Affinity Labeling of Steroid Binding Sites

SYNTHESIS OF 21-BROMOACETYLA MINOPROGESTERONE AND STUDY OF 20β-HYDROXYSTEROID DEHYDROGENASE*

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To further study the steroid binding site of 20β-hydroxy-steroid dehydrogenase (EC 1.1.1.53) by affinity labeling, 21-[2-'3H]bromoacetylaminoprogesterone was synthesized. The steroid inactivates the enzyme in a time-dependent and irreversible manner which follows pseudo-first order kinetics. Incorporation of 2 mol of 21-[2-'3H]bromoacetylaminoprogesterone accompanies inactivation of 1 mol of enzyme. Amino acid analysis of the 6 N HCl hydrolysate of the radiolabeled enzyme identified dicarboxymethylhistidine as the main alkylation product. The stability of the amide bond in 21-bromoacetylaminoprogesterone excludes the possibility that diacylation of the active site histidine residue occurs by a transacylation mechanism. When 20β-hydroxy steroid dehydrogenase is inactivated with 16α-[2-14C]iodoacetic acid, amino acid analysis of the acid hydrolysate of the radiolabeled enzyme shows that 1H and 14C are present in dicarboxymethylhistidine in a ratio of 1.03:1. A two-step mechanism involving a specific, site-directed, steroi d-medi ted alkylation followed by a rapid nonspecific alkylation step is proposed to account for diacylation of an active site histidine residue resulting from affinity labeling of 20β-hydroxy steroid dehydrogenase by 16α-bromoacetoxyprogesterone and 21-bromoacetylaminoprogesterone. 21-Bromoacetylaminoprogesterone, 21-acetylaminoprogesterone, and 16α-carboxyamidoprogesterone are not reduced in the presence of NADH when tested as substrates for the enzyme. Kinetic studies show that all three progesterone analogs are competitive inhibitors of cortisol reduction. However, 21-azidoprogesterone which is electronically and steric ally similar to 21-acetylaminoprogesterone is a substrate with a Vmax value of 10.3 nmol min⁻¹ µg⁻¹ and an apparent Km value of 7.1 x 10⁻⁶ M. 16α-Cyanoprogesterone, which is structurally analogous to 16α-carboxyamidoprogesterone, has a Vmax value of 9.25 nmol min⁻¹ µg⁻¹ and an apparent Km value of 6.7 x 10⁻⁶ M. A mechanism involving unfavorable hydrogen bonding interactions between the steroidal amides and amino acid residues at the enzyme active site is proposed to account for the inability of 20β-hydroxy steroid dehydrogenase to effect hydrogen transfer between NADH and these steroids.

Cortisone 21-iodoacetate (1) and 16α-bromoacetoxyprogesterone (2) irreversibly inactivate 20β-hydroxy steroid dehydrogenase (EC 1.1.1.53) from Streptomyces hydrogenans by alkylation of an active site histidyl residue. The major alkylation product obtained with each steroid is 1,3-dicarboxymethylhistidine. Earlier, we proposed two mechanisms to explain the dialkylation of a single histidine residue. Transacylation to an active site neighboring group of the initially formed steroid-histidine conjugate would displace hydroxyprogesterone and allow a second steroid bromoacetate molecule to enter the active site to react with the remaining imidazole ring nitrogen atom. Alternatively, dialkylation could occur by 2 molecules of steroid bromoacetate reacting with the active site histidine to form N,N-bis(steroid-carboxymethyl)-histidine conjugate (2). This report describes the synthesis of 21-bromoacetylaminoprogesterone, which does not undergo hydrolysis and therefore cannot undergo a transacylation reaction during affinity labeling of 20β-hydroxy steroid dehydrogenase. The progesterone analog was used to affinity label the enzyme in order to investigate the mechanism by which the active site histidine residue is dicarboxymethylated and also to gain further insight into the interaction between steroids and macromolecular steroid binding sites.

