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Allosteric Activation of Cytochrome P450 3A4 by α-Naphthoflavone: Branch Point Regulation Revealed by Isotope Dilution Analysis

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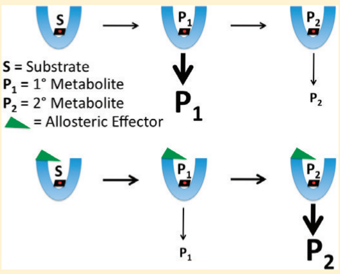
Supporting Information

ABSTRACT: Cytochrome P450 3A4 (CYP3A4) is the dominant xenobiotic metabolizing CYP. Despite great interest in CYP enzymology, two in vitro aspects of CYP3A4 catalysis are still not well understood, namely, sequential metabolism and allosteric activation. We have therefore investigated such a system in which both phenomena are present. Here we report that the sequential metabolism of Nile Red (NR) is accelerated by the heterotropic allosteric effector α-naphthoflavone (ANF). ANF increases the rates of formation for NR metabolites M1 and M2 and also perturbs the metabolite ratio in favor of M2. Thus, ANF has as an allosteric effect on a kinetic branch point. Co-incubating deuterium-labeled NR and unlabeled M1, we show that ANF increases k_{cat}/k_{off} ~1.8-fold in favor of the k_{cat} of M2 production. Steady-state metabolic experiments are analyzed using a kinetic model in which the enzyme and substrates are not in rapid equilibrium, and this distinction allows for the estimation of rates of catalysis for the formation of both the primary (M1) and secondary (M2) products, as well as the partitioning of enzyme between these states. These results are compared with those of earlier spectroscopic investigations of NR and ANF cooperativity, and a mechanism of ANF heteroactivation is presented that involves effects on substrate off rate and coupling efficiency.



Cytochrome P450 (CYP) enzymes catalyze the oxidation of xenobiotics and drugs and contribute significantly to their pharmacokinetic properties. One isoform in particular, cytochrome P450 3A4 (CYP3A4), is responsible for more than 50% of oxidative drug metabolism because of its high expression levels in the liver and the gut as well as its extreme substrate promiscuity.[1] CYP3A4 exhibits complex "atypical" kinetics that complicate prediction of in vivo metabolism on the basis of in vitro kinetic parameters.[2] These atypical kinetics reflect both homotropic and heterotropic allosteric effects. Despite the fact that α-naphthoflavone (ANF, 7,8-benzoflavone) is a paradigmatic allosteric effector of numerous CYPs, the mechanism(s) by which it elicits these effects with CYP3A4 remains unexplained. For example, Schwab et al.[3] first reported that ANF acts as an effector molecule of testosterone 6β-hydroxylase and 17β-estradiol 2-hydroxylase metabolism in human liver microsomes, with similar results in rifampicin-treated and untreated rabbit liver microsomes. Shou et al.[4] reported ANF and phenanthrene metabolism mediated by CYP3A4 microsomes. Their results were interpreted in terms of multiple-substrate binding, wherein both substrates affect each others' V_{Max}, but not each others' K_M, and thus have equal access to the active oxygen species. Although not applicable in all cases, this has subsequently become a common model for CYP allosterism.[5]

Our group, along with others, has presented evidence that multiple ANF molecules bind to CYP3A4 and that the first binding event is largely "silent" when observing changes in the ligand-dependent heme spin state via optical difference spectroscopy and EPR.[6] In a different approach, a fluorescently modified CYP3A4 was used to determine that testosterone (TST) binding does not compete with ANF, suggesting "specific" binding sites for different effector molecules distal from the active site.[7] This interpretation contrasts with the previous model for ANF heterotropic allosterism based on the ability of both substrates to share the capacious, fluid active site.[4,8] Thus, the location of ANF at low and high occupancy remains unclear. While crystallographic evidence demonstrates the possibility that multiple molecules can simultaneously bind in the CYP3A4 active site,[9] this has not been directly observed with ANF. Similarly, separate structural studies indicate a binding site for progesterone, which is distal from the heme,[10] but the analogous situation for ANF has not been directly observed.

In addition to the uncertainty about the location of ANF molecules bound to CYP3A4 under varying conditions, the mechanism by which ANF causes heterotropic functional effects on CYP3A4 has not been established. Non-Langmuir binding isotherms (e.g., sigmoidal) and non-Michaelis–Menten kinetics are often considered as evidence of multiple-ligand binding. However, multiple-ligand binding in CYPs might occur without "binding cooperativity" in the traditional sense and could instead reflect differential effects on the heme spin state by the first versus subsequent ligands that bind.[11,12] Heterotropic activation, as observed with ANF and CYP3A4, could also arise from specific perturbations by the effector molecule on any of the rate-determining steps in the P450 reaction cycle, regardless of its effect on substrate binding or substrate-induced spin-state changes. However, no studies have

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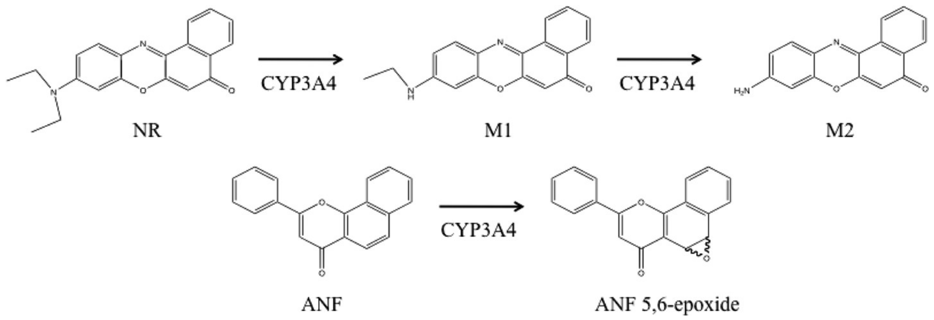
Scheme 1. NR and ANF Metabolites[a]



