Chemical Modification and Inactivation of Rat Liver Microsomal Cytochrome P-450c by 2-Bromo-4'-nitroacetophenone*

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The alkylating agent 2-bromo-4'-nitroacetophenone (BrNAP) binds covalently to each of 10 isoforms of purified rat liver microsomal cytochrome P-450 (P-450a–P-450j) but substantially inhibits the catalytic activity of only cytochrome P-450c. Regardless of pH, incubation time, presence of detergents, or concentration of BrNAP, treatment of cytochrome P-450c with BrNAP resulted in no more than 90% inhibition of catalytic activity. Alkylation with BrNAP did not cause the release of heme from the holoenzyme or alter the spectral properties of cytochrome P-450c, data that exclude the putative heme-binding cysteine, Cys-460, as the major site of alkylation. Two residues in cytochrome P-450c reacted rapidly with BrNAP, for which reason maximal loss of catalytic activity was invariably associated with the incorporation of ~1.5 mol of BrNAP/mol of cytochrome P-450c. Two major radiolabeled peptides were isolated from a tryptic digest of [14C]BrNAP-treated cytochrome P-450c by reverse-phase high performance liquid chromatography. The amino acid sequence of each peptide was determined by microsequence analysis, but the identification of the residues alkylated by BrNAP was complicated by the tendency of the adducts to decompose when subjected to automated Edman degradation. However, results of competitive binding experiments with the sulphydryl reagent 4,4'-dithiodipyridine identified Cys-292 as the major site of alkylation and Cys-160 as the minor site of alkylation by BrNAP in cytochrome P-450c.

Cytochrome P-450 refers to a family of membrane-bound heme-proteins that function as monooxygenases in the biotransformation of numerous xenobiotics (e.g. drugs, carcinogens, pesticides, etc.) and endogenous substrates, such as steroids, fatty acids, and certain vitamins (1). Ten isoforms of rat liver microsomal cytochrome P-450 (designated P-450a–P-450j) have been purified in our laboratory and characterized with respect to their role in xenobiotic biotransformation and their regulation by various physiologic and environmental factors (2-10).

The ability of cytochrome P-450c to catalyze the stereoselective metabolism of a wide variety of polycyclic aromatic hydrocarbons has been used to determine the spatial tolerance of the substrate-binding site of this 3-methylcholanthrene-inducible hemeprotein (22, 23). Although the amino acid sequence of cytochrome P-450c has been determined (16, 17), those amino acids that comprise the catalytic site and define the boundary of the substrate-binding site have not been established. We have now undertaken chemical modification studies to identify functionally important amino acid residues in cytochrome P-450 as an approach to gain further insight into the mechanism of catalysis by this unusual class of enzymes.

Inasmuch as cytochrome P-450c is noted for its efficient metabolism of polycyclic aromatic hydrocarbons (22-24), we had initially considered synthesizing a bromoacetyl derivative of such substrates in an attempt to alkylate one or more amino acid residues in the catalytic substrate-binding site. However, since we had previously identified a histidine residue in the catalytic site of epoxide hydrolase with BrNAP (25), we initiated the present studies on the interaction between the alkylating agent, BrNAP, and each of 10 isoforms of highly purified rat liver microsomal cytochrome P-450, as well as NADPH-cytochrome P-450 reductase.

**EXPERIMENTAL PROCEDURES**

**Chemicals**—BrNAP was obtained from Eastman and recrystallized from ethanol before use. 4-Nitroacetophenone, bromoacetic acid, 2-bromoacetophenone, and PDS were purchased from Aldrich, 2-bromoacetamido-4-nitrophenol from Sigma, and 1-chloro-2,4-dinitrobenzene and 2-bromo-2-phenylacetophenone from Eastman. Sodium cholate, CHAPS, octyl glucoside, dialauroylphosphatidylcholine, and

*1 The term cytochrome P-450 is used to refer to any or all forms of liver microsomal cytochrome P-450. Because a nomenclature for the various forms of cytochrome P-450 has not been established, we designate the rat hemeproteins based on the order in which they were purified. Rat liver cytochromes P-450a–P-450j have different amino acid sequences (7, 11-21). Cytochrome P-450c is inducible in liver microsomes by as much as 50-fold when rats are administered 3-methylcholanthrene (8, 9) or certain other xenobiotics (9, 10).