MATERIALS AND METHODS

Affinity labels were synthesized as described in the appendix.1 Cortisone, progesterone, and 11-deoxy cortisol were obtained from Steraloids Co. Nucleotides (NAD+, NADH), L-amino acids, Triton X-100, and inorganic chemicals were purchased from Sigma. 20β-Hydroxysteroid dehydrogenase from Streptomyces hydrogenans (specific activity of 16 to 18 units/mg) was purchased from Sigma or Boehringer-Mannheim. Glass-distilled water was used for all aqueous solutions. Organic chemicals and solvents were obtained

1 The details of the synthesis are presented in miniprint at the end of this paper. Full size photocopies are available from the Journal of Biological Chemistry, 9600 Rockville Pike, Bethesda, Md. 20014. Request Document No. 77M-1340, cite author(s) and include a check or money order for $1.00 per set of photocopies.
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from Fisher and distilled prior to use. 2,5 Diphenyloxazole and 1,4-bis(2-(5-phenyloxazolyl))benzene were obtained from New England Nuclear. [2-3H]Bromoacetic acid was purchased from Amersham/Searle. Dialysis were performed with Union Carbide dialysis tubes from Scientific Products.

Enzyme assays were conducted with the following solutions added to a final volume of 1.0 ml: 0.75 ml of 0.05 M potassium phosphate buffer, pH 6.5, 0.1 ml of cortisone (0.18 μmol) in ethanol; 0.1 ml of NADH (0.1 μmol) in 0.05 M phosphate buffer, pH 7.0; 0.05 ml of 200 hydroxysteroid dehydrogenase in 0.05 M phosphate buffer, pH 7.0. The slope of the initial linear decrease in absorbance at 340 nm (due to oxidation of NADH) as a function of time was used to calculate enzyme activity. Assays were conducted at 25 ± 1°C in a Beckman model 25 recording spectrophotometer. Least mean activation kinetics and stoichiometry of inactivation from radiolabeling experiments were obtained by least mean squares fit of data with a Monroe 1760 programmable calculator.

Radiolabeled enzyme samples for acid hydrolysis were treated with constant boiling 6 M HCl in evacuated sealed tubes at 110° for 48 h. Following lyophilization of the hydrolysis the residue was dissolved in 0.3 ml of 0.2 M citrate buffer, pH 2.2, and amino acid analysis was performed with a Beckman-Spinco model 120 automatic amino acid analyzer (3). The fractionated effluent (0.5-ml aliquots from each fraction) was obtained from the amino acid analyzer (flow rate: 1.7 ml/min/tube) and was used to elute amino acids with 21-bromoacetylaminoprogesterone was slower than with the amino acid conjugate was formed (9). Alkylation of the amino acids with 21-bromoacetylaminoprogesterone was slower than with the acid hydrolysis was used to detect a steroid amine. They contained starting material and no other detectable steroid component. Therefore, within the pH range from 3.0 to 25° for 72 h no detectable (i.e. 0.1% or greater) hydrolysis of the amide linkage occurred.

**RESULTS**

**Synthesis of 21-Bromoacetylaminoprogesterone**

21-Acetylaminoprogesterone (4), 21-adipinoprogesterone (5), and 16α-cyanoprogesterone (6) were synthesized in this laboratory and exhibited the published physical characteristics. Infrared spectra of all steroids in KBr pellets were obtained in a Beckman Aculab 4 spectrophotometer. A Beckman model 25 spectrophotometer was used to obtain ultraviolet spectra, and a Varian model T-60 nuclear magnetic resonance spectrometer provided nuclear magnetic resonance (NMR) spectra. Melting points were determined in a Mel-Temp or Electrothermal apparatus and are reported uncorrected. Elemental analyses were performed by Galbraith Laboratories, Knoxville, Tenn.

**Properties of 21-Bromoacetylaminoprogesterone**

Alkylation of Amino Acids—Incubations of 21-bromoacetylaminoprogesterone in 0.05 M potassium phosphate buffer, pH 7.0, 25°, with L-cysteine, L-methionine, and L-histidine were monitored by thin layer chromatography using 1-butanol/acetic acid/water (12:3:5) as eluent. The chromatograms were visualized under ultraviolet light, then sprayed with ninhydrin reagent and heated for 1 min at 110°. The appearance of a new spot with an Rf value intermediate between those of the steroid and amino acid, which absorbs ultraviolet light and gives a positive ninhydrin reaction, showed that a steroid-amino acid conjugate was formed (9). Alkylation of the amino acids with 21-bromoacetylaminoprogesterone was slower than with the bromoacetoxyprogesterone derivatives under similar conditions (2, 9) by a factor of 2 to 3.