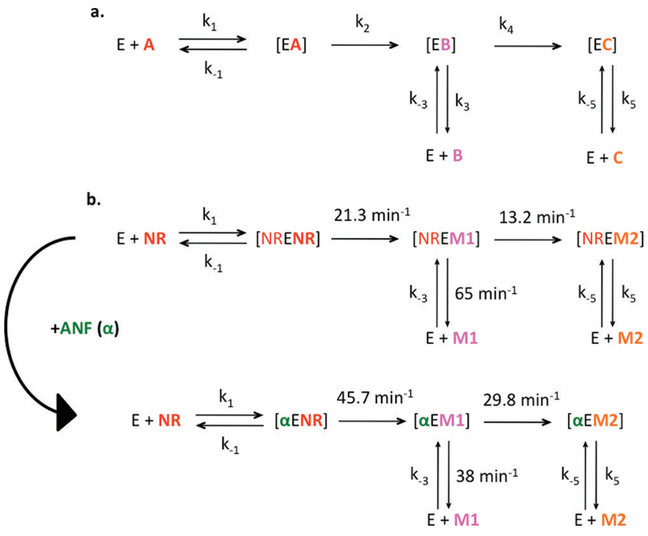
[a]NR is sequentially N-deethylated to its monoethyl and didesethyl metabolites. ANF is metabolized to the 5,6-oxide.

directly addressed the effect of ANF on the internal steps of the CYP reaction cycle. For example, the sequential oxidation of tertiary amines is a common route of drug metabolism, but the potential modulation by ANF or other allosteric effectors of branch points within sequential metabolism schemes has not been considered. Sequential or processive metabolism catalyzed by CYPs, of both endogenous and exogenous compounds, has been reported and was the subject of a recent review.[13]

To study allosteric effects on branch points within the CYP-catalyzed sequential metabolism of tertiary amines, here we use Nile Red (NR) as a fluorescent ligand and substrate for CYP3A4 and ANF as a heterotropic activator. The structures of NR, its metabolites, and ANF are shown in Scheme 1. Nile Red is sequentially metabolized via two N-deethylation reactions, allowing us to investigate an additional branch point between dissociation of the primary metabolite M1 (secondary amine) and oxidation to the secondary metabolite M2 (primary amine) (Scheme 2). In Scheme 2a, substrate A is NR in this work and B and C are M1 and M2, respectively. This is meant to show the general applicability of the experimental approach and the subsequently derived equations to any simple, two-step sequential metabolic pathway.

We have previously shown that NR is a fluorescent allosteric substrate of CYP3A4[14] and that ANF and NR simultaneously bind to CYP3A4.[15] Furthermore, single-molecule fluorescence experiments revealed a large ANF heterotropic effect resulting in a slower rate of dissociation of NR from the [CYP·ANF·NR] ternary complex than from the [CYP·NR] complex.[16] These, and other results, led us to further investigate NR sequential metabolism and heteroactivation afforded by ANF. Here, we present evidence that supports the hypothesis that heterotropic allostery can result from effector modulation of specific branch points in the P450 cycle, specifically, the ratio of sequential or processive Nile Red metabolism versus the dissociation of the primary metabolite. Our results show definitively different kinetic behavior for effector-free and ANF-bound CYP3A4.

Scheme 2. (a) Illustration of the General Case of Sequential Metabolism of Substrate A to Two Products, Primary Metabolite B and Secondary Metabolite C,[a] and (b) Estimated Rate Constants for NR Sequential Metabolism with and without ANF[b]



[a]For the specific case of NR metabolism, NR is A, M1 is B, and M2 is C. The scheme assumes irreversible chemical steps and reversible binding. Equations for global analysis of NR metabolism were derived using the steady-state approximation according to the King–Altman method.[26] Because of the sequential nature of NR metabolism, the concentration of the EB species is not in rapid equilibrium; it thus depends on catalysis rates k_2 and k_4, in addition to the dissociation rate constant for B ($K_{\mathrm{d,B}} = k_{-3}/k_3$).

[b]Catalytic rate constants are not microscopic rate constants because they include each of the rates in the P450 cycle and so represent a flux through a pathway with multiple branching points rather than a rate constant.

■ MATERIALS AND METHODS

Chemicals. All reagents were analytical grade and used without further purification unless otherwise specified. Solvents for LC–MS/MS were Optima LC–MS grade from Fisher. Solvents for flash chromatography and TLC were high-performance liquid chromatography (HPLC) grade and from EMD chemicals (ethyl acetate) and OmniSolv (hexanes). All water used other than that for LC–MS was deionized reverse osmosis Milli-Q quality from a Barnstead nanopureUV dispenser. Nile Red for kinetic and equilibrium experiments was a high-purity form from Sigma-Aldrich. Nile Red from TCI America was used for synthetic reactions. All other chemicals were from Sigma-Aldrich unless otherwise specified.

Protein Expression and Purification. Recombinant P450 3A4 was heterologously expressed in *Escherichia coli* C41(DE3) cells. C41(DE3) cells made competent using the $CaCl_2$ method were transformed with pCWOri+ encoding ampicillin resistance and a CYP3A4 gene based on the Nf14 construct, an engineered 3A4 gene with an N-terminal truncation, and additionally modified with a C-terminal histidine tag.[17,18] Plasmid was isolated from transformed DH5*α* cells using the Qiagen maxiprep kit. A single colony of freshly transformed C41(DE3) cells was used to inoculate a 5 mL starter culture incubated overnight at 37 °C in LB medium supplemented with a final concentration of ampicillin of 50 μg/mL. A 50 mL culture was inoculated from the starter culture, and 5 mL was added to each 500 mL of TB medium that was also supplemented with 50 μg/mL ampicillin and 0.5 mL of 1 M thiamine. After the mixture had been held for 4 h at 37 °C and shaken at 225 rpm, 0.5 mL of 1 M δ-ALA was added, and cells were induced with 0.5 mL of 1 M IPTG. The temperature and rate of revolution were reduced to 27 °C and 125 rpm, respectively, and after 40 h, cells were harvested. The innoculum was centrifuged for 30 min at 4 °C and 4500 rpm, and the resulting pellets were frozen at −80 °C. An aseptic technique was used throughout the cell culture procedure.

