Affinity Alkylation of the Active Site of C_{21} Steroid Side-Chain Cleavage Cytochrome P-450 from Neonatal Porcine Testis: A Unique Cysteine Residue Alkylated by 17-(Bromoacetoxy)progesterone†

Makoto Onoda,‡ Mitsuru Haniu,§ Kazutoshi Yanagibashi,‡ Frederick Sweet,‖ John E. Shively,*,§ and Peter F. Hall‡

Worcester Foundation for Experimental Biology, Shrewsbury, Massachusetts 01545, Washington University School of Medicine, St. Louis, Missouri 63110, and Beckman Research Institute of the City of Hope, Duarte, California 91010

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ABSTRACT: The affinity alkylating progesterone analogue 17-(bromoacetoxy)progesterone has been used to label the active site of a microsomal cytochrome P-450 enzyme from neonatal pig testis. The enzyme causes removal of the C_{20} and C_{21} side chains from the substrates progesterone and pregnenolone by catalyzing both 17-hydroxylase and $C_{17,20}$-lyase reactions, which produce the corresponding C_{19} steroidal precursors of testosterone. The progesterone analogue causes simultaneous inactivation of the two catalytic activities of the enzyme by a first-order kinetic process that obeys saturation kinetics. Progesterone and 17-hydroxyprogesterone each protect the enzyme against inactivation. The progesterone analogue is a competitive inhibitor of the enzyme with K_i values of 8.4 μM and 7.8 μM for progesterone and 17-hydroxyprogesterone, respectively. The enzyme inactivation and kinetic data are consistent with a theory proposing that the analogue and the two substrates compete for the same active site. The radioactive analogue 17-([^{14}C]bromoacetoxy)progesterone causes inactivation of the enzyme with incorporation of 1.5–2.2 mol of the analogue per mole of inactivated enzyme. When this experiment is carried out in the presence of a substrate, then 0.9–1.2 mol of radioactive analogue is incorporated per mole of inactivated enzyme. The data suggest that the analogue can bind to two different sites, one of which is related to the catalytic site. Radiolabeled enzyme samples, from reactions of the ^{14}C-labeled analogue with the enzyme alone or with enzyme in the presence of a substrate, were subjected to amino acid analysis and also to tryptic digestion and peptide mapping. Amino acid analysis revealed that two cysteine residues are modified by the analogue in the absence of substrate and one cysteine is modified in the presence of substrate. Peptide mapping of the tryptic digests gave two different radiolabeled peptides from the affinity-labeled enzyme. Each of the peptides contained one cysteine residue covalently modified with the affinity label. Incorporation of radiolabel into one of the peptides was inhibited by affinity labeling in the presence of substrate. This cysteine is located in Homology Region 1 (HR1), common to other microsomal cytochromes P-450. The other labeled cysteine (whose labeling is not protected by substrate) is located at the COOH terminus, proximal to the cysteine that is believed to be involved in the binding of the heme group (fifth ligand). Since the peptide derived from radiolabeled enzyme produced from the reaction of the analogue with enzyme in the presence of a substrate contains only the COOH-terminal *S*-([^{14}C]carboxymethyl)cysteine, it is concluded that a unique cysteine that is located in HR1 forms part of the active site of the enzyme and that the same active site catalyzes both 17-hydroxylase and $C_{17,20}$-lyase activities.

Conversion of the C_{21} steroids pregnenolone and progesterone to the corresponding C_{19} androgenic steroids by a side-chain cleavage process is catalyzed by a cytochrome P-450 enzyme in neonatal pig testis. This enzyme has recently been isolated in our laboratories and shown to be homogeneous by electrophoretic and immunochemical criteria (Nakajin & Hall, 1981; Nakajin et al., 1981a) and also by partial amino acid sequence analysis (Nakajin et al., 1981a). The enzyme distinguishes among various steroidal substrates as shown by enzyme kinetics, substrate-induced light absorption shifts of the Soret peak, and equilibrium dialysis (Nakajin et al., 1981b). The kinetic data suggest that both the 17-hydroxylase and the $C_{17,20}$-lyase steps occur at the same active site. These findings prompted us to employ affinity alkylation techniques for learning more about the nature of the catalytic site.

