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

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The mechanism by which 2-bromo-4'-nitroacetophenone (BrNAP) inactivates cytochrome P-450c, 
which involves alkylation primarily at Cys-292, is shown in the present study to involve an uncoupling 
of NADPH utilization and oxygen consumption from product formation. Alkylation of cytochrome P-450c 
with BrNAP markedly stimulated (≈30-fold) its rate of anaerobic reduction by NADPH-cytochrome P-450 reductase, as determined by stopped flow spectroscopy. This marked stimulation in reduction rate is highly 
unusual in that Cys-292 is apparently not part of the heme- or substrate-binding site, and its alkylation by 
BrNAP does not cause a low spin to high spin state transition in cytochrome P-450c. Under aerobic conditions the rapid oxidation of NADPH catalyzed by 
molecular oxygen, established that alkylation of 
alkylcytochrome P-450c with BrNAP uncouples the catalytic cycle prior to introduction of the second electron. The generation of superoxide anion by decomposition of the Fe**-O2 complex was consistent with the observations that, in contrast to native cytochrome P-450c, alkylation of cytochrome P-450c failed to form a 430 nm 
superoxide anion during the metabolism of 7-ethoxycoumarin. Alkylation of cytochrome P-450c 
with BrNAP did not completely uncouple the catalytic cycle such that 5-20% of the catalytic activity re- 
mained for the alkylated cytochrome compared to the native protein depending on the substrate assay. The 
uncoupling effect was, however, highly specific for cytochrome P-450c. Alkylation of nine other rat liver 
mitochondrial cytochrome P-450 isoforms with BrNAP caused little or no increase in hydrogen peroxide for- 

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In recent years considerable attention has been focused on the structure of individual isozymes of cytochrome P-450, a class of hemoproteins that function as monoxygenases in the metabolism of numerous xenobiotics and endogenous substrates. Information on the amino acid sequence of individual isozymes of cytochrome P-450 obtained by microsequence analysis and recombinant DNA techniques provides the necessary framework to identify specifically those amino acids that function in cytochrome P-450 to bind substrates and ligands, to catalyze oxidative biotransformations, and to interact with NADPH-cytochrome P-450 reductase. Inasmuch as attempts to crystallize mammalian cytochromes P-450 (which are integral membrane proteins) for x-ray crystallography have been unsuccessful, attempts to identify functional amino acids in cytochrome P-450 have involved a chemical modification approach. Such studies on the major phenobarbital-inducible cytochrome P-450 in rabbit, cytochrome P-450 LM2, have tentatively identified a tyrosine residue (modified with tetraniotromethane or N-acetylimidazole) as the 6th ligand trans to the heme-thiolate bond and suggest that the NH₂-terminal methionine and Lys-382 (both modified with fluorescein isothiocyanate) function in the interaction between cytochrome P-450 LM2 and NADPH-cytochrome P-450 reductase (1-4). In contrast to its proposed function in cytochrome P-450 LM2, a positively charged lysine in cytochrome P-450b (the major phenobarbital-inducible isozyme in rat) has been implicated in substrate binding (5). Studies with bromoacetyl and mercapto derivatives of camphor implicate Cys-56 in the substrate-binding site of cytochrome P-450cam (6, 7), a soluble hemoprotein purified from Pseudomonas putida. Highly reactive cysteine residues of as yet unidentified function have also been identified in cytochrome P-450cam (7-9).

BrNAP** was shown in the preceding study to alkylate 10 isozymes of purified rat liver microsomal cytochrome P-450 (cytochromes P-450a-P-450g) but to inhibit substantially (∼85%) the catalytic activity of only cytochrome P-450c (10). Microsequence analysis and competitive binding experiments with the sulfhydryl reagent PDS identified Cys-160 and Cys-292 as the minor and major sites, respectively, of alkylation of cytochrome P-450c by BrNAP. In the present paper the

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Inactivation of Cytochrome P-450c by 2'-Bromo-4-nitroacetophenone

Mechanism by which BrNAP inactivates cytochrome P-450c has been examined.

**EXPERIMENTAL PROCEDURES**

**Chemicals**—The sources of BrNAP and other reagents and the synthesis of [14C]BrNAP are described in the preceding paper (10). Horseradish peroxidase, cumene hydroperoxide, catalase, superoxide dismutase, and 3-(4-hydroxyphenyl)propionic acid were purchased from Sigma, and 7-ethoxyresorufin was purchased from Pierce Chemical Co. Dialysisrophiophatidylcholine (Calbiochem) was used as an aqueous solution which was sonicated immediately before use.

**Proteins**—Cytochromes P-450a-P-450f and NADPH-cytochrome P-450 reductase were purified to electrophoretic homogeneity as described (11, 12). Before use, acetylated cytochrome c was treated with EDTA and subjected to gel exclusion chromatography on Sephadex G-25 as described (13).