Cell pellets were thawed and resuspended in ice-cold resuspension buffer [100 mM KP_i, 20% glycerol, 10 mM β-mercaptoethanol (BME), and 2% Emulgen 911 (pH 7.4)] supplemented with protease inhibitor cocktail for His-tagged proteins (EDTA-free), DNase I, and 2 mg/mL lysozyme. The cell suspension was passed several times through a hand-held homogenizer and stirred at 4 °C for 1 h. The resulting suspension was centrifuged at 37000 rpm and 4 °C for 60 min in a Ti45 rotor. The resulting pellet was discarded, and the red supernatant was loaded directly onto a 30 mL Ni-NTA affinity column (Qiagen) equilibrated with wash buffer 1. After being loaded, the column was washed with 300 mL of wash 1 [50 mM KP_i, 300 mM KCl, 40 mM imidazole, 20% glycerol, 2 mM BME, and 0.2% Anapoe-$C_{10}E_9$ (pH 7.4)] followed by 300 mL of wash 2 [50 mM KP_i, 300 mM KCl, 40 mM imidazole, 20% glycerol, 2 mM BME, and 0.2% cholate (pH 7.4)] and 1000 mL of wash 3 [50 mM KP_i, 40 mM imidazole, 20% glycerol, 2 mM BME, and 0.2% cholate (pH 7.4)]. Protein was eluted with elution buffer (50 mM KP_i, 20% glycerol, 2 mM BME, 0.2% cholate, and 400 mM imidazole) and dialyzed with 10000 Da cutoff SnakeSkin pleated dialysis tubing (Thermo Scientific) against hydroxyapatite (HA) equilibration buffer (10 mM KP_i, 2 mM BME, 0.2% cholate, and 20% glycerol) at 4 °C; 50 μM TST was included in all buffers for Ni-NTA column chromatography but omitted from HA buffers. Dialyzed protein was loaded onto the HA column and then washed with 1000 mL of HA wash buffer [25 mM KP_i, 2 mM BME, and 20% glycerol (pH 7.4)]. Protein was eluted with 400 mM KP_i and 20% glycerol (pH 7.4) and dialyzed into storage buffer [100 mM KP_i, 20% glycerol, 1 mM EDTA, and 500 μM TCEP (pH 7.4)]. The protein was diluted to ∼20 μM, aliquoted, and stored at −80 °C for further use. Aliquots underwent no more than five freeze−thaw cycles each. Protein concentrations were determined by "dithionite difference spectra" (Fe^{3+}-H_2O vs Fe^{2+}-CO) using an extinction coefficient of 99 mM^{-1} cm^{-1}.[19,20] This method is similar to that of Omura and Sato (Fe^{2+} vs Fe^{2+}-CO)[21] but without the requisite anaerobic conditions in the reference cuvette. Protein was determined to be greater than 95% pure by reducing SDS−PAGE using a 10% acrylamide gel and staining with Coomassie brilliant blue.

Recombinant rat NADPH-cytochrome P450 (oxido)-reductase (rCPR) was expressed as a fusion protein with the *omp*A signaling peptide in the pOR263 plasmid and purified as previously described by Shen et al.[22] with small modifications. Briefly, cultures were grown as described for CYP3A4 expression except that LB was used and supplemented with 1 μg/mL riboflavin, 1 mM thiamine, and 100 μg/mL ampicillin. Expression was induced with 1 mM IPTG after 4 h, and cultures were grown for a further 16 h at 27 °C and shaking at 125 rpm. Cells were pelleted by centrifugation at 4 °C, briefly frozen at −80 °C, resuspended in cold lysis buffer [75 mM Tris (pH 8), 0.25 M sucrose, 0.5 mM EDTA, 200 μM FMN, 0.02 mg/mL lysozyme, 0.5 μg/mL DNase I, and 50 μM DTT, with standard protease inhibitor cocktail purchased from Sigma-Aldrich (St. Louis, MO)], and stirred for 1 h at 4 °C. Lysate was passed through a French pressure cell and centrifuged at 37000 rpm for 45 min. The supernatant was discarded and the pellet resuspended with homogenization/wash buffer [50 mM Tris (pH 7.7), 0.1 mM EDTA, 50 μM DTT, 10% glycerol, and 0.15% Triton X-100] and passed through a hand-held homogenizer. Centrifugation at 37000 rpm for 30 min afforded a yellowish-green supernatant that was loaded directly onto a 2′,5′-ADP Sepharose column equilibrated with wash buffer. The column was washed with 20 column volumes of wash buffer and eluted with a 0 to 6 mM $NADP^+$ gradient in wash buffer with 0.1% Triton X-100. rCPR was buffer exchanged into storage buffer [100 mM KP_i (pH 7.5), 0.1 mM EDTA, and 20% glycerol] via dialysis and size exclusion chromatography on a G-25 polyacrylamide sizing column equilibrated with storage buffer. rCPR was judged to be >95% pure by SDS−PAGE on an 8.25% reducing acrylamide gel stained with Coomassie. The rCPR concentration was determined by measuring the difference in absorbance between 456 and 550 nm of the purified protein in a solution of 0.01 mM $K_3Fe(CN)_6$ in 100 mM KP_i using an extinction coefficient of 21.1 mM^{-1} cm^{-1}.[23]

Rat cytochrome b_5 (rCyt b5) was a kind gift provided by A. Rettie (University of Washington).

Determination of S_{50} Using Optical Difference Spectroscopy. Low- to high-spin-state transitions upon binding of NR, metabolites, and ANF to CYP3A4 were performed as previously described for NR[14,15] using a dual-beam OLIS/Aminco DW2a spectrophotometer (OLIS, Bogart, GA). NR, metabolites, and ANF were titrated from DMSO stock solutions. The DMSO concentration did not exceed 2% (v/v).

