The anticarcinogenicity of some flavonoids has been attributed to modulation of the cytochrome P450 enzymes, which metabolize procarcinogens to their activated forms. However, the mechanism by which flavonoids inhibit some P450-mediated activities while activating others is a longstanding, intriguing question. We employed flash photolysis to measure carbon monoxide binding to P450 as a rapid kinetic technique to probe the interaction of the prototype flavonoid α-naphthoflavone with human cytochrome P450s 1A1 and 3A4, whose benzo[a]pyrene hydroxylation activities are respectively inhibited and stimulated by this compound. This flavonoid inhibited P450 1A1 binding to benzo[a]pyrene via a classical competitive mechanism. In contrast, α-naphthoflavone stimulated P450 3A4 by selectively binding and activating an otherwise inactive subpopulation of this P450 and promoting benzo[a]pyrene binding to the latter. These data indicate that flavonoids enhance activity by increasing the pool of active P450 molecules within this P450 macrosystem. Activators in other biological systems may similarly exert their effect by expanding the population of active receptor molecules.

Owing to their wide distribution in fruits, vegetables, and grain products, flavonoids are a regularly consumed component of the human diet (1, 2). Increased consumption of these phytochemicals is associated with decreased risk for colon, rectum, and lung cancers (3, 4). Anticarcinogenicity in rodents (5, 6) has usually been invoked to explain such inhibitory or stimulatory effects (14, 15). Most of these studies have assessed the effect of ANF on P450-mediated hydroxylation of the polycyclic aromatic hydrocarbon benzo[a]pyrene (BP), an environmental pollutant present in cigarette smoke and polluted air that is carcinogenic in experimental animals (16).

The P450 system metabolizes BP to a variety of products including activated metabolites that covalently bind to cellular macromolecules and initiate carcinogenesis (17, 18). Among the human P450s primarily responsible for metabolizing BP (18) are P450s 3A4 and 1A1. P450 3A4 is a major liver P450 that also metabolizes many important drugs (19), whereas P450 1A1 is normally undetectable in human liver but present in lung (20), where it is induced by cigarette smoking and is associated with lung cancer (19, 21). ANF inhibits P450 1A1-mediated metabolism of BP by rat liver microsomal P450s (22, 23) and human P450 1A1 (24, 25), yet stimulates BP metabolism by both human liver microsomal P450s (26–29) and P450 3A4 (14, 25, 29, 30). Classical mechanisms such as competitive inhibition or noncompetitive modulation of substrate binding have usually been invoked to explain such inhibitory or stimulatory effects (14, 15).

In order to clarify the mechanism of the opposing effects of ANF on P450s 3A4 and 1A1, we sought to identify a differential mode of interaction of ANF with these P450s. To address this question we applied a rapid kinetic technique, CO binding to P450 after laser induced flash photolysis of the P450 heme Fe-CO bond, as a sensitive probe of P450 conformation and dynamics. This unique approach contrasts with classical static measurements, which reflect the average properties of a macromolecule, and successfully revealed subtle and otherwise undetectable mechanistic details for several hemeproteins (31). Thus, a higher rate of CO binding indicates a wide ligand access channel and/or a flexible protein, whereas a lower rate suggests a restricted access channel and/or rigid protein conformation. This kinetic approach was previously used to define P450-substrate interactions using both liver microsomes (32, 33) and individual P450s (34, 35).

**MATERIALS AND METHODS**

**Human P450s 3A4 and 1A1**—These P450s were expressed in SF9 insect cells using recombinant baculoviruses (36, 37) and prepared for flash photolysis as described (34, 35).

**CO Flash Photolysis**—Reactions were carried out using 0.8 nmol/ml of P450 3A4 (corresponding to 2.2 mg/ml cell homogenate protein) or 0.3 nmol/ml of P450 1A1 (corresponding to 1.8 mg/ml cell homogenate protein) and 20 μM CO at 23°C. BP or ANF (Sigma) were added (from a 20 mM stock solution in dimethyl sulfoxide) to yield a final concentration of 20 μM, because initial experiments showed that this concentration produced maximal effects on the CO binding kinetics. Where indicated, lower concentrations of ANF were used. The mixture was then incubated for 20 min prior to addition of CO. Photodissociation of the P450 heme Fe-CO complex and monitoring of CO binding kinetics at 450 nm were performed as described (32). When both ANF and BP were present, identical curves were obtained regardless of their order of addition.