**Activation of Cytochrome P-450c**—Cytochrome P-450c (5 μM) was treated with sodium sulfite (50 μM) followed by DTT (500 μM) to regenerate sulfhydryl groups, dialyzed, diluted to 1 μM with potassium phosphate buffer (100 mM, pH 7.4), and treated with BrNAP (1.5-10 μM) as described (10). Except for experiments involving PDS, alkylation of cytochrome P-450c was determined by dithionite reduction of low spin ferric cytochrome P-450c (Type I binding spectrum), based on an extinction coefficient of 126 mM" cm" (16). Binding of [14C]BrNAP to cytochrome P-450c was determined by a glass-fiber binding method as described (14).

**Catalytic Activity**—The catalytic activity of cytochrome P-450c toward several substrates was measured at 37°C in a reconstituted system containing saturating amounts of NADPH-cytochrome P-450 reductase and optimal amounts of dialysisrophiophatidylcholine, as described (10).

**Substrate-binding Spectra**—The binding of benz(a)pyrene, azoxalzalone, and 7-ethoxyresorufin to cytochrome P-450c was determined by difference spectroscopy as described (14), except that dual compartment cuvettes were used to blank out the absorption spectra of the substrate itself (15). One compartment of both the reference and sample cuvette containing cytochrome P-450c (1 μM) in potassium phosphate buffer (100 mM, pH 7.4). The other compartment of each cuvette contained liver microsomes from 3-methylcholanthrene-induced rats, solubilized and inactivated with 150 mM KOH, as described by Estabrook et al. (16). Benz(a)pyrene (1 μM), azoxalzalone (500 μM), or 7-ethoxyresorufin (400 μM) was added to the sample cuvette compartment containing cytochrome P-450c and to the reference cuvette compartment containing KOH-solubilized microsomes.

**NADPH Oxidation, H2O2, Formation, and Superoxide Anion Production**—The rate of NADPH oxidation was measured spectrophotometrically at 37°C from the decrease in absorbance at 340 nm, based on an extinction coefficient of 6.26 mM" cm" (17). Hydrogen peroxide formation was determined by the fluorimetric method of Omura and Sat0 (22). An equal volume of solvent was added to the corresponding compartment in the opposite cuvette. The percent conversion of low spin ferric cytochrome P-450c to ferrous cytochrome P-450c was monitored between 550 and 590 nm by difference spectroscopy (23, 24), after addition of 10 μM of cumene hydroperoxide (10 mM in 100 mM potassium phosphate buffer, pH 7.4) to native or BrNAP-alkylated cytochrome P-450c (1 μM in 100 mM potassium phosphate buffer, pH 7.4). The O-dealkylation of 7-ethoxyresorufin was measured fluorimetrically as described (25, 26) in 1 ml incubation mixture containing 0.1-0.2 nmol of cytochrome P-450c, 15 nmol of dialysisrophiophatidylcholine, and 10 nmol of 7-ethoxyresorufin in 100 mM potassium phosphate buffer, pH 7.4. Reactions were initiated with cumene hydroperoxide (100-500 μM) and stopped with 2 ml of ice-cold acetone after a 1-ml incubation period at 24°C. Samples were clarified by centrifugation before the fluorescence intensity of resorufin was measured at 585 nm, with excitation at 530 nm (25, 26).

**Circular Dichroism Spectra**—Circular dichroism (CD) spectra of native and BrNAP-modified cytochrome P-450c (50 mM potassium phosphate buffer, pH 7.4, containing 20% glycerol) were measured at 24°C with a Jasco J700A spectropolarimeter. For determination of the enzyme-induced CD spectrum of pyrene, 1.0 ml of a solution containing 3.8 nmol of native or BrNAP-modified cytochrome P-450c (in the above buffer) was titrated with 5-μl portions of pyrene solution (0.5 mg/ml in 95% ethanol).

**Anaerobic Reduction of Cytochrome P-450c**—Rates of reduction of native and BrNAP-alkylated cytochrome P-450c by NADPH-cytochrome P-450 reductase were determined at 447 nm under anaerobic conditions in a stopped-flow spectrophotometer (2-cm light path) at 10°C. Details of the preparation of anaerobic solutions and of the operation of the stopped-flow apparatus are as described by Oprian et al. (27). The final concentrations after mixing the two anaerobic reaction mixtures, each saturated with carbon monoxide, were 1 μM cytochrome P-450c, 1 μM NADPH-cytochrome P-450 reductase, 30 μM dialysisrophiophatidylcholine, 100 mM potassium phosphate buffer (pH 7.4), 8% glycerol, 100 μM NADPH, and, when present, 250 μM 7-ethoxyresorufin.