Stability—21-Bromoacetylaminoprogesterone (90 μg in 0.2 ml of ethanol) was incubated at 25° in each of the following solutions: 0.05 M potassium bicarbonate buffer, pH 10.5; 0.05 M boric acid/potassium chloride/sodium hydroxide buffer, pH 9.0; 0.05 M potassium phosphate buffer, pH 7.0; 0.05 M potassium biphthalate buffer, pH 4.0; and 0.05 M sodium acetate buffer, pH 3.0. After 72 h, each of the solutions was extracted with 30 ml of methylene chloride and evaporated under reduced pressure at 40°. The residues were dissolved in 0.2 ml of ethanol and 0.1 ml of solution, containing 24 to 30 μg of steroid, was analyzed by thin layer chromatography with ammonium chloroform/methanol (9:1) as eluent. The chromatograms were visualized under ultraviolet light, then sprayed with ninhydrin reagent (sensitive to 0.01 μg of amine) to detect a steroid amine. They contained starting material and no other detectable steroid component. Therefore, within the pH range of 3.0 to 25° for 72 h no detectable (i.e. 0.1% or greater) hydrolysis of the amide linkage occurred.

**Evaluation of 20β-Hydroxysteroid Dehydrogenase for Esterase and Amidase Activity—20β-Hydroxysteroid dehydrogenase (0.25 mg) was incubated at 25° in 5 ml of buffer with 200 μg of either 16α-acetoxyprogesterone or 21-acetylaminoprogesterone added in 0.2 ml of ethanol. During 72 h the enzyme activity in the incubation mixtures decreased by 20%. Each of the mixtures was extracted with five 10-ml portions of methylene chloride. The pooled methylene chloride extracts were concentrated to dryness under reduced pressure at 40° and the residues were dissolved in 0.2 ml of ethanol. A 0.04-ml aliquot containing 30 to 40 μg of steroid from the 16α-acetoxyprogesterone incubation was analyzed by thin layer chromatography developed with benzene/ethyl acetate (92:8) and showed a single spot with an Rf value identical with 16α-acetoxyprogesterone. 16α-Hydroxyprogesterone (i.e. 0.5 to 1.0% of the steroid chromatographed), which is well sepa-

![Fig. 1. A, synthesis of 21-bromoacetylaminoprogesterone; B, synthesis of 16α-carboxamidoprogesterone.](http://www.jbc.org/Downloadedfrom)
rated from 16α-acetoxyprogesterone, it was not detected on the chromatograms. Similarly, a 0.04-ml aliquot of the residue from the 21-acetylamino progester one incubation was chromatographed with ammoniacal chloroform/methanol (9:1). A single spot due to 21-acetylamino progesterone was detected by ultraviolet absorbance. No free amino progesterone steroid (i.e., more than 0.1% of steroid chromatographed) was detected when the plate was treated with ninhydrin reagent.

Evidence that 21-Bromoacetylaminoprogesterone Binds at Active Site of 20β-Hydroxysteroid Dehydrogenase

Substrate Characteristics—Mixtures of 20β-hydroxysteroid dehydrogenase (10−7 M) with 21-bromoacetylamino progesterone or 21-acetylamino progesterone present in concentrations of up to 10−4 M did not produce detectable NADH (10−4 M) oxidation during a 30-min period. A solution containing 1.8 × 10−4 M cortisone, 2.5 × 10−5 M 21-acetylamino progesterone, 1 × 10−4 M NADH, and 1 × 10−7 M enzyme produced a 33% attenuation in reaction velocity of cortisone reduction compared with a control mixture which did not contain the steroid amide.

Competitive Inhibition—21-Acetylamino progesterone and 21-bromoacetylamino progesterone were used as competitors of cortisone reduction to kinetically characterize the type of enzyme inhibition obtained with 21-bromoacetylamino dehydrogenase (see "Materials and Methods"). Converging lines in the Dixon plots of the resulting kinetic data indicated that the acetylamino steroids are competitive inhibitors with K values of 4.5 × 10−4 M and 7.7 × 10−4 M, respectively. These results suggest that 21-acetylamino progesterone and 21-bromoacetylamino progesterone bind at the enzyme active site. Since these steroids are not substrates, K values could not be determined. The reader may refer to Table II for km values of other enzyme substrates.