Bromoacetoxy analogues of progesterone are useful as affinity alkylating probes because the steroid ring system is rigid and also because a large variety of the substrate analogues are available as reagents (Warren et al., 1975; Yasukochi et al., 1979). The active site of the cytochrome P-450 enzyme from neonatal pig testis catalyzes a side-chain cleavage reaction at the C_{17} position of progesterone. Therefore, we decided to use the 17-bromoacetoxy analogue of progesterone, presuming that there may be a nucleophilic amino acid at the enzyme active site within reach of the reactive bromoacetoxy side chain as the steroid binds at the active site. The bromoacetoxy group reacts with amino acids that contain a nucleophilic functional group such as histidine (imidazole), lysine (ϵ-amino), and cysteine (sulfhydryl) (Warren et al., 1975; Yasukochi et al., 1979). As the steroid analogue reversibly binds to the active site of an enzyme, the bromoacetoxy side chain can react with and thereby modify a nearby amino acid. The reaction leads to attachment of the (bromoacetoxy)progesterone analogue to the active site of a progesterone-specific enzyme because

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* Address correspondence to this author.
‡ Worcester Foundation for Experimental Biology.
§ Beckman Research Institute of the City of Hope.
‖ Washington University School of Medicine.

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displacement of the bromine atom by the nucleophilic amino acid residue forms a covalent bond between the amino acid and the analogue. The modified amino acid can be identified in an amino acid analyzer. Also, tryptic digestion of enzyme that has been affinity alkylated with a radioactive analogue can provide a radioactive peptide fragment from the active site, identified by the modified amino acid residue. The present study shows that 17-(bromoacetoxy)progesterone labels the active site of cytochrome P-450 from neonatal pig testis by affinity alkylation.

Materials and Methods

Preparation, Characterization, and Assays of P-450 Enzyme. A cytochrome P-450 enzyme that catalyzes the side-chain cleavage of C_{21} steroids was isolated from microsomes obtained from neonatal pig testis. The methods for purification and characterization of the enzyme have been previously reported by us (Nakajin & Hall, 1981). Measuring the 17-hydroxylase and $C_{17,21}$-lyase activities involves incubating the enzyme with [^{14}C]progesterone. After separation and measurement of the radioactivity of 17-hydroxyprogesterone and the androsterone products, both of the enzyme activities can be calculated from these data, as we previously described (Nakajin et al., 1981a). In the present studies, both of the enzyme activities were measured in each experiment. Flavoprotein reductase that was used in the enzyme assays was prepared from pig liver by an established method (Yasukochi et al., 1979). Reduction to the cytochrome *c* for the enzyme assays was performed by a standard method (Omura & Takesue, 1970; Onoda & Hall, 1982).

Preparation of the Progesterone Analogue 17-(Bromoacetoxy)progesterone. 17-(Bromoacetoxy)progesterone was prepared and characterized as described elsewhere (Sweet & Samant, 1980). The purity of the steroidal reagent was established by melting point, mixed melting point, and thin-layer chromatography. For the radiolabeling studies, 17-([^{14}C]-bromoacetoxy)progesterone was synthesized with ^{14}C in the bromoacetoxy side chain. The identity and radiochemical purity of the radioactive analogue were confirmed by its co-crystallization to a constant specific activity with authentic, nonradioactive 17-(bromoacetoxy)progesterone. The specific activity of 17-([^{14}C]bromoacetoxy)progesterone was derived from measuring the radioactivity by liquid scintillation spectrometry and the optical density at A_{239} (ϵ = 16 500 cm^{-1} M^{-1}) of the compound in an ethanol solution.

17-(Bromoacetoxy)progesterone was stable for several days in solution, and it showed no evidence of deterioration when stored for months at −20 °C in the dark in a desiccator. Acetoxyprogesterone, which does not react with amino acids as does its bromoacetoxy analogue, was substituted for the bromoacetoxy analogue in the control enzyme incubations.

Inactivation of Cytochrome P-450 by 17-(Bromoacetoxy)progesterone. Cytochrome P-450 (44 nM) was incubated at 25 °C in potassium phosphate buffer (50 mM, pH 7.25, containing 1 mM EDTA)[1] with 17-([^{14}C]bromoacetoxy)-progesterone or 17-acetoxyprogesterone (5 μM), in a total volume of 0.9 mL. Periodically, aliquots were removed from the incubation mixtures, and the enzyme activity was measured at 37 °C as previously described (Nakajin & Hall, 1981). In each experiment, both 17-hydroxylase and $C_{17,20}$-lyase activities were measured. The two enzyme activities were each determined in triplicate for each time point during the enzyme inactivation. All the experiments were accompanied by controls in which 17-acetoxyprogesterone was substituted for the bromoacetoxy analogue in the enzyme incubations. Inactivation of the enzyme was arrested by addition of 2-mercaptoethanol (500 μM), which rapidly reacts with bromoacetoxy groups, converting them to unreactive [(2-hydroxyethyl)mercapto]acetoxy groups (Warren et al., 1975). Each of the steroid analogues was added in ethanol (10-μL) solutions to the incubation mixtures.