**RESULTS AND DISCUSSION**

**Lack of Substrate Protection**—The observation of residual catalytic activity upon alkylation of cytochrome P-450c by BrNAP described in the preceding paper (10) argued against the possibility that BrNAP alkylated an amino acid in the substrate-binding site of cytochrome P-450c. To test this possibility further, various substrates were examined for their ability to protect cytochrome P-450c from alkylation and inactivation by BrNAP. Preincubation of cytochrome P-450c (1 μM) with benz(a)pyrene (80 μM), azoxalzalone (250 μM), testosterone (125 μM), or aniline (2 mM) for 10 min failed to block the subsequent alkylation and inactivation of cytochrome P-450c by BrNAP, even at concentrations of BrNAP.
(1.5–3 μM) that only partially inactivated the enzyme. This lack of substrate protection provides further evidence that BrNAP does not alkylate an amino acid residue in the substrate-binding site of cytochrome P-450c.

Substrate Binding—It was shown in the preceding paper (10) that, like native cytochrome P-450c, BrNAP-alkylated cytochrome P-450c is a predominantly low spin ferric hemoprotein. Spectral studies with native and alkylated cytochrome P-450c were performed to determine the ability of various substrates to cause a low to high spin conversion (which gives an indirect measure of substrate binding to cytochrome P-450). At the final concentrations indicated, benzo[a]pyrene (1 μM), zoxazolamine (500 μM), and 7-ethoxycoumarin (400 μM) produced Type I spectra (trough at 420 nm, peak at 390 nm) with native cytochrome P-450c (1 μM), causing a 100, 29, and 18% low spin to high spin transition, respectively. Virtually identical results were obtained with alkylated cytochrome P-450c, suggesting that BrNAP does not block the binding of substrates to cytochrome P-450c. As previously reported for native cytochrome P-450c (28), BrNAP-alkylated cytochrome P-450c (1 μM) apparent bound benzo[a]pyrene (0.05–1.0 μM) stoichiometrically, indicating that the high affinity with which cytochrome P-450c binds benzo[a]pyrene was not affected by chemical modification of the hemoprotein. Addition of 10 μM BrNAP to cytochrome P-450c (under which conditions alkylation occurs) gave little or no evidence of a Type I spectrum. This result is in agreement with the previously reported low spin state of alkylated cytochrome P-450c and provides additional evidence that BrNAP does not bind to the substrate-binding site of cytochrome P-450c.

Kinetics of Zoxazolamine 6-Hydroxylation—The effects of alkylation of cytochrome P-450c on the kinetic parameters for 6-hydroxylation of zoxazolamine were examined. Alkylation of cytochrome P-450c caused a marked decrease in Vmax (from 151 to 3.6 nmol/min/nmol of cytochrome P-450c) as well as a modest decrease in apparent Km (110 to 42 μM). Although the kinetic parameters of native and alkylated cytochrome P-450c were measured under slightly different incubation conditions (to compensate for the large difference in zoxazolamine turnover number), the results clearly demonstrated that the mechanism by which BrNAP inactivates cytochrome P-450c does not involve a decrease in the affinity with which cytochrome P-450c binds substrates, a conclusion supported by studies of the substrate-binding spectra described above.

CD Spectra—Fig. 1 shows the observed CD spectra of native and BrNAP-alkylated cytochrome P-450c at a concentration of 3.8 μM in the absence (left panel) and presence (right panel) of 11.5 μM pyrene. The molar ellipticity [θ] corresponding to the Soret band in native cytochrome P-450c (without bound pyrene) is about −10 × 10^4 degrees cm^2/dmol. For comparison [θ] values of −16 × 10^4, −13 × 10^4, and −7.3 × 10^4 degrees cm^2/dmol have been observed for rabbit liver cytochrome PB P-450 (29), rabbit liver cytochrome P-450 LM2 (30), and bacterial cytochrome P-450cam (31), respectively, all low-spin ferric hemoproteins. Variations in spin state and ligandation of cytochrome P-450cam markedly affect the position and intensity of this CD band (31). Increases in the intensity of this negative band have also been suggested to correspond to an opening up of the heme-binding pocket in cytochromes P-450 (30). In the present case, modification of rat liver cytochrome P-450c with BrNAP produced no significant changes in the CD spectrum between 220 and 250 nm (data not shown) and only a small enhancement of the negative Cotton effect at ~415 nm (Fig. 1). The observation that this Cotton effect changes only slightly upon modification with BrNAP (Fig. 1) suggests that alkylation with BrNAP produces little change in the immediate environment of the heme moiety in cytochrome P-450c. This result is consistent with our previous finding that the spectra and ligand-binding characteristics of cytochrome P-450c were unaltered by alkylation of the hemoprotein with BrNAP.