*2 The abbreviations are: BrNAP, 2-bromo-4'-nitroacetophenone; [14C]BrNAP, 2-bromo-4'-nitro[14C]acetophenone; TPCK, L-1-p-tosyl-L-2-phenylglycine chloromethyl ketone; CHAPS, 3-[[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; DTT, dithiothreitol; HPLC, high performance liquid chromatography; octyl glucoside, octyl β-D-glucopyranoside; PDS, 4,4'-dithiodipyridine.
DTT were obtained from Calbiochem, and Emulgen 911 was from Kao-Atlas Co., Tokyo, Japan. All organic solvents were purchased from Burdick and Jackson.

**Synthesis of Bromoacetyl Compounds—**

[\[^{14}C\]BrNAP (5.6 Ci/mol) was synthesized from 4-nitro[carboxyl-\[^{14}C\]]benzoic acid as previously described (25). Thin layer chromatography of the compound indicated a single radioactive spot (Rf = 0.75, 2-(bromoacetyl)-antranec; 0.2, 2-(bromoacetyl)-fluorene; and 0.2-3, 9- (2-bromoacetyl)-phenanthrene) were obtained by distillation of the corresponding acetyl hydrocarbons according to the general procedure of Hoppe and Mosetig (26). Typically, 1 g of acetyl derivative in 30 ml of ether was cooled in an ice bath, and 1 eq of bromine in 5 ml of dichloromethane was added with stirring over a 10-min period. After additional stirring for 50 min, solvents were removed under vacuum, and the residues were dissolved in benzene. The benzene solutions were passed through a small bed of Florisil and the desired products crystallized from benzene/hexane. Structures and purities were established by NMR and mass spectrometry.

**Purification of Microsomal Enzymes—** Cytochromes P-450a (2), P-450b, P-450c, and P-450e (4) were purified as described from liver homogenates. NADPH-cytochrome P-450 reductase was isolated by a combination of the methods of Dignam and Brobel (27) and Yasukochi and Masters (28). The purified flavoprotein had a specific activity of 80,000 units/mg of protein, and the extract had a radiochemical purity of >97%.

**Chemical Modification of Rat Cytochrome P-450c—**

After additional stirring for 50 min, solvents were removed under vacuum, and the residue was dissolved in benzene. The benzene solutions were passed through a small bed of Florisil and the desired products crystallized from benzene/hexane. Structures and purities were established by NMR and mass spectrometry.

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was assayed for catalytic activity under optimal conditions such that product formation was directly proportional to cytochrome P-450 concentration and incubation time. Initial experiments established that neither BrNAP nor the BrNAP-DTT adduct interfered with the detection or stability of the reaction products.

The catalytic activity of NADPH-cytochrome P-450 reductase was measured by the rate of reduction of cytochrome c, as described by Phillips and Langdon (29). In the second method, activity was measured at 37 °C by the ability of the flavoprotein to support the cytochrome P-450c-dependent hydroxylation of benzo[a]pyrene in a reconstituted system containing 0.014, 0.04, or 0.22 nmol of NADPH-cytochrome P-450 reductase, 0.02 nmol of cytochrome P-450c, and 31 nmol of dilauroylphosphatidylcholine in 1 ml of potassium phosphate buffer (100 mM, pH 7.4) containing 20% glycerol and 100 μM EDTA. The rate of reduction of cytochrome P-450c was determined by automated Edman degradation with a gas phase sequenator as described (33). The phenylthiohydantoins were identified by reverse-phase HPLC on an Altex Ultrasound ODS column (34).

RESULTS AND DISCUSSION

Alkylation of Cytochrome P-450 Isozymes by BrNAP.—The effects of treating each of 10 isozymes of highly purified cytochrome P-450 or NADPH-cytochrome P-450 reductase with 5- or 50-fold molar excess of BrNAP and the results (Table I) confirmed that the selective inactivation of cytochrome P-450c is unrelated to the substrate rather than the hemeprotein under investigation. To test this possibility, benzo[a]pyrene was also used as a substrate to measure the catalytic activity of cytochrome P-450b before and after treatment with a 50-fold molar excess of BrNAP. Table I shows that alkylation with BrNAP slightly activated the catalytic activity of cytochrome P-450b toward benzenzamine and benzo[a]pyrene. Additionally, zoxazolamine was used as a substrate for both cytochromes P-450c and P-450b, and the results (Table I) confirmed that the selective inactivation of cytochrome P-450c is unrelated to the substrate but reflects instead a property unique to cytochrome P-450c.