To test the enzyme for possible nonspecific alkylation, an experiment was carried out with bromoacetic acid (100 μM) replacing the bromoacetoxy analogue. During the time course of the affinity alkylation studies (3–4 h), no observable loss of enzyme activity occurred due to the bromoacetic acid.

To establish that 17-(bromoacetoxy)progesterone undergoes a reversible binding step at the active site of the enzyme, the following series of experiments was conducted. Enzyme assays were carried out with radioactive progesterone or 17-hydroxyprogesterone as the substrate, and various concentrations of 17-(bromoacetoxy)progesterone were used for testing the analogue as a reversible inhibitor during short-term incubations (i.e., less than 30 min). The analogue was found to be a competitive inhibitor of the enzyme with K_i values of 8.4 μM and 7.8 μM for progesterone and 17-hydroxyprogesterone, respectively.

A series of kinetic experiments was carried out with 17-(bromoacetoxy)progesterone to determine whether inactivation of the enzyme obeys saturation kinetics. The conditions of these enzyme inactivation experiments were similar to those described above except that the concentrations of the analogue in the incubation mixtures were varied between 5 and 80 μM. The results of these double-recipricol plots were linear with intercepts of approximately 8.3–10.3 μM.

Stoichiometry of Inactivation of Cytochrome P-450 by Affinity Radioalkylation with 17-([^{14}C]Bromoacetoxy)-progesterone. Cytochrome P-450 (44 nM) was incubated in the above-described buffer system with 17-([^{14}C]bromoacetoxy)progesterone (5 μM) or with an equivalent concentration of 17-acetoxyprogesterone in a total volume of 45 mL and at 25 °C. At various times, aliquots (0.9 mL) were removed and treated with 2-mercaptoethanol, and both enzyme activities were measured. Larger volumes of corresponding aliquots were treated with 2-mercaptoethanol (0.5 mL, 25 μmol), and the resulting mixtures were dialyzed until the radioactivity in the retentates was at the background level. Then the retentates were lyophilized, and the resulting residues were dissolved in small volumes of water. The ^{14}C content of the solutions was measured by liquid scintillation spectrometry, and the protein content was measured by amino acid analysis. The stoichiometry of inactivation of the enzyme was calculated from the incorporated ^{14}C radioactivity and the protein content, and then these data were correlated with the enzyme inactivation data.

Amino Acid Composition and Peptide Mapping of ^{14}C-Labeled Enzyme. Amino acid analysis of the affinity-alkylated ^{14}C-labeled enzyme was performed on 1 μg of samples, which were hydrolyzed in 6 N HCl (containing 0.2% of 2-mercaptoethanol) at 110 °C for 24–48 h. Samples of the ^{14}C-labeled enzyme were treated with 4-sulfophenyl isothicyanate (SPITC, 5 mg) in TEA/propanol/water (6/44/50 v/v/v) for 12 h at 55 °C to prevent the formation of a tryptic core. The resulting mixture was dialyzed against water, followed by 0.2 M ammonium bicarbonate (pH 8.0). The

[1] Abbreviations: EDTA, ethylenediaminetetraacetic acid sodium salt; HPLC, high-performance liquid chromatography; PTH, phenylthiohydantoin; scc, side-chain cleavage; SPITC, 4-sulfophenyl isothiocyanate; TEA, triethylamine; TFA, trifluoroacetic acid; TPCK, *N*-tosylphenylalanine chloromethyl ketone.

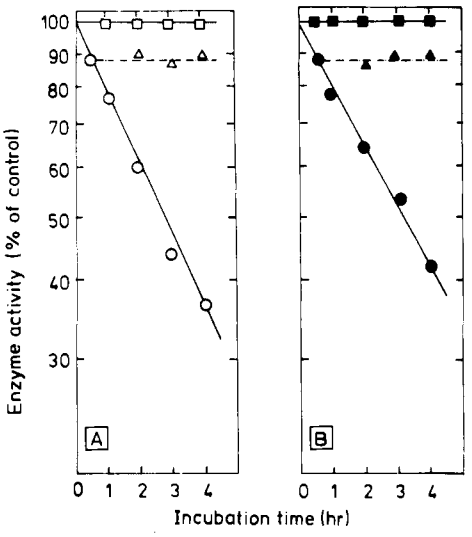

FIGURE 1: Inactivation of cytochrome P-450 enzyme with 17-(bromoacetoxy)progesterone. The enzyme (44 nM) was incubated with 17-(bromoacetoxy)progesterone (5 μM) at 25 °C, as described under Materials and Methods. At the times indicated, aliquots were removed from the incubation mixtures for measuring 17-hydroxylase activity (○ in panel A) and $C_{17,20}$-lyase activity (● in panel B). Both activities were measured in control incubations that contained 17-acetoxyprogesterone (5 μM) instead of the bromoacetoxy analogue (□ in panel A and ■ in panel B). When 2-mercaptoethanol (50 μM) was added to an incubation mixture that had lost more than 10% of its enzyme activity, the inactivation process was arrested (△ in panel A and ▲ in panel B).