Inactivation of 20β-Hydroxysteroid Dehydrogenase with 21-Bromoacetylamino progesterone

Kinetics of Inactivation—Fig. 2 represents the results obtained when 20β-hydroxyprogesterone dehydrogenase (1.25 mg) was incubated at 25° in buffer (0.05 M potassium phosphate, pH 7.0, containing 10% glycerol and 0.001 M EDTA) with 3.6 μmol of 21-bromoacetylamino progesterone. Aliquots from the incubation mixture were assayed at 6-h intervals. Inactivation of the enzyme under these conditions had a t½ of 48 h. Addition of 2-mercaptoethanol (15 μM excess relative to steroid) to the incubation mixture (Fig. 3) prevented further inactivation and did not restore enzyme activity. The results with 2-mercaptoethanol are similar to those obtained with bromoacetoxyprogesterone derivatives (2, 7–9).

Alkylation of Histidine at Active Site of 20β-Hydroxysteroid Dehydrogenase—The enzyme (0.025 μmol) was dissolved in 50 ml of buffer (0.05 M phosphate, pH 7.0, containing 10% glycerol and 0.001 M EDTA). An aliquot (2.5 ml) of the solution was removed and 0.125 ml of ethanol was added to serve as a control. 21-(2H)Bromoacetylamino progesterone (3.6 μmol in 2.5 ml of ethanol) was added to the remaining 47.5 ml of enzyme solution and the resulting incubation mixture kept at 25° and assayed every 6 h. At times when the enzyme was approximately 20%, 30%, and 50% inactivated, 10-ml aliquots were removed from the incubation mixture and treated with 15 μM excess of 2-mercaptoethanol to stop the reaction. The remaining incubation mixture was allowed to reach approximately 70% inactivation and then 2-mercaptoethanol was added. Each sample was dialyzed against water until the dialysate contained radioactivity at the "background" level. The contents of each dialysis bag were lyophilized and the residues dissolved in 2 ml of water. An aliquot (0.2 ml) of retentate was removed for protein determination (10), and 0.1 ml was removed for 2H radioactivity quantitation. The results presented in Fig. 3 show that 2 mol of 2H-carboxymethyl groups per mol of enzyme are plotted along the ordinate and abscissa, respectively.

One-half of the 70% inactivated enzyme sample was hydrolyzed with 6 N HCl and authentic 1,3-dicarboxymethylhistidine, 1-carboxymethylhistidine, and 3-carboxymethylhistidine were added to the hydrolysate prior to amino acid analysis. Fractions were collected from the amino acid analyzer and quantitated for radioactivity. The data were quantitated. The per cent of enzyme activity and corresponding calculated moles of [2H]carboxymethyl groups per mol of enzyme are plotted along the ordinate and abscissa, respectively.

FIG. 2 (left). Inactivation of 20β-hydroxyprogesterone dehydrogenase by 21-bromoacetylamino progesterone. Enzyme (1.25 mg) was dissolved in 50 ml of buffer (0.05 M phosphate buffer, pH 7.0, containing 10% glycerol and 0.001 M EDTA) and kept at 25°. An aliquot (2.5 ml) was removed to serve as control (Δ) and was replaced with 2.5 ml (0.6 μmol) of 21-bromoacetylamino progesterone in ethanol (Φ). At various time intervals, 0.05 ml of each mixture was assayed for enzyme activity as described under "Materials and Methods." In one experiment one-half of the reaction mixture was removed and 2-mercaptoethanol was added in a molar ratio of 1:1 with respect to 21-bromoacetylamino progesterone (Φ). Assays of both incubation mixtures were continued. The enzyme activity in the incubation mixtures (relative to activity of the control) is plotted on a logarithmic scale along the ordinate, and the incubation time is plotted on a linear scale along with abscissa. The values are means of at least two experiments.

FIG. 3 (right). Decrease in enzyme activity associated with stoichiometry of 2H-carboxymethyl incorporation. Aliquots of the incubation mixture containing radiolabeled enzyme were taken at various time points during inactivation and treated with 2-mercaptoethanol (see Fig. 2) and then exhaustively dialyzed against distilled water. The resulting protein solution was concentrated to dryness and the residue was dissolved in an accurately measured volume of distilled water. Protein content and radioactivity in the solutions were quantitated. The per cent of enzyme activity and corresponding calculated moles of [2H]carboxymethyl groups per mol of enzyme are plotted along the ordinate and abscissa, respectively.
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Steroid Directed versus Nondirected Alkylation of Active Site Histidine Residue