Treatment with BrNAP for 30 min at room temperature also resulted in a concentration-dependent alkylation of NADPH-cytochrome P-450 reductase, the extent of which was comparable to the cytochrome P-450 c isoforms tested (Table I). Treatment of NADPH-cytochrome P-450 reductase with 50 μM BrNAP (but not 5 μM) caused a substantial loss of catalytic activity, determined either by the rate of reduction of cytochrome c or by the hydroxylation of benzo[a]pyrene in a reconstituted system containing cytochrome P-450c and dilauroylphosphatidylcholine. The reason why alkylation of NADPH-cytochrome P-450 reductase inhibited catalytic activity toward cytochrome c (83% inhibition) more than toward cytochrome P-450b/benzo[a]pyrene (58% inhibition) is unknown, although it has long been known that the ability of NADPH-cytochrome P-450 reductase to reduce artificial electron acceptors, such as cytochrome c, can be dissociated from its ability to reduce cytochrome P-450 (36).

It should be emphasized that residual BrNAP in alkylated preparations of cytochrome P-450c cannot inactivate NADPH-cytochrome P-450 reductase when the two proteins are reconstituted. Cytochrome P-450c was treated with BrNAP and combined with the flavoprotein only after the excess BrNAP was alkylated with DTT or removed by exhaustive dialysis. Even if some of the excess BrNAP survived these treatments, the 10-50-fold dilution of cytochrome P-450c in the reconstituted system would lower the BrNAP level well below that required to inactivate NADPH-cytochrome P-450 reductase (see Table I). Furthermore, all cytochrome P-450 isozymes would suffer a loss of catalytic activity if NADPH-cytochrome P-450 reductase became alkylated and inactivated by any residual BrNAP during the reconstitution experiments.

Spectral Properties of Alkylated Cytochrome P-450c.—The effects of alkylation with BrNAP on the spectral properties of cytochrome P-450c were determined to examine the possibility that BrNAP inactivated cytochrome P-450c simply by disrupting the heme moiety of cytochrome P-450c. Both non-alkylated and alkylated cytochrome P-450c displayed an ab-
**TABLE I**

**Effect of treatment with BrNAP on the catalytic activity of purified cytochrome P-450 isozymes and NADPH-cytochrome P-450 reductase**

Cytochrome P-450 isozymes and NADPH-cytochrome P-450 reductase (1 μM) were treated with 5 or 50 μM ["C"]BrNAP for 30 min at 22°C, and the reactions were terminated by the addition of 200 μM DTT. Aliquots of the samples were assayed for catalytic activity and covalent binding as described under "Experimental Procedures."

<table>
<thead>
<tr>
<th>Protein</th>
<th>Substrate</th>
<th>Reaction</th>
<th>Covalent binding</th>
<th>Catalytic activity remaining[a]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>mol/mol protein</td>
<td></td>
</tr>
<tr>
<td>P-450a</td>
<td>Testosterone</td>
<td>7α-Hydroxylation</td>
<td>0.9 1.3</td>
<td>103 99</td>
</tr>
<tr>
<td>P-450b</td>
<td>Benzphetamine</td>
<td>N-Demethylation</td>
<td>0.6 1.6</td>
<td>114 129</td>
</tr>
<tr>
<td>P-450c</td>
<td>Benzo[a]pyrene</td>
<td>3- and 9-Hydroxylation</td>
<td>1.6 2.9</td>
<td>18 13</td>
</tr>
<tr>
<td>P-450d</td>
<td>Zoxazolamine</td>
<td>6-Hydroxylation</td>
<td>1.1 2.2</td>
<td>131 128</td>
</tr>
<tr>
<td>P-450e</td>
<td>Zoxazolamine</td>
<td>O-Deethylation</td>
<td>0.9 2.3</td>
<td>111 117</td>
</tr>
<tr>
<td>P-450f</td>
<td>Testosterone</td>
<td>6α-Hydroxylation</td>
<td>2.0 3.6</td>
<td>129 110</td>
</tr>
<tr>
<td>P-450g</td>
<td>Testosterone</td>
<td>6β- and 15α-Hydroxylation</td>
<td>2.1 3.7</td>
<td>117 54</td>
</tr>
<tr>
<td>P-450h</td>
<td>Benzphetamine</td>
<td>N-demethylation</td>
<td>1.9 3.5</td>
<td>163 139</td>
</tr>
<tr>
<td>P-450i</td>
<td>Androstane disulfate</td>
<td>15β-Hydroxylation</td>
<td>1.5 3.0</td>
<td>107 102</td>
</tr>
<tr>
<td>P-450j</td>
<td>Aniline</td>
<td>4-Hydroxylation</td>
<td>1.0 2.3</td>
<td>103 91</td>
</tr>
<tr>
<td>Reductase</td>
<td>Cytochrome c</td>
<td>Reduction</td>
<td>1.1 3.2</td>
<td>86 17</td>
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<td></td>
<td>Cytochrome P-450c/</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>benzo[a]pyrene</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