retentate was then incubated with trypsin (previously treated with TPCK, 1% by weight) for 24 h at 37 °C. Peptide mapping was performed on a RPC8 SynChropak column, as previously described (Yuan et al., 1983a). The same procedure was performed on the control enzyme. Radioactivity was measured in aliquots (10%) from each peptide fraction to locate the ^{14}C-labeled peptides. The ^{14}C-labeled peptides were rechromatographed on an Ultrasphere C8 column prior to amino acid sequence analysis. Amino acid analysis of the peptides was performed as described above. Microsequence analysis of the peptides (0.3–nmol) was performed as previously described (Shively, 1981; Hawke et al., 1982, 1985).

RESULTS

Inactivation of Cytochrome P-450 by 17-(Bromoacetoxy)progesterone. 17-(Bromoacetoxy)progesterone inactivates the cytochrome P-450, C_{21} steroid side-chain cleavage enzyme by a time-dependent and irreversible process that follows first-order kinetics (Figure 1). Inactivation of both the 17-hydroxylase and also the $C_{17,20}$-lyase enzyme activities occurs simultaneously and at identical rates. Statistical analysis of eight separate determinations showed that the kinetics of loss of the two activities are the same. The $t_{1/2}$ value was 3 ± 0.3 h for inactivation of the enzyme under the conditions described in Figure 1. All of the enzyme inactivation data were corrected to 100% according to the level of the enzyme activity in the 17-acetoxyprogesterone control incubation. Loss of the enzyme activity is completely and rapidly arrested by addition of 2-mercaptoethanol.

Inactivation of Cytochrome P-450 Obeys Saturation Kinetics. A series of inactivation experiments was conducted in which the concentration of the cytochrome P-450 enzyme in each incubation mixture was the same (44 nM) and the concentration of 17-(bromoacetoxy)progesterone in the incubation mixture was varied from 5 to 80 μM. Double-reciprical plots (not shown) of the inactivation rate constants for loss of 17α-hydroxylase activity and $C_{17,21}$-lyase activity as functions of inactivator concentrations were linear. Kitz and Wilson (1962) plots of these data show that the changes in the rate of enzyme inactivation as a function of inhibitor concentration obey saturation kinetics (Kitz & Wilson, 1962). Also, the K_i value for the analogue obtained by extrapolation of the curves to the abscissa intercept ($-1/K_i$) in the double-reciprical plots is approximately 8.3–10.3 μM.

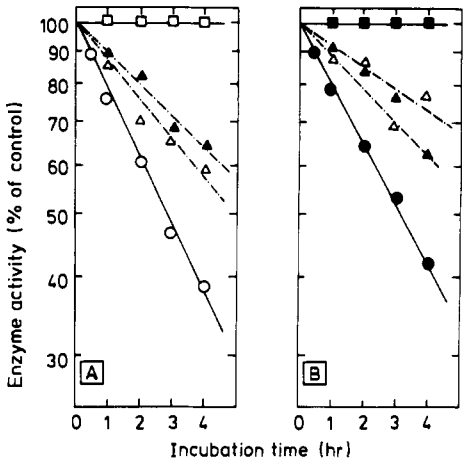

FIGURE 2: Protection of cytochrome P-450 by substrates against inactivation by 17-(bromoacetoxy)progesterone. The incubations were carried out under the conditions specified in Figure 1 except that 1.1 μM of the substrate progesterone (△) or 17-hydroxyprogesterone (▲) was included in the incubations, together with the enzyme and 17-(bromoacetoxy)progesterone. Panel A: 17-Hydroxylase activity. Panel B: $C_{17,20}$-lyase activity. The symbols represent control incubations of cytochrome P-450 with 17-acetoxyprogesterone (□, ■), incubations of cytochrome P-450 with 17-(bromoacetoxy)progesterone (○, ●), incubations of cytochrome P-450 with progesterone and 17-(bromoacetoxy)progesterone (△), and incubations of cytochrome P-450 with hydroxyprogesterone and 17-(bromoacetoxy)progesterone (▲).