Reconstituted System and Kinetic Assays. Determination of apparent kinetic constants K_M and V_{Max} using heterologously expressed human CYP3A4 followed the procedure of Shaw et al.[24] with the following modifications. Two 5× buffers, termed here "dilution buffer" (3 mM GSH and 50 mM potassium HEPES) and "reaction buffer" (12 mM GSH and 200 mM potassium HEPES), were used. Both buffers were at pH 7.4 and 37 °C. To the dilution buffer was added a 100× solution of detergent and lipid containing 2 mg/mL L-α-dilauroyl-*sn*-glycerophosphocholine, L-α-dioleoyl-*sn*-glycero-3-phosphocholine, and L-α-dilauroyl-*sn*-glycero-3-phosphoserine, at a 1:1:1 weight ratio per milliliter (Avanti Polar Lipids, Inc., Alabaster, AL) and 0.5 mg/mL CHAPS. This 100× stock solution was stored in screw-top Eppendorf tubes, kept frozen until use, and sonicated to clarity in 30 s intervals alternating between a bath sonicator (model 1510 from Branson operating at 40 kHz) at room temperature and wet ice. To the lipid-containing dilution buffer were then added CYP3A4, rCPR, and rCyt b5 in a 1:2:1 molar ratio. Final concentrations of enzyme in the 100 μL incubation were 30 nM CYP3A4, 60 nM rCPR, and 30 nM rCyt b5. Dilution buffer containing the enzymes was incubated for 10 min on ice with gentle mixing by hand. Twenty microliters of 5× reaction buffer was added to a 200 μL 96-well thin-walled PCR plate, to which was added 0.5 μL of NR, M1, and/or ANF stock solutions in DMSO. All incubations were in 1.5% DMSO. The PCR plate was then placed in a Rainin thermoshaker equipped with a PCR plate adapter and equilibrated at 37 °C. The rate of rotation was set at 1000 rpm. Control incubations contained the appropriate amount of DMSO. Water was added to the dilution buffer such that 68.5 μL of water and enzyme containing dilution buffer were added to each well, giving a volume of 90 μL. Wells were mixed by slow pipetting and incubated for 3 min at 37 °C while being shaken before addition of 10 μL of a freshly prepared 10× NADPH stock in H_2O, to give a final concentration of 1 mM, and a total reaction volume of 100 μL. After 6 min, the reactions were quenched into an equal volume (100 μL) of ice-cold ACN in a 250 μL conical bottom 96-well plate containing carbamazepine as internal standard. This plate was then spun at 4000 rpm in a Sorvall Legend XTR centrifuge (Thermo Scientific) for 10 min, and the supernatant was transferred to a separate 96-well plate for LC−MS/MS analysis. Standard curves were created by subjecting known amounts of NR, M1, M2, and ANF to identical incubation conditions in the absence of NADPH. The ANF metabolite was identified as the 5,6-epoxide on the basis of daughter ion spectra,[25] and the concentration was estimated on the basis of an ANF standard curve.

Isotope Dilution Experiments. Isotope dilution experiments were performed following the same procedure for the NR kinetic assays with the exception that deuterated NR and unlabeled M1 were included in incubations. Deuterated NR and the deuterated metabolites produced by CYP3A4 were monitored in the d_2-(M+H+2) channel corresponding to the incorporation of two deuterium atoms relative to unlabeled NR. Several corrections were applied to the raw peak intensity as follows. The percentage of d_2-NR versus total labeled NR was used to calculate the total amount of labeled NR and labeled metabolites. The amount of natural abundance ^{13}C causes a percentage of unlabeled M1 to appear in the d_2-M1 (M+H+2) channel, and this was corrected in both the d_2-M1 and d_2-M2 channels relative to unlabeled M1 and M2 intensities. The amount of unlabeled NR remaining in labeled NR (1.2%) was corrected in the M1 and M2 channels relative to d_2-M1 and d_2-M2 intensities. Standard curves and LC−MS/MS conditions were the same as those used for the kinetic experiments.

LC−MS/MS Metabolite Analysis. Metabolite identification and quantitation were performed on an API 4000 triple quadrupole mass spectrometer in positive electrospray mode (MDS SCIEX, Concord, ON), coupled to two LC-10ADvp pumps with a SCL-10ADvp controller (Shimadzu, Columbia, MD) with a CTC PAL (Leap Technologies, Carrboro, NC) autosampler. The flow rate was 0.5 mL/min over a Zorbax 5 μm, SB-C_{18} 2.1 mm × 150 mm column (Agilent) with a gradient elution profile starting at 50% solvent A (H_2O with 0.1% FA) and 50% solvent B (ACN with 0.1% FA) and increasing to 95% B over 9 min, holding at 95% B for 3 min, and then returning to the initial conditions. Retention times and MRM mass transitions are listed in Table 1. The desolvation temperature was 600 °C and the spray voltage 5.5 kV, and the declustering potential, collision energy, and entrance and exit potentials were optimized for each analyte.

Equations for Fitting NR Sequential Metabolism. Equations for the metabolism of a single substrate to sequential products can be derived from first principles on the basis of both the Henri−Michaelis−Menten and Briggs−Haldane assumptions.[26] The derivation based on the Briggs−Haldane assumptions is enumerated in the Supporting Information, and the equations used for data fitting follow (A, B, and C represent NR, M1, and M2, respectively).