As shown in the right panel of Fig. 1, the binding of pyrene to cytochrome P-450c induced a CD spectrum of the otherwise optically inactive symmetrical hydrocarbon. The maximal effect was produced at a pyrene concentration of ~7.8–11.5 μM, which presumably corresponds to saturation of the enzyme (final concentration, 3.8 μM). An induced CD spectrum for pyrene, accompanied by a decrease in magnitude of the negative Cotton effect associated with the Soret band, has been observed by Imai (32) upon binding of pyrene to high-spin rabbit cytochrome P-448, which appears to be the rabbit homolog of rat cytochrome P-450d (33). Modification of rat liver cytochrome P-450c with BrNAP substantially decreased the size of the negative bands associated with a chiral pyrene complex (Fig. 1). There was also a less pronounced change in the Cotton effect associated with the Soret band upon binding.
of pyrene to the modified, as compared to the native, enzyme. Although detailed interpretation is not possible, these spectral differences must reflect small but significant changes in the microenvironment of the pyrene bound to the native and BrNAP-modified enzyme. They constitute the only spectroscopic differences observed to date between native and BrNAP-modified cytochrome P-450c.

Interaction with NADPH-Cytochrome P-450 Reductase—

Two types of experiments were performed to compare the interaction of native and BrNAP-alkylated cytochrome P-450c with NADPH-cytochrome P-450 reductase. The rate of benzo[a]pyrene hydroxylation catalyzed by alkylated cytochrome P-450c was one-sixth (13-16%) of the rate catalyzed by native cytochrome P-450c, regardless of whether these hemoproteins were reconstituted with saturating (2-11 molar eq) or limiting amounts (0.7 molar eq) of NADPH-cytochrome P-450 reductases. These results suggest that alkylation with BrNAP does not alter the affinity with which cytochrome P-450c interacts with NADPH-cytochrome P-450 reductase for benzo[a]pyrene metabolism.

In the second experiment designed to compare the interaction between native and BrNAP-alkylated cytochrome P-450c with NADPH-cytochrome P-450 reductase, the effect of alkylation with BrNAP on the rate of reduction of cytochrome P-450c by NADPH-cytochrome P-450 reductase was studied under anaerobic conditions by stopped-flow spectroscopy. Fig. 2 shows the time course of reduction of native and alkylated cytochrome P-450c by NADPH-cytochrome P-450 reductase, as measured by the increase in absorbance at 447 nm. Although the kinetics of reduction of both native and alkylated cytochrome P-450c was apparently a biphasic process the two proteins differed significantly in that the rate of reduction of alkylated cytochrome P-450c was markedly greater than that of native cytochrome P-450c.

The rate constants for the fast (k₁) and slow (k₂) phase of reduction of native and BrNAP-alkylated cytochrome P-450c were calculated as described by Oprian et al. (27), based on the assumption that both phases conform to a first-order process (with appropriate correction for the contribution of k₂ to the calculation of k₁). As shown in Table I, alkylation of cytochrome P-450c with BrNAP markedly stimulated by ~30-fold both the fast and slow phases of reduction. By stimulating k₁ and k₂ to a similar extent, alkylation of cytochrome P-450c had little effect on the relative contribution of the fast and slow phases of reduction to the total absorbance change observed. Determination of the total absorbance change indicated that, under these experimental conditions, NADPH-cytochrome P-450 reductase was capable of reducing more than 90% of both native and alkylated cytochrome P-450c.

These results indicate that alkylation with BrNAP facilitates the initial one-electron reduction of cytochrome P-450c and establish that BrNAP does not inactivate cytochrome P-450c by blocking the introduction of the first electron.

Fig. 2 also shows the time course of reduction of cytochrome P-450c in the presence of 7-ethoxycoumarin. The rate of reduction of cytochrome P-450c (which, compared to other forms of cytochrome P-450 studied, is extremely slow) was greatly stimulated (~40-fold, Table I) by the presence of 7-ethoxycoumarin. In contrast, 7-ethoxycoumarin failed to stimulate the already rapid rate of reduction of BrNAP-alkylated cytochrome P-450c. Indeed, in the presence of 7-ethoxycoumarin, the rate of reduction of alkylated cytochrome P-450c appeared to be slightly retarded and conformed to a single first-order reaction (Table I). It is interesting that the marked stimulation of the rate of reduction of cytochrome P-450 by covalent modification with BrNAP is similar to the rate enhancement associated with substrate binding to cytochrome P-450c. Thus, the data in Fig. 2 and Table I suggest that BrNAP-alkylated cytochrome P-450c mimics substrate-bound native cytochrome P-450c in its rate of reduction by NADPH-cytochrome P-450 reductase. It has been argued that a conversion from low spin to high spin hemoprotein is the mechanism by which substrates stimulate the reduction of cytochrome P-450 by NADPH-cytochrome P-450 reductase (35-37). The rapid reduction of alkylated cytochrome P-450c, which is a low spin hemoprotein, clearly indicates that electron transfer between NADPH-cytochrome
Rates of reduction of native and BrNAP-alkylated cytochrome P-450c by NADPH-cytochrome P-450 reductase in the presence and absence of 7-ethoxycoumarin

Reduction of native and BrNAP-alkylated cytochrome P-450c was measured at 10 °C under anaerobic conditions in a stopped-flow spectrophotometer as described under “Experimental Procedures.” Absorbance changes and rate constants for the two phases were calculated assuming two first-order reactions as described by Oyrian et al. (27) with appropriate correction in the determination of k2 for the slower reaction. The values shown represent the mean and range for 2–7 reaction traces.