Protection by Substrates against Inactivation of Cytochrome P-450. 17-(Bromoacetoxy)progesterone (5 μM) was incubated either with the enzyme alone or with the enzyme in the presence of progesterone (or 17-hydroxyprogesterone). The concentration of substrate in each of the incubation mixtures was 1.1 μM, conditions under which the enzyme was calculated to be approximately 50% saturated with substrate. The resulting kinetics of enzyme inactivation is shown in Figure 2. Each of the substrates protects both of the catalytic activities against inactivation by 17-(bromoacetoxy)progesterone, with retardation in the rate of enzyme inactivation by a factor of 2. These data suggest that the substrates protect the enzyme against inactivation by competing with the analogue for the same active site.

Competitive Inhibition of Cytochrome P-450: 17-(Bromoacetoxy)progesterone vs. Progesterone or 17-Hydroxyprogesterone. In a series of assays with the cytochrome P-450 enzyme, radioactively labeled progesterone or 17-hydroxyprogesterone was used as a substrate and nonradioactive 17-(bromoacetoxy)progesterone served as an inhibitor during short-term incubations (less than 30 min). Under these conditions, less than 10% of the enzyme activity was lost by affinity alkylation by the inhibitor. Thus, enzyme, inhibitor, one of the two substrates, and the cofactor NADPH were incubated for 20 min. The concentrations of substrates and inhibitor were appropriately varied. The assay mixtures were extracted with methylene chloride, and the extracts were chromatographed to separate the substrate from products and to measure enzyme activity (see Materials and Methods). 17-(Bromoacetoxy)progesterone was found to be a competitive inhibitor of the enzyme with K_i values of 8.4 μM (with pro-

Table I: Amino Acid Compositions of Affinity-Labeled 17α-Hydrolase from Porcine Testis[a]

amino acid	residues/molecule −substrate	residues/molecule +substrate	amino acid	residues/molecule −substrate	residues/molecule +substrate
Asx	46	48	Ile	27	24
Thr	26	25	Leu	59	59
Ser	47	44	Tyr	14	11
Glx	53	52	Phe	24	22
Pro	29	36	Lys	37	37
Gly	47	45	His	14	15
Ala	37	42	Arg	29	30
Val	27	25	Trp	2	0
Met	7	11	CM-Cys	2	1

[a] The samples (10 μg) were hydrolyzed in 6 N HCl containing 0.2% 2-mercaptoethanol for 48 h 110 °C under vacuum. The recovery of cysteine and cystine under these conditions is essentially zero. The recovery of tryptophan is variable (50% of theoretical). The expected hydrolysis product of affinity-labeled cysteine is *S*-(carboxymethyl)cysteine (CM-Cys). The yield of this residue is quantitative for these hydrolysis conditions. It was assumed that the protein contains 527 residues/molecule (58 000 daltons).

Table II: Amino Acid Analysis of Affinity-Labeled Tryptic Peptides[a]

amino acid	residues/mol T-60	residues/mol T-69	amino acid	residues/mol T-60	residues/mol T-69
Asx	1.73 (2)	4.22 (5)	Met	0.36 (1)	(0)
Thr	1.05 (1)	(0)	Ile	0.69 (1)	0.66 (1)
Ser	1.06 (1)	1.29 (1)	Leu	1.76 (2)	3.20 (6)
Glx	2.72 (4)	3.12 (3)	Tyr	(0)	(0)
Pro	1.19 (1)	3.21 (4)	Phe	(0)	1.47 (2)
Gly	(0)	3.11 (2)	Lys	0.59 (1)	1.03 (1)
Ala	(0)	(0)	His	(0)	(0)
CM-Cys	0.30 (1)	0.25 (1)	Arg	1.87 (2)	(0)
Val	1.15 (1)	1.02 (2)			

[a] Approximately 0.5 μg of each peptide was hydrolyzed in 6 N HCl containing 0.2% 2-mercaptoethanol for 24 h at 110 °C. The values in parentheses are predicted from sequence analysis. Cysteine was recovered as the *S*-carboxymethyl derivative, the expected hydrolysis of the *S*-acetoxyprogesterone cysteine derivative. The low yield of leucine and valine for peptide T-69 is probably due to incomplete hydrolysis of the sequence Leu-Val-Leu present in the peptide.