Table 1. Retention Times and Mass Transitions for Metabolite Identification and Quantification

compound	retention time (min)	mass transition (*m*/*z*)
Nile Red	7.43	319 → 275
d_2-Nile Red	7.43	321 → 277
M1	5.21	291 → 247
d_2-M1	5.19	293 → 249
M2	2.81	263 → 190
d_2-M2	2.78	265 → 192
ANF	5.63	273 → 115
ANF oxide	3.54	289 → 131
carbamazepine	1.54	237 → 193

$$\frac{d[B]}{dt}=\frac{V_{Max,B}\left(\frac{[A]}{K_{m,A}}\right)-V_{Max,C}\left(\frac{k_2}{k_3+k_4}\right)\left(\frac{[A]}{K_{m,A}}\right)-V_{Max,C}\left(\frac{[B]}{K_{m,B}}\right)}{1+\left(\frac{[A]}{K_{m,A}}\right)+\left(\frac{k_2}{k_3+k_4}\right)\left(\frac{[A]}{K_{m,A}}\right)+\left(\frac{[B]}{K_{m,B}}\right)+\left(\frac{k_4}{k_5}\right)\left(\frac{k_2}{k_3+k_4}\right)\left(\frac{[A]}{K_{m,A}}\right)+\left(\frac{k_4}{k_5}\right)\left(\frac{[B]}{K_{m,B}}\right)+\frac{[C]}{K_{d,C}}} \quad (1)$$

$$\frac{d[C]}{dt}=\frac{V_{Max,C}\left(\frac{k_2}{k_3+k_4}\right)\left(\frac{[A]}{K_{m,A}}\right)+V_{Max,C}\left(\frac{[B]}{K_{m,B}}\right)}{1+\left(\frac{[A]}{K_{m,A}}\right)+\left(\frac{k_2}{k_3+k_4}\right)\left(\frac{[A]}{K_{m,A}}\right)+\left(\frac{[B]}{K_{m,B}}\right)+\left(\frac{k_4}{k_5}\right)\left(\frac{k_2}{k_3+k_4}\right)\left(\frac{[A]}{K_{m,A}}\right)+\left(\frac{k_4}{k_5}\right)\left(\frac{[B]}{K_{m,B}}\right)+\frac{[C]}{K_{d,C}}} \quad (2)$$

The grouping of terms in eqs 1 and 2 has specific relevance to the sequential metabolism of NR as shown in Scheme 2a. Specifically, M2 formation can be sequential or dissociative, represented by the two terms in the numerator of eq 2. The dissociative M2 formation rate (numerator, on the right) depends on the concentration of M1 in solution and the M1−CYP3A4 binding affinity. The sequential M2 formation rate (numerator, on the left) depends on the NR concentration in solution and the NR−CYP3A4 binding affinity, in addition to the M1 formation and M1 dissociation rates. In eq 1, the numerator contains one term for M1 formation but also contains the two terms for M1 depletion (i.e., M2 formation). Several assumptions have been made to facilitate the use of these complex equations. First, it was assumed that there is no significant product inhibition, so the term dependent on $K_{d,C}$ can be ignored in the denominator. Also, the k_4/k_5 ratio can thus be taken to be zero, and two further terms can be ignored. Because the experimental conditions are such that NR is in excess and the M1 concentration is low, combined with the fact that NR has a much tighter binding affinity than M1, we have ignored all dissociative enzyme−substrate complexes (i.e., those complexes that do not depend on NR concentration). Terms that include $K_{M,B}$ are thus ignored. These assumptions allow the use of the steady-state rate equations to analyze NR sequential metabolism under carefully controlled conditions, but care should be taken to recognize situations in which these assumptions are invalid (e.g., when B or C is at a high concentration or if metabolites B and C have a tighter binding affinity than parent molecule A). As we will show, NR metabolism is highly sequential and NR has tighter spectral binding affinity than its metabolites, so we make these assumptions cautiously, but not without justification.

Isotope Dilution Equations. Analysis of the metabolism of labeled NR co-incubated with unlabeled M1 is used to quantify sequential metabolism in CYP3A4 following a published procedure for the sequential metabolism of testosterone in CYP2C11.[27] In a similar fashion, we have used the amount of labeled and unlabeled NR metabolites to define two quantities, *A*′ and *B*′, as follows:

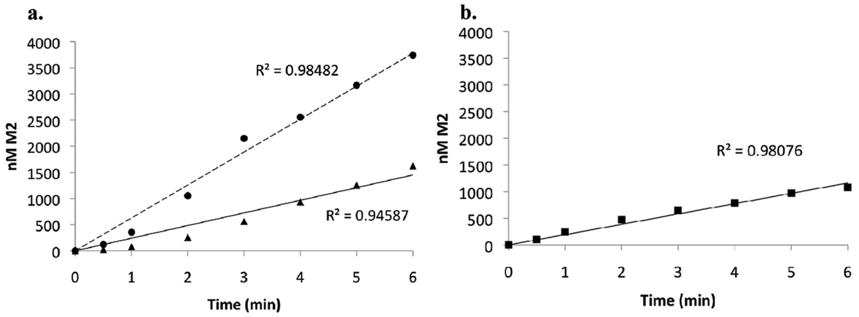


Figure 1. Incubations of NR (a) and M1 (b) were quenched at successive time points. The formation of M2 from NR (▲) shows signs of a lag in formation rate, indicative of the sequential steps required for production of M2 from NR. When M1 is incubated with CYP3A4, no lag in M2 formation is evident (■). When 12 μM ANF is included in the NR incubation (···●···), the lag is seen to decrease as judged by the R^2 value and the amount of M2 produced is increased more than 2-fold at 6 min.

$$A' = \frac{d_2\text{-M1}}{d_2\text{-M1} + d_2\text{-M2}} \tag{3}$$

$$B' = \frac{\text{M2/M1}}{1 - e^{-\text{M2/M1}}} \tag{4}$$

Concentrations of deuterated and nondeuterated metabolites were measured at the termination of 6 min incubations using the same procedure as in the kinetic experiments. A' and B' were then used to calculate the "branching ratio", $k_{\text{off}}/k_{\text{cat}}$ (k_3/k_4 from Scheme 2a).

$$\frac{A'B'}{1 - A'B'} = \frac{k_{\text{off}}}{k_{\text{cat}}} \tag{5}$$

Please see ref 27 for the full derivation of the equations listed above.