<table>
<thead>
<tr>
<th>Cytochrome P-450c</th>
<th>Fast phase, k1</th>
<th>Slow phase, k2</th>
<th>Amount as fast phase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native</td>
<td>1.8 (1.3–2.2)</td>
<td>0.2 (0.1–0.2)</td>
<td>51 (45–57)</td>
</tr>
<tr>
<td>BrNAP alkylated</td>
<td>52 (48–61)</td>
<td>6.8 (4.6–10)</td>
<td>42 (40–44)</td>
</tr>
<tr>
<td>Native + 7-ethoxycoumarin</td>
<td>75 (67–98)</td>
<td>8.0 (2.8–19)</td>
<td>33 (25–39)</td>
</tr>
<tr>
<td>BrNAP alkylated + 7-ethoxycoumarin</td>
<td>20 (18–22)</td>
<td>——</td>
<td>&gt;90</td>
</tr>
</tbody>
</table>

* Computer-assisted simulation of the data indicated a good fit to a single first-order reaction.

![Graph](image)

**Fig. 3. NADPH oxidation and superoxide anion formation by a reconstituted system containing native or BrNAP-alkylated cytochrome P-450c.** Native or BrNAP-alkylated cytochrome P-450c (0.05 nmol) was reconstituted with NADPH-cytochrome P-450 reductase (0.05 nmol) and dilauroylphosphatidylcholine (15 nmol) in 1 ml of potassium phosphate buffer (50 mM, pH 7.4). Reactions were initiated with NADPH (0.5 mM). NADPH oxidation (left panel) was measured spectrophotometrically at 37 °C from the decrease in absorbance at 340 nm. Superoxide anion formation (right panel) was determined spectrophotometrically by measuring the rate of reduction of acetylated cytochrome c (1.6 mg/ml) at 550 nm in the presence and absence of superoxide dismutase (SOD, 400 units/ml). The superoxide dismutase-inhibitable reduction of acetylated cytochrome c was used to quantitate superoxide anion production, values of which are given in Table II.

P-450 reductase and cytochrome P-450c can be stimulated without a spin-state transition.

**Stoichiometry**—The ability of covalent modification with BrNAP to stimulate the reduction of cytochrome P-450c by NADPH-cytochrome P-450 reductase (Fig. 2 and Table I) was confirmed in a series of experiments designed to study the stoichiometry of the reactions catalyzed by native and alkylated cytochrome P-450c. As shown in Fig. 3, alkylation of cytochrome P-450c with BrNAP markedly accelerated the rate of NADPH oxidation in an incubation mixture containing all components of the reconstituted system except substrate. This result eliminates the possibility that the apparent accelerated rate of reduction of alkylated cytochrome P-450c under anaerobic conditions (Fig. 2) was due to a change in the affinity of the reduced protein for carbon monoxide rather than a change in the rate of reduction of the protein. Fig. 3 also shows that this rapid rate of NADPH oxidation by alkylated cytochrome P-450c was associated with the formation of superoxide anion. In contrast, native cytochrome P-450c exhibited little NADPH oxidase activity and generated little superoxide anion. It should be emphasized that superoxide anion production was measured as the superoxide dismutase-inhibitable reduction of acetylated cytochrome c (monitored at 550 nm). Although alkylation of cytochrome c preferentially decreases its rate of reduction by NADPH-cytochrome P-450c reductase over that by superoxide anion (20), acetylated cytochrome c can nevertheless be reduced by NADPH-cytochrome P-450c reductase (at 1–2% the rate of unmodified cytochrome c). Based on estimates of the amount of superoxide anion generated during the first minute of reaction, alkylation of cytochrome P-450c with BrNAP increased the initial rate of superoxide anion formation approximately 50-fold (Table II). BrNAP-alkylated cytochrome P-450c continued to generate large amounts of superoxide anion, even in the presence of the substrate zoxazolamine (Table II).

The stoichiometry of the reactions catalyzed by native and BrNAP-alkylated cytochrome P-450c in the presence and absence of zoxazolamine, catalase, or superoxide dismutase is shown in Table III. Alkylation of cytochrome P-450c markedly stimulated both NADPH oxidation and hydrogen peroxide formation and inhibited zoxazolamine 6-hydroxylation in a reconstituted system containing NADPH-cytochrome P-450 reductase, NADPH, and lipid. The relatively good agreement between the rate of formation of H2O2 (Table III) and that of superoxide anion (Table II) indicates that the pathway to H2O2 formation by alkylated cytochrome P-450c is predominantly via superoxide anion.