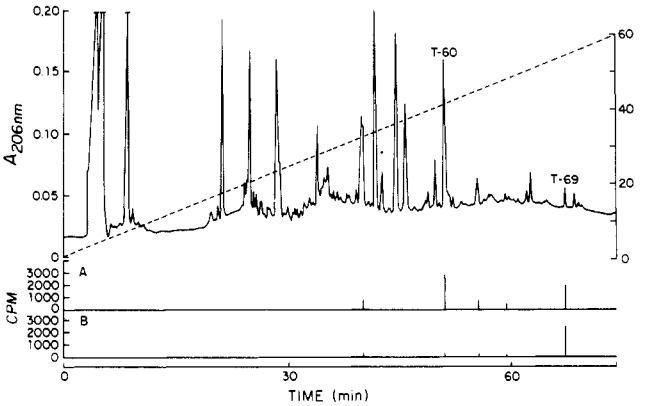

FIGURE 3: Tryptic peptide mapping of affinity-labeled cytochrome P-450 enzyme, radioalkylated in the presence and absence of substrate. The enzyme (5 nmol) was treated with 17-([^{14}C]bromoacetoxy)-progesterone for 2 h, quenched with 2-mercaptoethanol, and dialyzed. Aliquots were taken for measuring radioactivity and for amino acid analysis. The ^{14}C-labeled sample (4 nmol) was treated with SPITC to modify lysine residues. After dialysis, the sample (1 nmol) was treated with trypsin (2% by weight, 24 h), and the resulting peptides were separated by reverse-phase HPLC on a SynChropak RPC8 column (4.6 × 250 mm) using a linear gradient from 100% solvent I (0.1% TFA) to 100% solvent II (TFA/water/acetonitrile, 0.1/9.9/90) over 90 min at a flow rate of 0.8 mL/min. The peaks were manually collected, and a 10% aliquot was counted for radioactivity (inset A). Similarly, the sample treated with 17-([^{14}C]bromoacetoxy)progesterone in the presence of substrate (100-fold excess) was treated with SPITC, trypsinized, and mapped by reverse-phase HPLC. The map was practically identical except that the radioactive peak corresponding to peptide T-60 was absent. Aliquots (10%) were counted for each peak (inset B).

gesterone) and 7.8 μM (with 17-hydroxyprogesterone).

Stoichiometry of Inactivation and Identification of Cysteine at the Active Site of Cytochrome P-450. The enzyme (22–110 nM) was incubated with 17-([^{14}C]bromoacetoxy)progesterone (5 μM) in the presence or absence of a substrate (200 μM). Aliquots from the affinity radioalkylation reaction at 1, 2, and at 3 h were treated with 2-mercaptoethanol (500 μmol), and the resulting mixtures were dialyzed against water until the radioactivity levels in the retentates were at a constant level. The stoichiometry of incorporation of the ^{14}C-labeled analogue during enzyme inactivation was determined by measuring the radioactivity in the retentates and performing amino acid analysis on a second sample of the retentate. Quantitative amino acid analysis was used to determine the number of moles of the amino acid that was modified by the analogue. The results revealed that 1.5–2.2 mol of ^{14}C label was incorporated per mole of enzyme protein that was inactivated by the ^{14}C analogue in the absence of a substrate. However, 0.9–1.2 mol of ^{14}C label was incorporated per mole of the enzyme that was inactivated by the analogue in the *presence* of a substrate. *S*-([^{14}C]Carboxymethyl)cysteine was the only modified amino acid identified by amino acid analysis. This derivative is expected from the hydrolysis of the conjugate formed by reaction of cysteine with 17-([^{14}C]bromoacetoxy)progesterone. The yields of *S*-([^{14}C]carboxymethyl)cysteine were in agreement with the stoichiometry determined by measuring the radioactivity. The results from a typical experiment are shown in Table I.

Approximately 4 nmol of the ^{14}C-labeled enzyme [previously incubated with 17-([^{14}C]bromoacetoxy)progesterone alone] was treated with SPITC. By modifying the ϵ-NH_2 groups of lysine, the reagent converts the protein to a more soluble form and prevents the formation of an insoluble tryptic core (unpublished observations for this enzyme). The SPITC-modified protein was digested with trypsin, and the resulting peptides were separated by reverse-phase HPLC. Examination of aliquots from each fraction revealed two ^{14}C-labeled peptides (T-60 and T-69, Figure 3). When the same procedure was repeated with the ^{14}C-labeled enzyme that had been incubated with the analogue in the *presence* of a substrate, then only one ^{14}C-labeled peptide was obtained, corresponding to T-60 (Figure 3).