Single Isotope Labeling Experiment—Inactivation of 20β-hydroxysteroid dehydrogenase (2.5 \times 10^{-7} \text{ M}) with nonradioactive 16α-bromoacetoxypregesterone (3.75 \times 10^{-3} \text{ M}) in the presence of [2-3H]bromoacetic acid (3.75 \times 10^{-3} \text{ M; containing 5 mCi of isotope}) in 40 ml of 0.05 M phosphate buffer, pH 7.0, at 25°, exhibited nearly identical inactivation kinetics as in the absence of bromoacetic acid (2). The respective molar ratios of bromoacetic acid to enzyme and steroid to enzyme were 15,000:1 and 150:1. The control incubation contained the same molar ratios of [2-3H]bromoacetic acid, enzyme, and 16α-acetoxyprogesterone as above. After the enzyme was 90% inactivated, 2-mercaptoethanol was added to the reaction and the control mixtures. The resulting mixtures were separately dialyzed, lyophilized, digested with 6 N HCl at 110°, and prepared for amino acid analysis as described above. Authentic 1,3-dicarboxymethylhistidine was added to each sample prior to analysis. Tritium quantitation of the fractionated effluent from the analyzer column revealed that 40% of the total emergent radioactivity (4500 dpm) was coincident with the dicarboxymethylhistidine fraction (the elution profile was similar to that in Fig. 4). The remaining radioactivity, constituting less than 10% of the total, did not correspond to either 1- or 3-carboxymethylhistidine or indeed to any known carboxymethyl amino acids and was unevenly distributed across the amino acid profile. By contrast, analysis of the control sample showed no significant peak of radioactivity associated with dicarboxymethylhistidine or any other known carboxymethyl amino acids.

Double Isotope Labeling Experiment—16α-[2-3H]Bromoacetoxypregesterone (26 mCi/mmol), [2-14C]iodoacetic acid (250 &micro;Ci/mmol), and 20β-hydroxysteroid dehydrogenase were incubated in a solution containing the same molar ratios and under similar conditions to those described for the single isotope labeling experiment above. Following enzyme inactivation of 22%, 47%, 59%, 75%, and 81% of initial activity a 15 molar excess of 2-mercaptoethanol relative to the steroid was added to each solution and they were exhaustively dialyzed against water to remove radioactivity which was not covalently bound to the protein. The enzyme inactivation kinetic data from this experiment, which are plotted in Fig. 5, show that the presence of [2-14C]iodoacetic acid does not alter the rate of affinity labeling. Each of the dialyzed aliquots was quantitated for 3H and 14C radioactivity and protein content. The resulting data, plotted as per cent enzyme activity as a function of moles of 3H-carboxymethyl groups and 14C-carboxymethyl groups per mol of enzyme protein, are shown in Fig. 6. These results show that inactivation of 20β-hydroxysteroid dehydrogenase by [2-3H]bromoacetoxypregesterone in the presence of a large excess of [14C]iodoacetic acid is accompanied by the incorporation of approximately 1 mol of 2-3H-carboxymethyl groups and 1 mol of 14C-carboxymethyl groups per mol of inactivated enzyme protein. Furthermore, incubation of the enzyme with equivalent amounts of 16α-acetoxyprogesterone and [2-14C]iodoacetic acid neither inactivates the enzyme, nor does [2-14C]iodoacetic acid alkylate the enzyme (see Refs. 1 and 9).

A doubly radiolabeled and 75% inactivated enzyme sample was prepared for amino acid analysis according to the procedure described above for the single isotope labeling experi-
ment. The fractionated effluent from the amino acid analyzer was quantitated for $^3$H and $^{14}$C activity. The resulting elution profiles are shown in Fig. 7. The fractions which contained dicarboxymethylhistidine possessed a constant ratio of $^3$H to $^{14}$C. There was no correspondence between $^3$H and $^{14}$C activity throughout the rest of the elution profile and the total radioactivity in all other fractions was less than 10% of that found in the dicarboxymethylhistidine fractions.