[a] Molar ratio of BrNAP.

In the absence of BrNAP, the turnover numbers (nmol of product formed/nmol of cytochrome P-450/min) of each isozyme tested were comparable to previously published values (2-7).

Inasmuch as the ligand-binding characteristics of cytochrome P-450 are dependent on an intact heme-thiolate bond (37, 38), the unaltered ligand-binding properties make it extremely unlikely that BrNAP alkylates Cys-460, the cysteine residue that putatively serves as the fifth ligand of the heme iron (17, 39, 40). Since the sixth ligand to the heme iron is thought to be important in determining the spin state of cytochrome P-450 (37, 38), the unaltered spin state of alkylated cytochrome P-450 suggests that BrNAP does not alkylate the (as yet unidentified) sixth ligand of the heme iron. The results of these spectral studies indicate that the inactivation of cytochrome P-450c by BrNAP does not simply involve a conversion to cytochrome P-420 or a dissociation of the heme moiety from cytochrome P-450c, but apparently involves the alkylation of a residue(s) that alters the catalytic effectiveness of cytochrome P-450c.

The Effects of BrNAP Concentration and Time of Incubation—The relationship between the loss of catalytic activity toward benzo[a]pyrene and the covalent binding of BrNAP to cytochrome P-450c, as a function of the concentration of BrNAP, is shown in Fig. 1. Treatment of cytochrome P-450c (1 μM) with various concentrations of BrNAP for 30 min at 22°C, and the reactions were terminated by the addition of 200 μM DTT. Aliquots of the samples were assayed for catalytic activity and covalent binding as described under "Experimental Procedures."

![Fig. 1. Inactivation and alkylation of cytochrome P-450c as a function of BrNAP concentration. Cytochrome P-450c (1 μM) was treated with up to a 100-fold molar excess of ["C"]BrNAP for 30 min at 22°C, and the reactions were terminated by the addition of 200 μM DTT. Aliquots of the samples were assayed for catalytic activity and covalent binding as described under "Experimental Procedures."](image-url)
The effect of various chemicals on the catalytic activity of cytochrome P-450c

Cytochrome P-450c (1 μM) was pretreated with various chemical reagents or the solvent (acetonitrile, 2.5% final concentration) for 30 min at 22 °C. An aliquot was removed, treated with 200 μM DTT, and the sample was analyzed for benzo[a]pyrene metabolism. The remainder of the sample was treated with [14C]BrNAP (5 mol/mol of cytochrome P-450c) for 30 min at 22 °C, and the reaction was terminated with 200 μM DTT. Covalent binding was determined as described under “Experimental Procedures.”