Peptides T-60 and T-69 were subjected to amino acid analysis and microsequence analysis (Tables II and III). Both peptides contain a single cysteine residue and correspond to peptides from the enzyme that was previously sequenced by us. The Edman cycle that was expected to contain ^{14}C-labeled cysteine did not give a PTH derivative, suggesting that the derivative was not stable under the conditions used in Edman chemistry. However, each of the ^{14}C-labeled, cysteine-containing peptides yielded only *S*-([^{14}C]carboxymethyl)cysteine

Table III: Sequence Analysis of Affinity-Labeled Tryptic Peptides[a]

cycle	T-60 (pmol)	T-69 (pmol)	cycle	T-60 (pmol)	T-69 (pmol)
1	Ile (135)	Phe (186)	13	Cys[c]	Cys[c]
2	Leu (146)	Asp (30)	14	Val (14)	Leu (242)
3	Pro (98)	Leu (338)	15	Arg[d] (+)	Val (186)
4	Ser (15)	Glu (112)	16	Glu (8)	Gly (138)
5	Gln (49)	Leu (301)	17	Arg (+)	Asn (113)
6	Thr (20)	Pro (140)	18	Asn (+)	Pro (60)
7	Leu (32)	Asp (24)	19		Ser (28)
8	Glu (26)	Asp (25)	20		Leu (156)
9	Asn (26)	Gly (131)	21		Val (66)
10	Met (16)	Gln (166)	22		Leu (252)
11	Lys[b] (10)	Leu (268)	23		Gln (86)
12	Gln (27)	Pro (117)	24		Ile (92)

[a] Approximately 200 pmol of T-60 and 500 pmol of T-69 were sequenced. [b] Lys was identified as the (sulfophenyl)carbamyl derivative (see Materials and Methods). [c] Cysteine residues were determined separately by sequence analysis on the corresponding tryptic peptides from the S-carboxymethylated protein. [d] Detected but not quantitated (+).

on acid hydrolysis (Table II), a result consistent with the modification of cysteine by 17-([^{14}C]bromoacetoxy)progesterone.

Discussion

17-(Bromoacetoxy)progesterone inactivates the C_{21} side-chain cleavage P-450 enzyme from porcine testis (Figure 1). Inactivation of both the 17-hydroxylase and the $C_{17,20}$-lyase activities proceeds simultaneously and at equal rates, consistent with our previous kinetic evidence which suggested that the two enzyme activities occur at the same active site (Nakajin et al., 1981b). The fact that 2-mercaptoethanol promptly stops the enzyme inactivation process indicates that the analogue acts by way of the chemical reactivity of the bromoacetoxy moiety.

The inactivation of the cytochrome P-450 by 17-(bromoacetoxy)progesterone was found to obey saturation kinetics. During these kinetic studies, the loss of both the 17-hydroxylase and the $C_{17,20}$-lyase activities followed nearly identical patterns, underscoring the conclusion that the two activities occur at the same active site. The results firmly demonstrate that the process by which 17-(bromoacetoxy)progesterone inactivates the enzyme is affinity alkylation.

Progesterone and 17-hydroxyprogesterone protect both activities of the enzyme against inactivation by 17-(bromoacetoxy)progesterone. This protection is evidenced by a retardation in the rate of inactivation of enzyme when either of the two substrates and 17-(bromoacetoxy)progesterone are coincubated with the enzyme (Figure 2). These findings suggest that the substrates and the analogue mutually compete for binding at the same active site of the enzyme.

The analogue was found to be a competitive inhibitor during short-term incubations of the enzyme. This is consistent with the protection experiments and reinforces the hypothesis that the analogue competes with the two substrates for the same active site. Another test of this hypothesis would be a demonstration that 17-(bromoacetoxy)progesterone serves as a substrate for the enzyme. However, the bromoacetoxy substituent at the 17-position of progesterone makes it impossible for the analogue to serve as a substrate for the enzyme.

Reaction of the enzyme with 17-([^{14}C]bromoacetoxy)-progesterone provided the stoichiometry of enzyme inactivation and identification of the amino acid residue that is modified. When the enzyme was incubated with the radioactive analogue alone, 1.5–2.2 mol of the ^{14}C-labeled analogue was incorporated for each mole of enzyme that was inactivated. However,