**FIG. 5.** Inactivation of 20$\beta$-hydroxysteroid dehydrogenase by 16$\alpha$-[2$'$$'$-3H]bromoacetoxyprogesterone in the presence of [2-$^{14}$C]iodoacetic acid. The enzyme (1.75 mg) was dissolved in 70 ml of buffer (0.05 M phosphate buffer, pH 7.0, containing 10% glycerol) and kept at 25°C. An aliquot (11 ml) of this mixture was removed and 6.0 $\times$ 10$^{-4}$ mmol of 16$\alpha$-acetoxyprogesterone were added so that the resulting mixture served as a control (O). To 59 ml of enzyme solution was added 1 ml (6.0 $\times$ 10$^{-3}$ mmol) of [2-$^{14}$C]iodoacetic acid (specific activity 8.36 mCi/mmol) and 12.3 ml were removed and the resulting mixture served as a second control (■). To 47.7 ml of enzyme-iodoacetic acid solution were added 2.3 ml (1.1 $\times$ 10$^{-3}$ mmol) of 16$\alpha$-[2$'$$'$-3H]bromoacetoxyprogesterone (specific activity 25.98 mCi/mmol) (○). At various time intervals 0.1 ml of each solution was assayed for enzyme activity, and at 78%, 53%, 41%, 25%, and 19% activity levels aliquots were removed and further inactivation was stopped by addition of a 15:1 molar excess of 2-mercaptoethanol relative to steroid and iodoacetic acid. Enzyme activity in the incubation mixture (relative to activity in the controls) is plotted on a logarithmic scale along the ordinate and incubation time is plotted on a linear scale along the abscissa.

**FIG. 6.** Stoichiometry of $^3$H- and $^{14}$C-carboxymethyl incorporation associated with decrease in enzyme activity. Aliquots of radiolabeled enzyme were removed from the incubation mixture at various time points during inactivation with 16$\alpha$-[2$'$$'$-3H]bromoacetoxyprogesterone and [2-$^{14}$C]iodoacetic acid in the presence of [2-$^{14}$C]iodoacetic acid and were treated with 2-mercaptoethanol and then exhaustively dialyzed against distilled water. The retentates were concentrated to dryness and the residues were dissolved in an accurately measured volume of distilled water. Protein content and radioactivity in the solutions were quantitated in duplicate in two separate experiments. The per cent of enzyme activity and corresponding calculated moles of 2$'$$'$-H-carboxymethyl and 2$'$$'$-C-carboxymethyl groups present per mol of enzyme are plotted along the ordinate and abscissa, respectively.

21-Aceto versus 21-Acetammonopropylgesterone and 16a-Carboxamido versus 16a-Cyanopropylgesterone as Substrates of 20$\beta$-Hydroxysteroid Dehydrogenase

Assays conducted under standard conditions (see "Materials and Methods") with varying amounts of C-21 or C-16 substituted progesterone derivatives, and at two different enzyme concentrations, produced the kinetic results summarized in Tables I and II. The presence of $5 \times 10^{-3}$ m acylaminosteroid in the assay mixture did not produce a measurable consumption of NADH, even after 1 h at 25°C. Kinetic studies of these steroids with cortisone as a substrate showed 21-acetylaminopropylgesterone to be a competitive inhibitor as described above;
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**Table I**

| Inhibitor                           | $K_v$ value | $V_{max}$ |
|-------------------------------------|-------------|-----------|
| Progesterone                        | 0.395       | 11.58     |
| Cortisone                           | 5.10        | 12.52     |
| Cortisone 21-iodoacetate            | 10.0        | 10.0      |
| 21-Iododeoxycortisone               | 14.0        | 20.0      |
| 21-Azidoprogesterone                | 7.1         | 10.5      |
| 16α-Acetoxyprogesterone             | 12.5        | 0.93      |
| 16α-Cyanoprogesterone               | 67.0        | 9.25      |

$^a$ Data were taken from Ref. 2.

$^b$ Data were taken from Ref. 1.

**Discussion**

Cortisone 21-iodoacetate (1) and 16α bromoacetoxypregesterone (2) were previously synthesized to affinity label the oxidoreductase 20β-hydroxysteroid dehydrogenase. Each of these steroids produced incorporation of 2 mol of 3H-carboxyterone (2) were previously synthesized to affinity label the basis on the fact that base-catalyzed hydrolysis of bromoacetoxyprogesterone-amino acid conjugates readily occurs (8).