<table>
<thead>
<tr>
<th>PRETREATMENT ACTIVITY</th>
<th>BOUND [14C]BrNAP</th>
<th>PRETREATMENT ACTIVITY</th>
<th>BOUND [14C]BrNAP</th>
</tr>
</thead>
<tbody>
<tr>
<td>compound</td>
<td>molar ratio</td>
<td>% remaining</td>
<td>molar/mol cytochrome P-450c</td>
</tr>
<tr>
<td>BrNAP</td>
<td>10:1</td>
<td>15–21</td>
<td>0.3</td>
</tr>
<tr>
<td>4-Nitroacetophenone</td>
<td>10:1</td>
<td>95</td>
<td>1.6</td>
</tr>
<tr>
<td>Bromoacetic Acid</td>
<td>5:1</td>
<td>91</td>
<td>98</td>
</tr>
<tr>
<td>2-Bromoacetophenone</td>
<td>10:1</td>
<td>22</td>
<td>0.2</td>
</tr>
<tr>
<td>2-(2-Bromoacetyl)-naphthalene</td>
<td>50:1</td>
<td>22</td>
<td>0.3</td>
</tr>
<tr>
<td>9-(2-Bromoacetyl)-anthracene</td>
<td>50:1</td>
<td>78</td>
<td>1.5</td>
</tr>
</tbody>
</table>

The results of experiments on the alkylation of cytochrome P-450c by BrNAP were not significantly influenced by the half-life or the solubility of the alkylating agent in aqueous solution. Measurement of the stability of [14C]BrNAP in potassium phosphate buffer (100 mM, pH 7.4) indicated the alkylating agent decomposed with a half-life of 4.8 h; hence, less than 10% of the BrNAP would have decomposed during the course of a 30-min incubation. The apparent solubility of [14C]BrNAP in potassium phosphate buffer (100 mM, pH 7.4) at 22 °C was determined to be ~600 μM, as described under “Experimental Procedures.” This value far exceeds the highest concentration of BrNAP tested (i.e. 10 μM).

Three important findings are illustrated in Fig. 1. First, the loss of catalytic activity toward benzo[a]pyrene and the covalent binding of BrNAP to cytochrome P-450c show good correspondence only at very low concentrations of BrNAP (≤5 μM). Second, treatment of cytochrome P-450c for 30 min at room temperature does not lead to a complete loss of catalytic activity. Even cytochrome P-450c treated with 100 μM BrNAP (i.e. 20 × the concentration effecting maximal inactivation) retained ~15% of its catalytic activity toward benzo[a]pyrene. Third, maximal loss of catalytic activity was associated with the incorporation of approximately 1.5 mol of BrNAP/mol of cytochrome P-450c. This latter finding indicates that at least two amino acid residues in cytochrome P-450c are extremely reactive toward BrNAP, at least one of which is responsible for the loss of catalytic activity. Several variables (pH, time, BrNAP concentration) were examined in an unsuccessful attempt to identify reaction conditions that gave selective binding of BrNAP to a single residue in cytochrome P-450c. These experiments did reveal, however, that the rate of inactivation and alkylation of cytochrome P-450c by BrNAP increased with increasing pH over the range pH 6.5 to 8.5 (results not shown).

Antibody Inhibition Experiments—Antibody inhibition experiments were performed to test the possibility that the 10–15% catalytic activity remaining after cytochrome P-450c was alkylated with BrNAP was due to a contaminating isozyme in the cytochrome P-450c preparation. The catalytic activity of alkylated cytochrome P-450c was determined with benzo[a]pyrene as substrate in the presence and absence of monoclonal antibody C8 (1.5 mol of IgG/mol of cytochrome P-450c) (30). The monoclonal antibody inhibited >97% of the residual benzo[a]pyrene hydroxylase activity catalyzed by alkylated cytochrome P-450c. Similar results were obtained with 7-ethoxycoumarin and zoxazolamine as substrates. Inasmuch as monoclonal antibody C8 reacts with a single peptide (M, = 56,000) in “Western blots” of rat liver microsomes (30), the antibody inhibition experiment demonstrated that a contaminating isozyme of cytochrome P-450 is not responsible for the 10–15% residual catalytic activity of BrNAP-treated cytochrome P-450c. Finally, the catalytic effectiveness of cyto-
Chemical Modification of Rat Cytochrome P-450c