**Data Analysis**—A kinetic difference method was applied to kinetically distinguish the individual P450 species from the total P450 species (32). This approach evaluates the difference between the kinetic profiles.
Enzyme Assay—BP hydroxylation was assayed by measuring fluorescent phenolic metabolites (38). The P450 3A4 and BP concentrations were the same as those used in the flash photolysis experiments, whereas the ANF concentration was 0, 1, 5, or 20 μM. The reaction volume was 0.125 ml and included NADPH-cytochrome P450 reductase (39) in 2-fold molar excess over P450. A 3-min assay was chosen after establishing the linearity of the reaction rate.

RESULTS AND DISCUSSION

Fig. 1A depicts the time course for CO binding to P450 1A1 in the absence and the presence of the substrate BP and the inhibitor ANF. These compounds decreased the overall reaction rate (as gauged by half-time) in the order ANF (0.20 s), BP (0.15 s), and no addition (0.075 s). However, precise kinetic definition of the effects of these agents requires further analysis. We have previously shown that P450s 3A4 (34) and 1A1 (35) are each comprised of multiple, kinetically distinguishable species that are unresolvable by standard multienzyme analysis. The kinetics of the individual P450 species, however, can be resolved using a kinetic difference method (32, 34, 35), which entails analysis of a curve generated by subtracting the kinetic data in the absence of effector from that in the presence of effector. The resulting kinetic difference profile thus reflects the kinetic properties of the effector-specific P450 species. Fig. 1 (B and C) shows the difference curves for the data in Fig. 1A along with the least squares fits to the kinetic difference equation. This procedure yielded \( k_1 \) and \( k_2 \), which represent the CO binding rate constants for a effector-specific P450 1A1 species in the absence and the presence of the effector, respectively.

These results revealed (Table I) that BP decreased the rate of CO binding to its target P450 1A1 species by 3-fold (from 19.7 to 6.8 s\(^{-1}\)), whereas ANF similarly decelerated CO binding by 3.5-fold (from 19.4 to 5.4 s\(^{-1}\)). Because the experiments with BP and ANF yielded similar \( (p > 0.05) \) values for \( k_1 \) (19.7 and 19.4 s\(^{-1}\)) and \( a \) (0.0071 and 0.0084), these results indicate that both compounds interact with same P450 1A1 species.

We next assessed the effect of ANF on BP binding to its target P450 1A1 species by measuring the kinetics of P450 1A1 pre-equilibrated with both BP and ANF. The progress curve was identical to (data not shown) and the kinetic parameters were indistinguishable \( (p > 0.05) \) from those obtained with ANF alone (Table I). The observation that BP had no effect on the CO binding kinetics when ANF was present, in conjunction with the known metabolism of ANF by this P450 (25), indicates that ANF competitively inhibits BP binding.

Time course curves for CO binding to P450 3A4 are illustrated in Fig. 2A. The addition of either BP or ANF decelerated the overall rate, although ANF was clearly more potent. Data analyses by the kinetic difference method (Fig. 2, B and C) revealed (Table I) that BP decreased the rate of CO binding to a P450 3A4 species by 4.5-fold (from 19.1 to 4.2 s\(^{-1}\)), whereas ANF decreased the rate of its target species by 16-fold (from 37.7 to 2.4 s\(^{-1}\)). Furthermore, the large difference between the

### Table I

| P450     | Effector                        | \( k_1 \) | \( k_2 \) | \( a_1 \) | \( a_2 \) |
|----------|---------------------------------|----------|----------|---------|---------|
| P450 1A1 | Benzo[a]pyrene                  | 0.0127 ± 0.0023 | 6.8 ± 0.8 | 0.0071 ± 0.0021 | 19.7 ± 5.0 |
| P450 1A1 | α-Naphthoflavone                | 0.0035 ± 0.0010 | 5.4 ± 2.5 | 0.0084 ± 0.0009 | 19.4 ± 2.5 |
| P450 1A1 | Benzo[a]pyrene + α-Naphthoflavone | 0.0029 ± 0.0012 | 4.2 ± 1.7 | 0.0074 ± 0.0011 | 21.0 ± 5.2 |
| P450 3A4 | Benzo[a]pyrene                  | 0.0064 ± 0.0014 | 4.2 ± 1.0 | 0.0068 ± 0.0011 | 19.1 ± 2.8 |
| P450 3A4 | α-Naphthoflavone                | 0.0274 ± 0.0042 | 2.4 ± 0.4 | 0.0186 ± 0.0041 | 37.7 ± 2.1 |
| P450 3A4 + α-Naphthoflavone | Benzo[a]pyrene  | 0.0134 ± 0.0052 | 9.4 ± 0.4 | 0.0160 ± 0.0021 | 2.2 ± 0.04 |
corresponding $k_1$ values (19.1 and 37.7 s$^{-1}$, $p < 0.05$) clearly shows that BP and ANF interact with different P450 3A4 species, termed species I and II, respectively. In addition, the $\alpha_1$ values reflect the amounts of these two species and show that the ANF-reactive species is more predominant than the BP-reactive species ($\alpha_1 = 0.0186$ and 0.0068, respectively).