Synthesis of Deuterated NR. Deuterium was incorporated into NR in 10% D_2SO_4, 20% anhydrous ACN, and 70% D_2O. The reaction mixture was refluxed under a dry nitrogen atmosphere for 2.5 h, and reaction time points were monitored by LC–MS at 30 min intervals. LC–MS utilized a H_2O/ACN gradient (0.1% formic acid) and a 2.1 mm × 30 mm, 3 μm AQUASIL C_{18} column (Thermo). Mass spectra were recorded in positive ionization mode scanning from m/z 50 to 500. When d_0-NR was ~99% depleted, the reaction was quenched when the mixture was cooled to 4 °C and anhydrous K_2CO_3 was added. The crude product was extracted extensively with cold ethyl acetate, washed with brine, dried over anhydrous $MgSO_4$, and filtered. EtOAc was concentrated with a rotovap, and the crude product was adsorbed onto silica. The product was purified using flash chromatography (hexanes/EtOAc gradient from 1:0 to 1:1). TLC on silica was monitored via UV detection: 2:1 hexanes/EtOAc; R_f = 0.35. Peak integrations for NR (m/z 319 → 275) and its deuterated products from LC–MS/MS chromatograms in MRM mode were performed under conditions identical to those used in the isotope dilution experiments and indicated the following m/z distribution: 1.2% 319-d_0, 18.7% 320-d_1, 48.3% 321-d_2, 26.9% 322-d_3, 4.4% 323-d_4, and 0.5% 324-d_5.

Synthesis of M1. M1 was synthesized following the protocol of Olofson et al.[28] Briefly, 10 equiv of 1-chloroethyl chloroformate and 1 equiv of NR (TCI America) were refluxed in anhydrous toluene under a dry nitrogen atmosphere for 18 h. After concentration, the resulting crude chloroethyl carbamate ester was refluxed in 200 mL of methanol with 1 equiv of hydrochloric acid and monitored by TLC. The reaction was quenched by addition of solid $NaHCO_3$, and cold EtOAc was added. The combined organic fractions were washed with brine, dried over anhydrous $MgSO_4$, filtered, concentrated, and adsorbed onto silica for purification by flash column chromatography as described for deuterated NR (hexanes/EtOAc gradient from 1:0 to 1:1). The purity was assessed using HPLC as described above: TLC (2:1 hexanes/EtOAc) R_f = 0.21; ^{1}H NMR (500 MHz, CD_3CN) δ 8.6 (ddd, 1H, J = 8.0, 1.2, 0.6 Hz), 8.17 (ddd, 1H, J = 8.0, 1.5, 0.5 Hz), 7.76 (td, 1H, J = 7.5, 1.3 Hz), 7.67 (td, 1H, J = 7.5, 1.3 Hz), 7.54 (d, 1H, J = 9.0 Hz), 6.67 (dd, 1H, J = 9.0, 2.4 Hz), 6.46 (d, 1H, J = 2.5 Hz), 6.26 (s, 1H), 5.5 (s, 1H), 3.23 (qd, 2H, J = 7.5, 1.9 Hz), 1.23 (t, 3H, J = 7.5 Hz); found m/z (M + H) 291.1134, within 0.3 ppm of theoretical monoisotopic $C_{18}H_{15}N_2O_2$.

Synthesis of M2. M2 was synthesized by modifying a reported synthesis for Nile Red from starting material Nile Blue.[29] Cresyl Violet acetate salt (Acros) was refluxed in 0.5% H_2SO_4 with 20% ACN for 2 h. The reaction was monitored by TLC, and the product was purified by flash column chromatography as described above: TLC (2:1 hexanes/EtOAc) R_f = 0.09; ^{1}H NMR (500 MHz, CD_3CN) δ 8.56 (ddd, 1H, J = 8.0, 1.2, 0.4 Hz), 8.13 (ddd, 1H, J = 8.0, 1.3, 0.4 Hz), 7.74 (td, 1H, J = 7.5, 1.4 Hz), 7.65 (td, 1H, J = 7.5, 1.3 Hz), 7.51 (m, 1H), 6.65 (dd, 1H, J = 8.5, 2.4 Hz), 6.5 (d, 1H, J = 2.4 Hz), 6.22 (s, 1H), 5.14 (s, 2H); found m/z (M + H) 263.0827, within 2.3 ppm of theoretical monoisotopic $C_{16}H_{11}N_2O_2$.

^{1}H NMR Spectroscopy of Synthetic Metabolites. ^{1}H NMR spectra were recorded on a Varian 500 MHz Inova NMR spectrometer (Varian, Palo Alto, CA) using acetonitrile-d_3 or DMSO-d_6 as the solvent. The saturation frequency was 499.735 MHz. Spectra were referenced to water. Proton assignments were assisted by ^{1}H COSY and ^{1}H ROESY homocorrelation two-dimensional NMR spectroscopy, which allowed the identification of the primary sites of incorporation of deuterium into d-NR as the two most upfield aromatic protons.

High-Mass Accuracy LC–MS/TOF. Synthetic NR metabolites and deuterated NR were analyzed on a Waters Synapt MS/TOF instrument operated in positive electrospray mode coupled to an Agilant UPLC system. Samples were introduced into the mass spectrometer via isocratic 80% ACN, 20% H_2O, and 0.1% FA, with a 2.1 mm × 30 mm, 3 μm AQUASIL C_{18} column (Thermo). Calibration was conducted with sodium formate scanning a mass range from m/z 50 to 1000. Leucine-enkephalin (m/z 556.2771, 500 pg/μL) was constantly infused at a flow rate of 10 μL/min as an internal standard.

■ RESULTS

Time Courses and Steady-State Kinetics of Nile Red Metabolism. NR is metabolized by CYP3A4 to two predominant products, M1 (monodesethyl-NR) and M2 (didesethyl-NR), but the binding characteristics of M1 and M2 have not been determined previously. The net N-dealkylation of NR results from initial CYP3A4-mediated hydroxylation of the aliphatic methylene carbon on the diethylamino group to afford a carbinolamine.[30,31] This carbinolamine then spontaneously decomposes to an aldehyde and secondary amine.[31] The secondary amine is subsequently oxidized in an analogous reaction, either prior to dissociation of M1 or after dissociation and rebinding of M1. NR sequential metabolism was monitored via LC−MS/MS. Chromatographic separation and unique mass transitions were used to unambiguously identify NR metabolites (Table 1), and the concentration was determined from standard curves prepared from synthetic metabolites.