Structure-Activity Relationships—Several compounds structurally related to BrNAP were tested at two concentrations (10 and 50 μM) for their ability to alkylate and inactivate cytochrome P-450c (1 μM), and the results are shown in Table II. The alkylation of cytochrome P-450c by these compounds was measured by their ability to block the subsequent covalent binding of [14C]BrNAP. As expected, 4-nitroacetophenone was inactive. Bromoacetic acid also failed to block the ability of BrNAP to alkylate and inactivate cytochrome P-450c, whereas 2-bromoacetophenone (removal of the nitro group) was moderately effective in preventing the binding of [14C]BrNAP. Although 2-(2-bromoacetyl)-napthalene was an effective blocking agent, 9-(2-bromoacetyl)-anthracene was not. Substitution of the bromoacetyl group of 2-bromoacetophenone with a phenyl group (2-bromo-2-phenylacetophenone) caused a reduction of reactivity. Interestingly, 2-bromoacetamido-4-nitrophenol, which reacts rapidly with a cysteine residue in cytochrome P-450cam (47), failed to inactivate cytochrome P-450c and to block the subsequent binding of BrNAP.

For most of the above-mentioned compounds (the first eight listed in Table II), there was good correspondence between the ability of each compound to inhibit the catalytic activity of cytochrome P-450c toward benzo[a]pyrene and to block the subsequent binding of BrNAP to cytochrome P-450c. However, this relationship was not upheld by the last four compounds listed in Table II. Treatment of cytochrome P-450c with 2-(2-bromoacetyl)-fluorene or 2-, 3-, or 9-(2-bromoacetyl)-phenanthrene effectively blocked the subsequent binding of BrNAP to cytochrome P-450c but inhibited the catalytic activity of cytochrome P-450c by only ~60% (compared to ~80% inhibition by BrNAP). The experiments with the last four compounds listed in Table II were repeated, and the results confirmed the ability of 2-(2-bromoacetyl)-fluorene and the three bromoacetyl derivatives of phenanthrene to block the subsequent binding of BrNAP to cytochrome P-450c without inactivating cytochrome P-450c to the same extent as BrNAP.

It is noteworthy that many of the compounds listed in Table II have the potential to undergo substrate-like interactions with cytochrome P-450c. We have shown previously that cytochrome P-450c metabolizes naphthalene and anthracene at the 1,2-position and metabolizes phenanthrene at the 1,2-, 3,4- and 9,10-positions (23, 48). Consequently, 2-(2-bromoacetyl)-naphthalene, 9-(2-bromoacetyl)-anthracene, and 2-, 3-, and 9-(2-bromoacetyl)-phenanthrene are substrate analogs that might be expected to position the bromoacetyl group in the vicinity of the active site of cytochrome P-450c.

The results in Table II show that the structure of the substituent influences the ability of the bromoacetyl derivatives to alkylate cytochrome P-450c at the same sites(s) as BrNAP. This dependence on the structure of the alkylating agent suggests that the residue(s) alkylated by BrNAP are not freely exposed on cytochrome P-450c, but are possibly located in a microenvironment that limits the access of certain compounds, such as the bromoacetyl derivative of anthracene. More importantly, however, compounds capable of binding to the same residue(s) as BrNAP do not always inactivate cytochrome P-450c to the same extent as BrNAP. Furthermore, none of the compounds tested inhibited the catalytic activity of cytochrome P-450c more than 90%. The data provide further evidence that the residues alkylated by BrNAP do not include an essential amino acid (i.e., an amino acid that when alkylated completely inactivates the enzyme). The data suggest that the alkylation of cytochrome P-450c by BrNAP and related compounds decreases its catalytic efficiency and that the catalytic activity of alkylated cytochrome P-450c is considerably reduced (45, 46), alkylation with BrNAP results in only ~85% inactivation of cytochrome P-450c. The catalytic activity of purified cytochrome P-450c was measured by their ability to block the subsequent binding of BrNAP to cytochrome P-450c.
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the extent to which the catalytic competency of cytochrome P-450c is compromised is dependent on the chemical structure of the alkylating agent. It is conceivable that some of the compounds listed in Table II do not alkylate cytochrome P-450c but block the subsequent binding of BrNAP in a competitive manner. However, the inability of benzo[a]pyrene and other substrates to block the subsequent binding of BrNAP to cytochrome P-450c (see above) makes this an unlikely possibility.