The next question is: because BP and ANF interact with different P450 3A4 species, how does ANF activate BP metabolism? To address this question we measured CO binding in the presence of both BP and ANF (Fig. 2A, d). The resulting CO binding rate was less than that in the presence of BP alone but greater than that observed for ANF alone. Thus, BP, in contrast to its decelerating effect on species I, increased the rate of the ANF-bound species II. Application of the kinetic difference method by subtraction of the curve in the presence of ANF alone (Fig. 2A, c) from that in the presence of both BP and ANF (Fig. 2A, d) yielded a difference curve (Fig. 2D) representing BP binding to a single ANF-bound P450 species (Table I). The results firstly show that BP increased the CO binding rate of its target ANF-bound P450 3A4 species II by 4-fold (from 2.2 to 9.4 s$^{-1}$), in contrast to the BP-induced rate deceleration for species I. The $k_1$ value (2.2 s$^{-1}$) is furthermore indistinguishable ($p > 0.05$) from the $k_1$ value (2.4 s$^{-1}$) of ANF-bound species II, confirming that BP targets the latter.

These results suggest that ANF stimulates BP metabolism by P450 3A4 (14, 25, 29, 30) by promoting BP binding to P450 3A4 species II. To examine the relationship between BP binding and metabolism, we measured both CO binding kinetics and BP hydroxylation at different levels of ANF-bound species II by varying the ANF concentration. Kinetic experiments were performed in the presence of BP + ANF and ANF alone to yield kinetic difference curves similar to that in Fig. 2D. Kinetic difference analyses yielded $\alpha_1$ values that reflect the amount of ANF-bound species II at each ANF concentration. A plot of $\alpha_1$ versus BP hydroxylation activity (Fig. 3) revealed that these are well correlated ($r = 0.98$) and establishes a functional link between BP binding to species II and BP metabolism. The data further show that ANF is an exceptionally potent activator of BP hydroxylation. For example, the relatively low activity (1.8 pmol min$^{-1}$ nmol$^{-1}$) in its absence was markedly enhanced (82.1 pmol min$^{-1}$ nmol$^{-1}$) in the presence of 20 $\mu$M ANF. However, 20 $\mu$M ANF enhanced BP binding to the total P450 3A4 by only 2.4-fold, based on $\alpha_1$ values of 0.0068 and 0.0160 for the BP targeted P450 species in the absence and the presence of ANF, respectively (Table I). These results indicate that in the absence of ANF, BP binds P450 3A4 species I, which has little catalytic activity. In contrast, in the presence of ANF, BP binds the highly active ANF-bound species II.

The results are summarized in Fig. 4, which shows that ANF inhibits P450 1A1 by competitive binding and activates P450 3A4 by an allosteric enhancement mechanism: P450 species II, which normally does not bind BP, binds to ANF and undergoes a conformational change that promotes BP binding and metabolism. This model thus differs from the classical allosteric mechanism, which does not account for the conformational heterogeneity of proteins.

These data indicate that the pharmacological and toxicological effects of flavonoids arise from a dual mode of action, because P450-mediated drug and carcinogen metabolism can be inhibited via classical competitive inhibition or enhanced by...
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**FIG. 4. Schematic representation of P450 interactions with BP and ANF.** The binding of a single P450 1A1 species with either BP or ANF is depicted on the left. The top of the right side shows binding of BP to P450 3A4 species I (left column) and ANF binding to species II (right column). In addition, BP binds to ANF-bound species II but not to ANF-free species II. The conformational induction of selected P450 molecules to an active form. The latter mechanism is similar to the recently described activation of receptors by agonists (40, 41) and may prove to be generally applicable in protein interactions with small molecules. Such elucidation of the diverse mechanisms of flavonoid action enhances our understanding of the role of dietary flavonoids in modulating P450-mediated reactions and may aid in the identification and design of flavonoids as chemopreventive agents.