue is nonessential to enzyme activity. 16α-(Bromoacetoxy)estradiol 3-(methyl ether) in the presence or absence of evolutive cofactor inactivates the control and reactivated enzymes at equal rates. Further, 17β-estradiol increases the alkylation of cysteine when estrone 3-(bromoacetate) and NADPH are incubated with human placental estradiol 17β-dehydrogenase (Murdock & Warren, 1982). Our results clarify Murdock and Warren's observation and demonstrate that estrone 3-(bromoacetate) modifies cysteine by nonspecific, hydrophobic labeling when NADPH is present. Since the reactivated enzyme did contain some radiolabeled *S*-(carboxymethyl)cysteine, 16α-(bromoacetoxy)estradiol 3-(methyl ether) also modifies one or more cysteinyl residues that are not labeled by estrone 3-(bromoacetate) in the presence of cofactor.

The seemingly paradoxical increase in 1,3-bis(carboxymethyl)histidine observed in reactivated enzyme after inactivation by 16α-(bromo[2′-^{14}C]acetoxy)estradiol 3-(methyl ether) represents the radioalkylation of unlabeled 3-(carboxymethyl)histidine already present. Affinity alkylation studies by Sweet et al. (1978) on the production of 1,3-bis(carboxymethyl)histidine in 3α,20β-hydroxysteroid dehydrogenase from *Streptomyces hydrogenans* demonstrated this phenomenon. Incubation of the bacterial enzyme with 16α-(bromoacetoxy)progesterone and bromo[2-^{3}H]acetic acid resulted in the production of tritiated 1,3-bis(carboxymethyl)histidine, but incubation of the enzyme with bromo[2-^{3}H]acetate alone did not form carboxymethylated amino acids. Sweet et al. concluded that nonspecific alkylation of histidyl residues already modified by the affinity-labeling steroid produced the 1,3-bis(carboxymethyl)histidine. Our comparison of ninhydrin absorbance peaks demonstrates that most of the 3-(carboxymethyl)histidine initially formed when estrone 3-(bromoacetate) alkylated native enzyme was converted to radiolabeled 1,3-bis(carboxymethyl)histidine when 16α-(bromo[2′-^{14}C]-acetoxy)estradiol 3-(methyl ether) was incubated with reactivated enzyme. Thus, both estrogen alkylators identify one or more common histidyl residues under these conditions. Further, the reduced amount of radiolabeled 3-(carboxymethyl)histidine identified by 16α-(bromoacetoxy)estradiol 3-(methyl ether) in reactivated enzyme compared to control enzyme supports our premise for common histidyl residues. Since 16α-(bromoacetoxy)estradiol 3-(methyl ether) inactivates the control and reactivated enzymes at equal rates in both the presence and absence of cofactor, the common histidine probably lies outside of the enzyme active site.

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Affinity Labeling of $NADP^+$-Specific Isocitrate Dehydrogenase by a New Fluorescent Nucleotide Analogue, 2-[(4-Bromo-2,3-dioxobutyl)thio]-1,N^6-ethenoadenosine 2′,5′-Bisphosphate†

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ABSTRACT: A new reactive fluorescent adenine nucleotide analogue has been synthesized and characterized: 2-[(4-bromo-2,3-dioxobutyl)thio]-1,N^6-ethenoadenosine 2′,5′-bisphosphate (BDB-TεADP). This compound reacts irreversibly with $NADP^+$-specific isocitrate dehydrogenase. Biphasic kinetics of inactivation are observed that can be described in terms of a fast initial phase of inactivation resulting in partially active enzyme of 8–10% residual activity, followed by a slower phase leading to total inactivation. NADPH protects completely against the fast phase of the reaction, indicating that modification occurs at the coenzyme binding site, whereas isocitrate with $MnSO_4$ protects totally against the slow phase of reaction. The inactivation rate constants for both phases exhibit nonlinear dependence on BDB-TεADP concentration, consistent with the formation of a reversible complex with the enzyme prior to irreversible modification. Covalent incorporation of BDB-TεADP is limited and specific; only 0.99 mol of reagent/mol of subunit is incorporated when the enzyme is 98% inactivated in the absence of ligands. A maximum incorporation of 0.5 mol of reagent/mol of subunit is obtained in the presence of isocitrate and $MnSO_4$, while incorporation in the presence of NADPH equals the difference between the incorporation in the absence of ligands and that measured in the presence of isocitrate and $MnSO_4$. It appears that 0.5 mol of reagent/mol of subunit is responsible for the fast phase of inactivation and the remaining incorporation causes the slow phase. Under all conditions used in this study, isocitrate dehydrogenase has been shown to exist as a dimer by analytical ultracentrifugation and by cross-linking with dimethyl suberimidate followed by analysis on polyacrylamide gels in the presence of sodium dodecyl sulfate. It is proposed that, in the fast phase of inactivation, 2-[(4-bromo-2,3-dioxobutyl)thio]-1,N^6-ethenoadenosine 2′,5′-bisphosphate reacts at the coenzyme binding site of one subunit of dimeric isocitrate dehydrogenase, causing complete inactivation of the modified subunit and substantial inactivation of the other subunit. This new reagent is likely to have general applicability as an affinity label for other $NADP^+$ binding enzymes.

Chemical modification studies have previously been performed on pig heart $NADP^+$-specific isocitrate dehydrogenase [threo-D_s-isocitrate:$NADP^+$ oxidoreductase (decarboxylating), EC 1.1.1.42] using a variety of group-specific reagents. Cysteinyl, glutamyl, arginyl, lysyl, methionyl, and histidyl residues have all been implicated as essential groups [see Colman (1983a) for a recent review]. Of these, only a histidyl residue has clearly been shown to be in the coenzyme binding site (Ehrlich & Colman, 1978). Affinity labeling using purine nucleotide analogues that have reactive functional groups has the potential to yield more specific chemical modification than is usually achieved with group-specific reagents (Colman, 1983b). The presence of a 2′-phosphate has been demonstrated to be essential for binding of nucleotides to $NADP^+$-specific isocitrate dehydrogenase (Ehrlich & Colman, 1978; Mas & Colman, 1984), so it is critical that any potential affinity label for this enzyme has such a 2′-phosphate. Few purine nucleotide affinity labels with a 2′-phosphate have been described. We here report the synthesis of such an analogue: 2-[(4-bromo-2,3-dioxobutyl)thio]-1,N^6-ethenoadenosine 2′,5′-bisphosphate (BDB-TεADP)[1] (shown in Figure 1 as structure VI). This new reagent has many characteristics desirable for a purine nucleotide affinity label including solubility in water, negative charge at neutral pH, reasonable stability in the pH range generally optimal for enzymes, and high reactivity of the bromodioxobutyl moiety enabling potential covalent reaction with a variety of amino acids. The fluorescent properties of this new reagent offer a convenient means of introducing a fluorescent probe into the nucleotide binding site of an enzyme. Because of the location of the functional group adjacent

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[1] Abbreviations: BDB-TεADP, 2-[(4-bromo-2,3-dioxobutyl)thio]-1,N^6-ethenoadenosine 2′,5′-bisphosphate; PADPR, 2′-phosphoadenosine 5′-diphosphoribose; εPADPR, 2′-phospho-1,N^6-ethenoadenosine 5′-diphosphoribose; TεADP, 2-thio-1,N^6-ethenoadenosine 2′,5′-bisphosphate; MES, 2-(*N*-morpholino)ethanesulfonic acid; SDS, sodium dodecyl sulfate; NMR, nuclear magnetic resonance; Me_2SO, dimethyl sulfoxide.

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