Figure 1 shows NR metabolite formation versus time. Production of M2 from the substrate NR shows a subtle but reproducible lag in formation. No lag in M2 production is apparent when M1 is the substrate, nor is there a lag in production of M1 from NR. The inclusion of ANF in the NR incubation increases the M2 formation rate and also decreases the lag as evidenced by the R^2 values for the linear regression of M2 concentration versus time curves in Figure 1. These data suggest intuitively that some dissociative metabolism occurs during the conversion of NR to M2, and that ANF alters the internal reaction dynamics or branch points. More striking is the relative rate of formation of M2 with saturating NR versus saturating M1. M2 is formed slightly faster from NR than M1 in the absence of ANF, and significantly faster in its presence. This is an intriguing indication that NR activates its own metabolism and does so to a greater extent than M1 activates its own metabolism. Because the amount of M2 metabolite formed with saturating NR is comparable to the amount of M2 formed with saturating M1 and because significant amounts of M1 are also produced when NR is saturating, we surmise that NR can act as a homotropic activator. In effect, more M2 is made from NR than from saturating M1, the upstream metabolite, and this demands that NR activates its own metabolism to M2. Previous spectroscopic evidence has established the multiple-binding behavior of NR with and without ANF.[14,15] The ability of ANF, NR, and M1 to act as an allosteric effector would thus be rank ordered as follows: ANF > NR > M1.

Figure 2 shows the steady-state kinetics of NR turnover. Total metabolite formation was fit to the M−M equation to give K_M values for NR metabolism at 0 and 12 μM ANF. These K_M values were used to parametrize the B−H fitting equations. Formation of M1 and formation of M2 can be saturated by NR, and in fact, the apparent K_M values for M1 and M2, with NR as the substrate, are not experimentally distinguishable. Because formation of M1 and formation of M2 depend on NR concentration, it is likely that M2 production is highly sequential. Fitting the sequential metabolism to the B−H equations provides estimates of the individual catalytic rate constants (V_{Max}) based on enzyme partitioning to the NR- and M1-bound species. Results from Figure 2 are summarized in Table 2 and Scheme 2b.

Spin-State Perturbation. The equilibrium affinity of substrates and metabolites is a critical determinant of the kinetic behavior of sequential metabolism schemes. If the metabolites bind with sufficiently high affinity, a greater fraction of downstream products will arise from nondissociative metabolism. Conversely, if the metabolites bind with low affinity compared to substrate, this will strengthen the tendency for dissociative metabolism. Affinities of NR and ANF for CYP3A4 were determined via UV−vis titrations as done previously, but results are shown here for comparison with the NR metabolite spectra.[6,14] Like ANF and NR, both metabolites M1 and M2 yield type I difference spectra indicative of a perturbation to high-spin heme from the low-spin resting state. All ligands show pronounced sigmoidal behavior in the binding isotherms (Figure 3). Recovered S_{50} values from fits to the Hill equation are 7.3, 68, and 58 μM for NR, M1, and M2, respectively. The NR metabolites have a significantly lower affinity for CYP3A4 as judged by the spin-state titrations. One interpretation of the sigmoidal spin-state changes is that multiple molecules bind with the first having little or no effect on the spin state while the second, or subsequent, binding event causes a perturbation to the high-spin state. This does not necessarily indicate that the binding events are cooperative in the traditional definition, as pointed out previously.[11,12] When compared to the turnover kinetics, the spin-state results are quite striking, as NR turnover can be fit to a hyperbola with no indication of sigmoidicity (Figure 2), unlike ANF turnover, which is sigmoidal at low concentrations, prior to the apparent substrate inhibition (data not shown, results below).[32] An important result of these experiments is that M1 and M2 have significantly lower affinity for CYP3A4 than does NR.

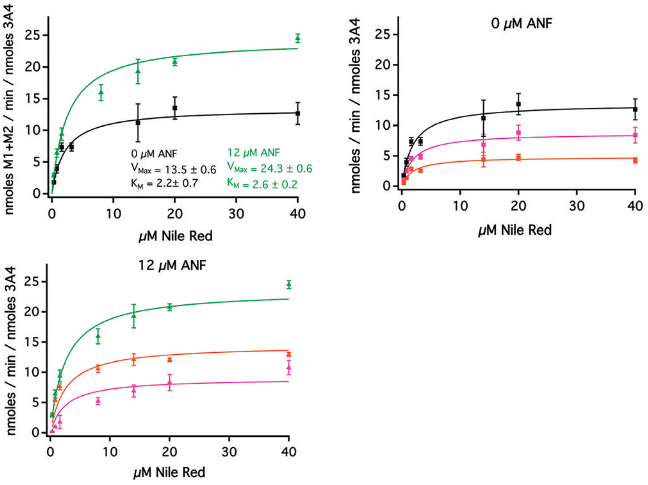


Figure 2. Steady-state metabolism of Nile Red. The first panel shows total metabolite production (M1 + M2) for 0 (black) and 12 μM ANF (green). The fit is to the M−M equation and illustrates that the ANF effect is on V_{Max} while K_M is not significantly different. The K_M values were used to parametrize the global fits. The second and third panels show M1 (pink), M2 (orange), and total metabolite (black or green) formation rates plotted vs NR concentration. The three data sets in each panel are fitted globally to the steady-state velocity equations as detailed in Materials and Methods. The stimulatory effect of ANF on M2 production is larger than the effect on M1 production, indicating the possible role of branch point modulation. See Table 2 for extracted rate constants.