Zoxazolamine-stimulated NADPH oxidation by native cytochrome P-450c to an extent that could be accounted for by

**Table I**

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Acetylated cytochrome c reduction</th>
<th>Superoxide anion production</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native P-450c</td>
<td>10.5</td>
<td>1.2 (0.3)</td>
</tr>
<tr>
<td>Native P-450c + superoxide dismutase</td>
<td>9.3</td>
<td>27.7 (14.2)</td>
</tr>
<tr>
<td>Alkylated P-450c</td>
<td>37.6</td>
<td>27.7 (14.2)</td>
</tr>
<tr>
<td>Alkylated P-450c + superoxide dismutase</td>
<td>9.9</td>
<td>28.4 (13.9)</td>
</tr>
</tbody>
</table>

* Values in parentheses are estimated initial rates (nmol of superoxide produced/min).
Effect of alkylation of cytochrome P-450c with BrNAP on NADPH oxidation, hydrogen peroxide formation, and zoxazolamine hydroxylation

Cytochrome P-450c (1 µM) was treated with 5 µM BrNAP for 30 min at 22 °C. Pretreatment of cytochrome P-450c with PDS was performed for 30 min at 22 °C (2 mol of PDS/mol of cytochrome P-450c) prior to treatment of the protein with BrNAP. Cytochrome P-450c (0.05 nmol) was combined with 0.05 nmol of NADPH-cytochrome P-450 reductase and 15 nmol of dilauroylphosphatidylcholine in 1.0 ml of 50 mM potassium phosphate buffer (pH 7.4) with or without zoxazolamine (250 nmol), superoxide dismutase (200 units/ml), or catalase (400 units/ml). Reactions were initiated with 0.25 mM NADPH, and NADPH oxidation was monitored spectrophotometrically at 37 °C. After 5 min, the reactions were terminated by the addition of 0.1 ml of 15% trichloroacetic acid, and H₂O₂ and 6-hydroxyzoxazolamine were quantified as described under "Experimental Procedures." Values represent the mean of duplicate determinations.

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Zoxazolamine</th>
<th>NADPH oxidation</th>
<th>H₂O₂ formation</th>
<th>6-Hydroxyzoxazolamine formation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experiment 1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P-450c</td>
<td></td>
<td>3.0</td>
<td>1.9</td>
<td></td>
</tr>
<tr>
<td>Alkylated P-450c</td>
<td></td>
<td>14.4</td>
<td>12.3</td>
<td>11.6</td>
</tr>
<tr>
<td>Experiment 2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P-450c</td>
<td></td>
<td>1.6</td>
<td>11.2</td>
<td></td>
</tr>
<tr>
<td>P-450c + catalase</td>
<td></td>
<td>0.0</td>
<td>11.3</td>
<td></td>
</tr>
<tr>
<td>P-450c + superoxide dismutase</td>
<td></td>
<td>1.7</td>
<td>11.1</td>
<td></td>
</tr>
<tr>
<td>PDS-treated P-450c</td>
<td></td>
<td>2.1</td>
<td>10.2</td>
<td></td>
</tr>
<tr>
<td>Alkylated P-450c</td>
<td></td>
<td>38.2</td>
<td>1.0</td>
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<tr>
<td>Alkylated P-450c + catalase</td>
<td></td>
<td>0.0</td>
<td>0.93</td>
<td></td>
</tr>
<tr>
<td>Alkylated P-450c + superoxide dismutase</td>
<td></td>
<td>39.0</td>
<td>0.7</td>
<td></td>
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<tr>
<td>PDS-pretreated, alkylated P-450c</td>
<td></td>
<td>2.0</td>
<td>10.4</td>
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</tbody>
</table>

formation of 6-hydroxyzoxazolamine, i.e. NADPH oxidation by native cytochrome P-450c was tightly coupled to product formation. In contrast, zoxazolamine failed to stimulate further the already high rate of NADPH oxidation or to retard the high rate of H₂O₂ formation by alkylated cytochrome P-450c, i.e. zoxazolamine was unable to reverse the uncoupling effect of chemical modification with BrNAP.

Inclusion of catalase or superoxide dismutase in the reconstituted system had a negligible effect on the 6-hydroxylation of zoxazolamine by either native or alkylated cytochrome P-450c. These data indicate that a free chemical reaction between zoxazolamine and the large amount of H₂O₂ or superoxide anion produced in the solution by alkylated cytochrome P-450c is not responsible for the residual catalytic activity of the modified hemoprotein. Table III also shows that the ability of BrNAP to bind to cytochrome P-450c, to stimulate NADPH oxidation and H₂O₂ formation, and to inhibit catalytic activity can all be blocked by pretreatment of cytochrome P-450c with PDS.