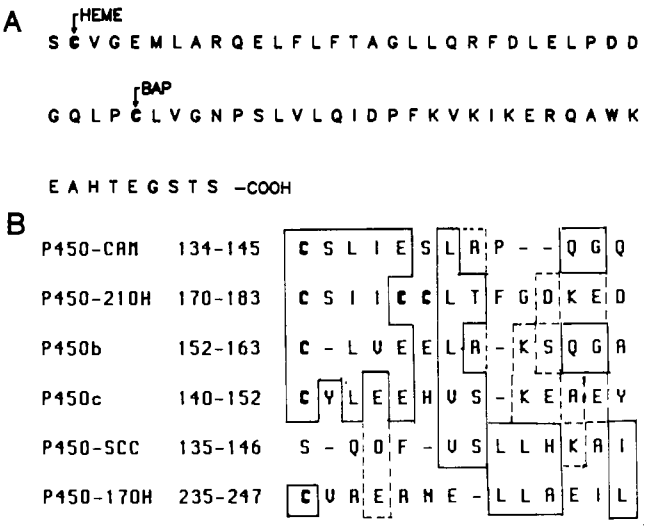

FIGURE 4: Location of cysteine residues from affinity-alkylated P-450 enzyme. (A) Location of the modified cysteine in ^{14}C-labeled peptide T-69 from Homology Region 2 (HR2) near the COOH terminus. During affinity alkylation, ^{14}C labeling of this peptide is not blocked by substrate; therefore, T-69 is not a candidate for the active site region (substrate-binding site). The location of the proposed heme-binding cysteine residue is also marked. (B) Location of the cysteine in peptide T-60 near Homology Region 1 (HR1). Sequences from a number of cytochromes P-450 are shown, beginning with the cysteine residue located near HR1 [the alignment is slightly different from that reported by Kawajiri et al. (1984)]. Data were from the following sources: bacterial cytochrome P-450$_{cam}$ was from Haniu et al. (1982), porcine adrenal 21-hydroxylase was from Yuan et al. (1983a), rat liver P-450b was from Kawajiri et al. (1984) and Yuan et al. (1983b), rat liver P-450c was from Yabusaki et al. (1984), bovine adrenal cytochrome P-450$_{scc}$ was from Morohashi et al. (1984), and porcine testicular 17-hydroxylase was from the present work. Note that cytochrome P-450$_{scc}$ begins with a serine and not a cysteine residue and that the numbering scheme is for the mature protein. Gaps were inserted to maximize homology. Basic residues, acidic residues, and the groups leucine/isoleucine/valine and serine/threonine are considered equivalent.

when the affinity radioalkylation of the enzyme was carried out in the presence of progesterone or 17-hydroxyprogesterone, only 0.9–1.2 mol of the analogue was incorporated. Evidently, a substrate protects one of two sites in the enzyme against reaction with the analogue.

Two different cysteine residues in the enzyme were modified when a substrate was absent during affinity radioalkylation. However, only one of them was modified when a substrate was present during the reaction. Tryptic mapping of both preparations of the enzyme that had been radioalkylated by 17-([^{14}C]bromoacetoxy)progesterone allowed the isolation of two different ^{14}C-labeled peptides. Peptides T-60 and T-69 were obtained in the absence of a substrate, but only peptide T-69 was produced when a substrate was present during affinity alkylation. Peptide T-69, found in both preparations of the enzyme, must have been derived from a site not protected by substrate against radioalkylation. The modified cysteine residue in T-69 can be placed by amino acid sequence analysis near the COOH-terminal end of the protein (Figure 4A). Thus, peptide T-69 is located near the heme binding cysteine in Homology Region 2 (HR2) (Kawajiri et al., 1984).

The cysteine residue in peptide T-60, derived from a site that is protected by a substrate against affinity alkylation, can be located by homology with bovine 17-hydroxylase, recently cloned and sequenced by Zuber et al. (1986). The cysteine in this region corresponds to HR1 (Kawajiri et al., 1984), a highly conserved region in all cytochromes P-450 sequenced to date (with the exception of bovine mitochondrial cytochrome P-450$_{scc}$). The location of this cysteine residue in the amino

acid sequence of several enzymes is shown in Figure 4B. Mitochondrial cytochrome P-450$_{scc}$ may have diverged from the microsomal enzymes early in evolution (Yabusaki et al., 1984) and, thus, may more likely have different amino acids in the active site region. This hypothesis is strengthened by the quite different catalytic requirements of the mitochondrial enzyme. Probably the cysteine of the microsomal enzymes is replaced by serine in C_{27} side-chain cleavage cytochrome P-450. The evidence for the location of peptide T-60 is based on amino acid sequence analysis of the porcine adrenal and testicular C_{21} side-chain cleavage enzymes, now known to have virtually identical sequences. More than 85% of the sequence of the adrenal enzyme and 50% of the testicular enzyme have been determined in our laboratories (M. Haniu and J. E. Shively, unpublished results).

This paper provides new evidence in support of the hypothesis that there is a region in cytochromes P-450 that is distinct from the heme-binding site but is at, or very close to, the substrate-binding site. Previously designated as HR1, this region is only weakly conserved in microsomal cytochromes P-450, which may be explained by the role of this substrate-binding region in accommodating the wide range of substrates metabolized by these enzymes.

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Registry No. L-Cys, 52-90-4; cytochrome P-450, 9035-51-2; 17α-hydroxylase, 9029-67-8; $C_{17,20}$-lyase, 9044-50-2; 17-(bromoacetoxy)progesterone, 61886-11-1; progesterone, 57-83-0; 17-hydroxyprogesterone, 68-96-2.

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