The second mechanism is plausible mechanism was that following steroid-directed reaction of 20P-hydroxysteroid dehydrogenase with Gp-bromoacetoxyprogesterone, reactivation of the enzyme can be accomplished by adjusting the incubation mixture to pH 9.0, conditions which hydrolyze the steroid ester (11). 21-Bromoacetylamino acid conjugate is formed under these conditions inactivation with the structurally analogous cortisone 21-iodoacetate or 16α-bromoacetoxypregesterone. The $t_{1/2}$ of enzyme inactivation with 21-bromoacetoxypregesterone is 48 h, while under similar experimental conditions inactivation with the structurally analogous cortisone 21-iodoacetate occurs with a $t_{1/2}$ of 4 h (1).

16α-carboxyamidoprogesterone was found to have a $K_v$ of $5 \times 10^{-3} \text{ M}$. 21-Bromoacetylaminoprogesterone was synthesized to serve as starting material for the preparation of 21-bromoacetoxypregesterone (VI) because the blocked C-3 and C-20 keto groups permit isolation of this intermediate. The groundwork for establishing the structure of VI was recently reported (5), and the structure of V was confirmed on a similar basis. 21-Acetylamino-21-bromoacetoxypregesterone and 21-bromoacetoxypregesterone are stable in strongly acidic solutions (the latter steroid is synthesized in methanolic hydrogen bromide). The C-21 amide linkage also resists base-catalyzed hydrolysis even under strongly alkaline conditions.

One of our earlier stated criteria for evaluating the capability of an affinity-labeling steroid to react with an amino acid residue at the enzyme active site is the demonstration that the steroids are substrates for the enzyme (1, 3, 8, 9). Under optimally adjusted conditions of maximum steroid concentration, enzyme and co-factor concentrations, and prolonged periods of incubation, substrate activity of 21-bromoacetoxypregesterone could not be detected. Similarly, 21-acetylamino-21-bromoacetoxypregesterone is not a substrate for 20β-hydroxysteroid dehydrogenase. Therefore, both C-21 steroid amides were studied as inhibitors of cortisone reduction by the enzyme. Dixon plots of the kinetic results show that 21-bromoacetoxypregesterone and 21-acetylamino-21-bromoacetoxypregesterone are competitive inhibitors with $K_v$ values of $4.5 \times 10^{-5} \text{ M}$ and $7.7 \times 10^{-4} \text{ M}$, respectively. Therefore, the C-21 acetylamino steroids are capable of binding at the enzyme active site.

Incubations of 21-bromoacetoxypregesterone with cysteine, methionine, and histidine, under conditions similar to those reported for bromoacetoxypregesterone isomers (2, 7-9), showed that 21-bromoacetoxypregesterone alkylates nucleophilic amino acids much more slowly than do any of the steroid bromoacetates. This attenuation in alkylating activity may explain the longer period required for inactivation of 20β-hydroxysteroid dehydrogenase by 21-bromoacetoxypregesterone (Fig. 2) compared with that of the structurally analogous cortisone 21-iodoacetate or 16α-bromoacetoxypregesterone. The $t_{1/2}$ of enzyme inactivation with 21-bromoacetoxypregesterone is 48 h, while under similar experimental conditions inactivation with the structurally analogous cortisone 21-iodoacetate occurs with a $t_{1/2}$ of 4 h (1).

Amino acid analysis of the acid hydrolysate derived from 20β-hydroxysteroid dehydrogenase which had been radiolabeled with 21-[2-3H]bromoacetoxypregesterone revealed that affinity labeling produced 1,3-dicarboxyamidohistidine (Fig. 4) and no detectable monocarboxyamidohistidine. Similar results obtained earlier with analogous hydroxysteroid aromatases were rationalized on the basis that the ester linkage of these affinity labeling steroids is susceptible to hydrolysis and, therefore, a transacylation reaction could possibly be driving dialkylation to rapid completion (2). Evidence that this type of transacylation occurs during affinity labeling of chymotrypsin with substrates that contain an ester bond has been reported by Lawson and Schramm (12, 13). In the present case the amide linkage of 21-bromoacetoxypregesterone is not hydrolyzed under acidic or basic conditions. Moreover, 20β-hydroxysteroid dehydrogenase does not have esterase or amidase activity. The steroid-histidine conjugate (Fig. 6) resulting from alklylation by 21-bromoacetoxypregesterone is expected to be stable. Thus, our earlier proposed transacylation mechanism can be ruled out by analogy with the experimental results reported by Schramm and Lawson. They did not obtain the chymotrypsin-induced transacylation reaction with the N-substituted α-bromoacetamide substrates (14) as they had earlier obtained when the analogous esters...
Affinity Labeling were used for affinity labeling (12, 13).