Specificity of the Uncoupling Effect of BrNAP—The importance of the uncoupling effect on the mechanism by which BrNAP inactivates cytochrome P-450c was corroborated by measuring the formation of H₂O₂ by each of 10 cytochrome P-450 isozymes (P450a–P450j), both before and after alkylation with BrNAP. In the native state each cytochrome P-450 isozyme catalyzed H₂O₂ formation to a comparable extent (1.5–3.8 nmol/5 min) in a reconstituted system containing NADPH-cytochrome P-450 reductase, NADPH, and lipid (incubated as described in the legend to Table III). Treatment of cytochromes P450e–P450j with a 5-fold molar excess of BrNAP, which inactivates only cytochrome P-450c (10), markedly stimulated H₂O₂ formation by cytochrome P-450c, but had little or no effect on H₂O₂ production by the other nine isozymes tested (1.8–7.1 nmol/5 min).

Oxy-Ferrous Cytochrome P-450c Complex—Native and alkylated cytochrome P-450c were compared for their ability to form a Fe³⁺–O₂ complex (Fig. 4, complex III) during catalytic turnover with 7-ethoxycoumarin as substrate. During catalysis, native cytochrome P-450c produced a 430-nm absorbing chromophore, indicative of an oxy-ferrous cytochrome P-450 complex (21). As shown in Fig. 5, formation of this complex...
was undetectable with alkylated cytochrome P-450c.\textsuperscript{3} Alkylated cytochrome P-450c was only slightly less stable than native cytochrome P-450c during the 10-min incubation, as determined from the carbon monoxide-difference spectrum of each ferric hemoprotein (see Fig. 5). This indicated that the inability of alkylated cytochrome P-450c to form a spectrally detectable Fe\textsuperscript{II}O\textsubscript{2} complex was not due to its destruction by the large amounts of superoxide anion/H\textsubscript{2}O\textsubscript{2} generated.

**Interactions with Cumene Hydroperoxide—**It has been shown previously (23, 24, 39) that certain organic peroxides, such as cumene hydroperoxide, can substitute for NADPH-cytochrome P-450 reductase and NADPH by supporting cytochrome P-450-dependent metabolism. The ability of cumene hydroperoxide (100–500 \(\mu\)M) to support the O-dealkylation of 7-ethoxyresorufin by native and BrNAP-alkylated cytochrome P-450c was examined as described under “Experimental Procedures.” The rate of resorufin formation catalyzed by alkylated cytochrome P-450c was 10–20% of that catalyzed by native cytochrome P-450c, indicating that alkylation of cytochrome P-450c impairs its ability to function as a peroxidase.

The peroxidative reactions catalyzed by cytochrome P-450 (24, 40) are thought to involve the intermediary of a complex of cytochrome P-450 with oxygen at the oxidation state of hydrogen peroxide (Fig. 4, complex IV). Fig. 6 shows the difference spectra (350–500 nm) recorded over 15 min after addition of cumene hydroperoxide (100 \(\mu\)M) to cytochrome P-450c (1 \(\mu\)M). The spectra are characterized by a trough at 417 nm and a broad peak at -460 nm. The slow destruction of cytochrome P-450c by cumene hydroperoxide is the cause of the trough at 417 nm whereas the slow formation of a complex between cytochrome P-450c and cumene hydroperoxide accounts for the peak at -460 nm. The difference spectra recorded after addition of cumene hydroperoxide differ from the absolute spectrum of cytochrome P-450c in the reference cuvette (Fig. 6, dashed line), indicating they do not arise simply by the destruction of cytochrome P-450c in the sample cuvette. The complexation between cytochrome P-450c and cumene hydroperoxide differs in two respects from that reported for rabbit cytochrome P450 LM2 (24). First, the rate of complexation is relatively slow and, second, the absorbance maximum of the complex is centered around -460 nm rather than -440 nm (34). Neither of these two unusual features was observed with cytochrome P-450b which, like rabbit cytochrome P-450 LM2, rapidly formed a 440-nm absorbing complex with cumene hydroperoxide (results not shown).

Difference spectra recorded after the addition of cumene hydroperoxide to BrNAP-alkylated cytochrome P-450c are also shown in Fig. 6. Alkylation with BrNAP diminished the ability of cytochrome P-450c to form a complex with cumene hydroperoxide (based on the relatively small peak at -460 nm) and also retarded its rate of destruction by the peroxide (as indicated by the relatively small trough at 417 nm). The diminished ability of alkylated cytochrome P-450c to form a complex with cumene hydroperoxide explains, at least in part, the diminished capacity of alkylated cytochrome P-450c to function as a peroxidase in the O-dealkylation of 7-ethoxyresorufin.\textsuperscript{4} It is noteworthy that alkylation of cytochrome P-

\textsuperscript{3}In separate experiments we failed to observed any significant formation of a Fe\textsuperscript{III}O\textsubscript{2} complex with native or alkylated cytochrome P-450c in the presence of zoxazolamine (250 \(\mu\)M).