Alkylation with the Sulfhydryl Reagent, PDS—A cysteine residue(s), other than the heme-binding thiolate ligand, was a likely candidate for the site of alkylation in cytochrome P-450c by BrNAP (43, 44). The effect of pretreating cytochrome P-450c with the sulfhydryl reagent, PDS, on the subsequent binding of [14C]BrNAP was examined in an attempt to provide further evidence that cysteine is the major amino acid(s) alkylated by BrNAP. Pretreatment of cytochrome P-450c with as little as 2 molar eq of PDS significantly blocked the subsequent binding of BrNAP (Fig. 2).3 The subsequent binding of BrNAP was still blocked after the PDS-treated cytochrome P-450c was dialyzed to remove 4-thiopyridone, the side product formed during the alkylation of sulfhydryl groups by PDS. This result established that the ability of PDS to block the subsequent binding of BrNAP to cytochrome P-

3 Exposure of PDS-alkylated cytochrome P-450c to DTT was avoided to prevent reversal of the chemical modification (31, 43).

450c was not an artifact caused by the binding of BrNAP to 4-thiopyridone.

In addition to blocking the binding of BrNAP, pretreatment of cytochrome P-450c with PDS also blocked the inactivation of cytochrome P-450c by BrNAP (Fig. 2). These results also

450c (upper) and [14C]BrNAP-alkylated protein (lower). Cytochrome P-450c and PDS-treated cytochrome P-450c were alkylated with [14C]BrNAP, dialyzed against ammonium bicarbonate (200 mM, pH 8.0), and digested with TPCK-trypsin. The tryptic peptides were resolved by reverse-phase HPLC, as described under “Experimental Procedures.” A 20-μl aliquot was counted from each fraction (fraction sizes ranged from 0.5-1.0 ml) and plotted for each chromatogram (uncorrected for volume differences between samples).

![HPLC profile of tryptic peptides from PDS-pretreated, [14C]BrNAP-alkylated cytochrome P-450c (upper) and [14C]BrNAP-alkylated protein (lower).](image-url)

**Fig. 3.** HPLC profile of tryptic peptides from PDS-pretreated, [14C]BrNAP-alkylated cytochrome P-450c (upper) and [14C]BrNAP-alkylated protein (lower). Cytochrome P-450c and PDS-treated cytochrome P-450c were alkylated with [14C]BrNAP, dialyzed against ammonium bicarbonate (200 mM, pH 8.0), and digested with TPCK-trypsin. The tryptic peptides were resolved by reverse-phase HPLC, as described under “Experimental Procedures.” A 20-μl aliquot was counted from each fraction (fraction sizes ranged from 0.5-1.0 ml) and plotted for each chromatogram (uncorrected for volume differences between samples).

**TABLE III**

Microsequence analysis of BrNAP-labeled tryptic peptides

<table>
<thead>
<tr>
<th>Cycle</th>
<th>BrNAP-1</th>
<th>BrNAP-2</th>
<th>BrNAP-3</th>
</tr>
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<tbody>
<tr>
<td></td>
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<td>Asp (30)</td>
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<td>Ala (33)</td>
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<td>Asp (12)</td>
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<tr>
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<tr>
<td>13</td>
<td>Arg (+)</td>
<td>Arg (+)</td>
<td>Cys (+)</td>
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</table>

Total cpm/peptide: 3520, 5568, 2560

*The amounts of BrNAP-1, -2, and -3 sequenced were 200, 350, and 200 pmol, respectively. Yields at each cycle are given in pmol. A (+) indicates that the yield was too low to quantitate. No derivative was identified at cycle 10 for peptides BrNAP-1 and BrNAP-2, but cysteine is the known residue in this sequence (18). The amount of [14C]BrNAP incorporated per peptide is given as cpm/peptide. No cpm were recovered for the phenylthiohydantoin derivatives which were counted at each cycle. The low yields prevented identifications beyond cycle 13 for BrNAP-3. The occurrence of identical sequences for the separated peptides BrNAP-1 and BrNAP-2 may be due to the acid liability of the Cys-BrNAP adduct.
show that alkylation of cytochrome P-450c with PDS does not result in a loss of catalytic activity, as was previously observed by Kawalek et al. (31). One possible explanation for the ability of PDS to bind to the same amino acid residue(s) in cytochrome P-450c as BrNAP without inactivating the enzyme is that the PDS adduct decomposes when the heme-protein is reconstituted with NAPDH-cytochrome P-450 reductase, lipid, and NADPH. To investigate this possibility, we wished to determine whether 4-thiopyridone is released from the PDS-modified enzyme upon incubation with NADPH, NADPH-cytochrome P-450 reductase, and lipid. Because of the highly polar nature of 4-thiopyridone (a zwiterionic resonance structure can be drawn), direct extraction into organic solvents was not practical, and it was necessary to trap this compound by forming a less polar derivative. For this purpose, reaction with 1-chloro-2,4-dinitrobenzene, which forms a highly absorbing (330 nm) product, was employed (Scheme I).