The mechanism of dialkylation of a histidine residue at the active site of 20β-hydroxysteroid dehydrogenase by 21-bromoacetylaminoprogesterone, 16α-bromoaetoxyprogesterone, or cortisone 21-iodoacetate occurs in a two-step sequence represented by the equations in Fig. 8. Incubation of the enzyme for 12 h at 25° with [2-3H]bromoaetic acid alone does not produce loss in catalytic activity or formation of 1,3-[2-H]dicarboxymethylhistidine, but the presence of 16α-bromoaetoxyprogesterone promotes both of these reactions. When a mixture of 16α-[2'-3H]bromoaetoxyprogesterone and [2-14C]iodoacetate is incubated with 20β-hydroxysteroid dehydrogenase the kinetic data from enzyme inactivation (Fig. 6) are nearly identical with those reported earlier (2). Both 3H and 14C are incorporated in the enzyme protein. A 1:1 ratio of 3H-carboxymethyl and 14C-carboxymethyl groups is present in 1,3-dicarboxymethylhistidine derived from amino acid analysis of the doubly radiolabeled enzyme hydrolysate, and no monocarboxymethylhistidine can be detected. These results suggest that a slow steroid-directed alkylation step (k1, Fig. 8) is followed by a rapid and nonsteroid-directed alkylation reaction (k2 or k3, Fig. 8).

The fact that the steroid amides are not substrates for 20β-hydroxysteroid dehydrogenase deserves further consideration. 21-Azidoprogesterone (II, Fig. 1) was used as an isosteric and isoelectronic model of 21-acetylaminoprogesterone to gain insight into the reason for the lack of substrate behavior of the C-21 acetylaminosteroids. The similarity in chemical shifts of the C-21 proton magnetic resonance signal (7, 6.10) of II to the corresponding signal (7, 5.92) due to the C-21 protons of 21-acetylaminoprogesterone (4, 5) suggests that contributions by the azido and acetylamino groups to the electronic environment surrounding the C-20 carbonyl group are similar. Comparison of molecular models shows that the azido and acetylamino groups are spatially similar. 21-Azidoprogesterone was found to have a Vmax value of 10.5 nmol min⁻¹ mg⁻¹ and an apparent Km value of 7.1 × 10⁻⁵ M, which are characteristic of a good substrate for 20β-hydroxysteroid dehydrogenase (see Table II).

A possible connection between the hydrogen-bonding capacity of C-21 amide substituents and the apparent inability of the enzyme to effect transfer of a hydrogen atom between NADH and the C-20 carbonyl group is suggested by the infrared spectra of the C-21 acylamino steroids, which exhibit absorption bands at 3340 cm⁻¹ characteristic of strong intramolecular hydrogen bonding. Therefore, 16α-carboxamidoprogesterone (VIII, Fig. 1) was synthesized in order to further explore the fact that a hydrogen-bonding substituent has on a potential steroid substrate. Preparation of VIII from 16α-cyanoprogesterone (VII) was accomplished by hydrolysis of the cyano group with hydrochloric acid (Panel B, Fig. 1). Although VII exhibits good substrate characteristics with 20β-hydroxysteroid dehydrogenase (Table II), VIII possesses no detectable substrate activity. The amide is a competitive

![Fig. 8](http://www.jbc.org/Downloaded from http://www.jbc.org/)

**Fig. 8.** Mechanism of dialkylation of a histidine residue by 16α-bromoaetoxyprogesterone at the active site of 20β-hydroxysteroid dehydrogenase. The first reaction (k1) involves a slow steroid-directed alkylation step. The second step (k2 or k3) involves a rapid nonsteroid-directed alkylation of the remaining imidazole ring nitrogen atom. The structures shown are arbitrarily drawn since the specific nitrogen atoms involved in each step are not known.
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inhibitor, similar to 21-acetylamino progesterone. Both 16α- and 21-haloacetoxy groups on the steroid molecule can alkylate the same histidine residue because the reaction groups have access to a common region of space (2). By analogy, the corresponding 21-acetylamino and 16α-carboxyamido groups can assume similar conformations which enable the amide groups to interact with the active site histidine residue (Fig. 9) and inhibit hydrogen transfer between NADH and the C-20 carbonyl function.

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