\textsuperscript{4}It is interesting that alkylated cytochrome P-450c is relatively resistant to the destructive effects of cumene hydroperoxide (Fig. 7) and is similarly resistant to destruction by the large amount of hydrogen peroxide produced when alkylated cytochrome P-450c is reconstituted with NADPH-cytochrome P-450 reductase and NADPH (Fig. 6).

**CONCLUSIONS**

The present studies were undertaken to investigate the mechanism by which BrNAP, which alkylates primarily Cys-292 and to some extent Cys-160, alters the catalytic effectiveness of cytochrome P-450c. In the preceding paper, we eliminated the possibility that the inactivation of cytochrome P-450c by BrNAP involved alkylation of the putative heme-
binding thiolate, Cys-460, or disruption of heme binding to cytochrome P-450c.

Based on the currently accepted catalytic cycle (38) of cytochrome P-450 (see Fig. 4), BrNAP might be expected to inactivate cytochrome P-450c by blocking 1) access of the substrate to the catalytic site; 2) introduction of the first electron; 3) binding and activation of molecular oxygen; 4) introduction of the second electron; and/or 5) release of product.

The first possibility, namely that BrNAP inactivated cytochrome P-450c by blocking substrate binding, can be excluded based on several lines of evidence. Of the four substrates tested, none protected cytochrome P-450c from alkylation with BrNAP and, conversely, alkylation of cytochrome P-450c with BrNAP had no effect on substrate binding to cytochrome P-450c, based on spin state transitions induced by benzo[α]pyrene, zoxazolamine, or 7-ethoxy-coumarin. Addition of BrNAP to cytochrome P-450c failed to elicit the spin-state transition that would be anticipated if BrNAP bound to the substrate-binding site of cytochrome P-450c. Furthermore, if the alkylation of cytochrome P-450c were preceded by a substrate-like interaction with BrNAP, the kinetics of inactivation would be expected to conform to a pseudo-first-order process leading to complete inactivation of the protein, which is apparently not the case as shown in the preceding paper (10).

Experiments to test the possibility that alkylation with BrNAP blocked the first one-electron reduction of cytochrome P-450c by NADPH-cytochrome P-450 reductase led to the unexpected observation that alkylation with BrNAP actually stimulated (by ~30-fold) the rate of reduction of cytochrome P-450c. In the alkylated state, cytochrome P-450c efficiently oxidized NADPH and catalyzed the reduction of molecular oxygen to hydrogen peroxide via superoxide anion. The intermediacy of superoxide anion in the production of hydrogen peroxide established that alkylation of cytochrome P-450c causes the decomposition of complex III rather than complex IV (see Fig. 4). The decomposition of complex III indicates that alkylation of cytochrome P-450c with BrNAP uncouples the catalytic cycle prior to the introduction of the second electron, which is often the rate-determining step in cytochrome P-450-dependent reactions (40, 41). Addition of purified cytochrome b5 to a reconstituted system (2 mol of cytochrome b5/mol of cytochrome P-450c) had no effect on the rate of benzo[α]pyrene hydroxylation by native or BrNAP-alkylated cytochrome P-450c, suggesting that the uncoupling effect of BrNAP alkylation cannot be overcome by introducing the second electron via cytochrome b5 (results not shown). Experiments on formation of a putative oxy-cytochrome P-450 complex during substrate metabolism (Fig. 5) and experiments with cumene hydroperoxide (Fig. 6) provided additional evidence that alkylation of cytochrome P-450c impairs its ability to form a stable complex with activated oxygen.

Uncouplers have been defined as compounds that bind to cytochrome P-450 and cause an increase in the rate of NADPH utilization and oxygen consumption without undergoing oxidation themselves (41–46). Depending on the uncoupler, the oxygen may be reduced to superoxide anion (44, 46), hydrogen peroxide (45), or water (41–43). Previously identified uncouplers are compounds that form reversible enzyme-substrate complexes with cytochrome P-450 but, for chemical or steric reasons, cannot undergo oxidation. Although this definition serves to identify BrNAP as a cytochrome P-450 uncoupler, it is apparent that BrNAP is fundamentally different from all other known uncouplers in several respects. First, BrNAP does not simply behave as a pseudosubstrate for cytochrome P-450c, but binds covalently to a site (primarily Cys-292) that apparently lies outside the substrate-binding site. Second, alkylation does not cause a spin state transition, but precedes the subsequent binding of substrates and yet triggers the rapid reduction of cytochrome P-450c by NADPH-cytochrome P-450 reductase (under both anaerobic and aerobic conditions). Third, BrNAP binds covalently to each of 10 rat liver microsomal cytochrome P-450 isoymes, but selectively uncouples only cytochrome P-450c. The simplest explanation for decreased rate of product formation by the alkylated protein is that there is a competition between decomposition of the oxy-cytochrome P-450 complex and introduction of the second electron.

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Inactivation of Cytochrome P-450c by 2'-Bromo-4-nitroacetophenone