Incubation of cytochrome P-450c (modified with 2 mol of PDS/mol of protein) with either NADPH and NADPH-cytochrome P-450 reductase or NADPH alone caused the rapid release of 1.6–1.9 mol of 4-thiopyridone/mol of cytochrome P-450c. Product identification was made as described under “Experimental Procedures.” Thus, the lack of effect of PDS treatment on the enzymatic activity of cytochrome P-450c can be accounted for by regeneration of the free enzyme upon reaction of the PDS-modified protein with NADPH.

Identification of the Alkylated Residues—To identify directly the amino acid residues alkylated by BrNAP, cytochrome P-450c (1 μM) was treated with 5 molar eq of [14C]BrNAP for 30 min at room temperature, before or after pretreatment of the protein with PDS. After dialysis against ammonium bicarbonate buffer, the alkylated cytochrome P-450c preparations were digested with TPCK-treated trypsin and the peptides separated by reverse-phase HPLC (18), as detailed under “Experimental Procedures.” Three major and several minor radiolabeled tryptic peptides were identified in the cytochrome P-450c preparation treated only with [14C]BrNAP (Fig. 3). Pretreatment of cytochrome P-450c with PDS, which decreased the subsequent binding of [14C]BrNAP from 1.6 to 0.7 mol/mol of cytochrome P-450c, caused a marked decrease in the amount of radioactivity associated with the three major peptides but had little effect on the subsequent binding of [14C]BrNAP to the minor sites of alkylation (Fig. 3). Microsequence analysis (Table III) revealed that the amino acid sequences of BrNAP-1 and BrNAP-2 were identical (see footnote to Table III) and that they corresponded to peptide T-36 previously sequenced from unalkylated cytochrome P-450c (18). The amino acid sequence of BrNAP-3 was the same as T-46 for the unalkylated protein (18). Peptides BrNAP-1 and BrNAP-3 each contain one cysteine residue (Cys-160 and Cys-292; Fig. 4). The identification of the residues alkylated by BrNAP was complicated by the tendency of the adducts to decompose when the peptides were subjected to automated Edman degradation. Various attempts to stabilize the adduct proved unsuccessful, and, hence, the alkylated amino acid could not be identified directly. However, all of the amino acids except cysteine gave clear signals when BrNAP-1, -2, and -3 were subjected to Edman degradation. These results, together with the ability of PDS to decrease the binding of BrNAP to cytochrome P-450c (Fig. 2 and 3), are consistent with the alkylation of Cys-160 and Cys-292 in cytochrome P-450c by BrNAP. The overall recovery and specific activities of the peptides show that the major and minor alkylated cysteines are Cys-292 and Cys-160, respectively. In another alkylation experiment, the major radiolabeled peptides isolated were BrNAP-1 and BrNAP-2. Very little BrNAP-3 was detected (data not shown). This experiment further suggests that Cys-292 is the major alkylated amino acid.

Although the results of both experiments provide strong evidence that BrNAP alkylates primarily Cys-292 in cytochrome P-450c, they do not establish whether only this residue is involved in catalytic inactivation of the enzyme. Comparison of the amino acid sequence of BrNAP-1 and BrNAP-3 with the corresponding amino acid sequences in cytochromes P-450d, P-450b, and P-450e (19, 20) and in both cytochromes P-450b and P-450e (13–15) are shown for comparative purposes. Gaps are introduced to maximize homology.
NADPH-cytochrome P-450 reductase. Among the 10 isoenzymes of cytochrome P-450 tested, cytochrome P-450c is unique in that it is the subject of the inactivation by BrNAP. The mechanism by which BrNAP inactivates cytochrome P-450c is the subject of the accompanying paper and involves an uncoupling of the catalytic cycle with the consequent formation of superoxide anion.

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REFERENCES