ANF as a Substrate. To understand the allosteric effects of ANF on NR sequential metabolism, it is necessary to account for possible ANF metabolism in co-incubations that include ANF with either NR or M1. Therefore, we determined the apparent kinetic parameters for ANF turnover under our experimental conditions. The only ANF metabolite detected

Table 2. Measured and Calculated Kinetic Constants for NR Sequential Metabolism from Figure 2[a]

	A → B M1 rate (min^{-1})	A → C M2 rate (min^{-1})	"B → C" V_{MaxC} (min^{-1})	A → C/A → B M2 rate/M1 rate	A → C/"B → C" [EB]/([EA] + [EB]) fraction [EB]	k_{off}-M1 (min^{-1})	$k_{\text{off}}/k_{\text{cat}}$	V_{MaxB} (min^{-1})
0 μM ANF	8.9 ± 0.4	4.9 ± 0.2	13.2 ± 3.2	0.55	0.37	65	4.89	21.3 ± 2.2
12 μM ANF	8.9 ± 0.8	14.4 ± 0.8	29.8 ± 6.6	1.62	0.48	38	1.28	45.7 ± 6.9

[a] The first two columns show the V_{Max} values for M1 and M2 formation fit to the M–M equation and represent nominal rates of formation irrespective of enzyme species distribution. The third column shows the $V_{\text{Max,C}}$ values from the B–H global analysis, and they represent estimates of k_4 in both NR homotropic (0 μM ANF) and heterotropic (12 μM ANF) species. The next two columns show ratios that estimate the fraction of enzyme metabolizing NR and enzyme metabolizing M1. The k_3 (k_{off}-M1) can then be calculated, and a theoretical branching ratio $k_{\text{off}}/k_{\text{cat}}$, using $V_{\text{Max,C}}$ as k_4.

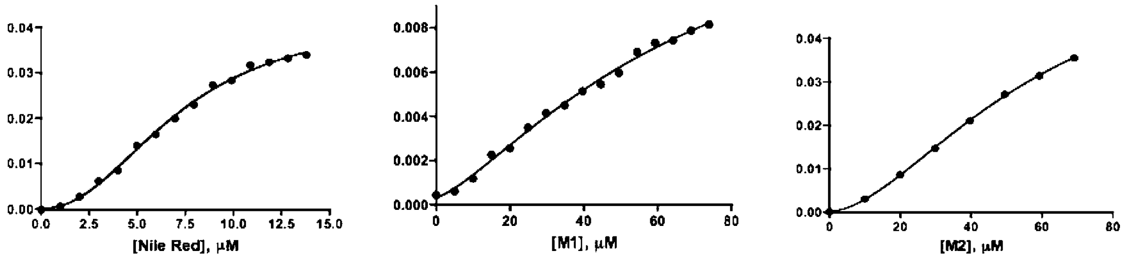


Figure 3. Plots of spin-state perturbation vs ligand concentration of NR and metabolites all yield sigmoidal binding titrations. Recovered S_{50} values from fits to the Hill equation are 7.3, 68, and 58 μM for NR, M1, and M2, respectively.

was the 5,6-oxide as indicated by the fragmentation pattern and m/z 289 → 131 mass transition as presented by Shou et al.[25] The velocity versus ANF concentration plots are complex, with a sigmoidal shape at low concentrations and apparent substrate inhibition at high concentrations. These results are consistent with those of Sligar et al.,[32] who showed that ANF binds to CYP3A4 with a stoichiometry of up to three ANF molecules per CY3A4. Our proposed stoichiometry is based on the pronounced sigmoidicity at low ANF concentrations and substrate inhibition at high ANF concentrations. This suggests the third ANF binding site causes some inhibition. The approximate S_{50} for the ANF turnover in the lower concentration range is 10 μM, which is significantly above the apparent K_{M} for NR (2–3 μM). The estimated V_{Max} is 0.02 nmol min^{-1} (nmol of CYP)$^{-1}$ for epoxidation of ANF obtained from the peak rate in the complex velocity versus ANF concentration plot (not shown). This rate is slower than some previously published results[33,34] on the basis of HPLC for estimating the ANF metabolite, and which used a different CYP:CPR:rCyt b5 ratio. In addition, modest changes in purification procedures or buffer components could contribute to the variable rates. The important result of this direct comparison is that ANF is metabolized much less efficiently than NR, and under the conditions used here, it does not compete with NR for metabolism. Therefore, in some of the subsequent experiments, the concentration of ANF is assumed not to change. In addition, the results indicate that the allosteric effects of ANF, described below, occur at concentrations that do not afford metabolites or significant heme spin-state changes.

ANF Acts as an Effector of NR Metabolite Formation. ANF increases the amount of M2 that is produced in the NR incubations while having a negligible effect on the amount of M1 produced (Figure 2). An important aspect of sequential metabolism of NR is that all M1 formed is not released into the medium, as some is retained on the enzyme and subsequently oxidized. That is to say, although apparent M1 formation rates are similar with and without ANF, the actual NR depletion rate, and thus M1 formation rate, must be increased in the presence of ANF even though the largest measurable experimental outcome is the dramatic increase in the level of M2 formation. In other words, the total amount of NR metabolism increases in the presence of ANF, with the largest effect being on M2 production. ANF exerts its effects mainly on V_{Max}, with the experimentally determined K_{M} values for NR total metabolite production increasing slightly as the ANF concentration increases. ANF, therefore, does not increase the affinity of CYP3A4 for NR but acts to increase the V_{Max} term in the velocity equations. Steady-state analysis of NR sequential metabolism indicates that ANF increases the velocity of both M1 and M2 formation. The steady-state model used here, however, does not include uncoupling, and the V_{Max} terms are thus aggregate kinetic constants that include multiple steps in the P450 catalytic cycle.

Isotope Dilution Analysis. Isotope dilution analysis provides a quantitative measure of the relative flux of an intermediate in the reaction cycle, such as [CYP·M1], forward to yield the secondary product [CYP·M2], compared to the dissociation of the [CYP·M1] complex.[27] The measured ratio of forward flux to dissociation is independent of the absolute rates of these processes. In Scheme 2, this represents the bifurcation of the [CYP·M1] complex to either of two fates, dissociation to free enzyme and M1 or further metabolism to the [CYP·M2] complex. The relative magnitude of the catalytic rate constant k_4 versus the off rate for the [CYP·M1] complex, k_3, defines the amount of sequential metabolism and will be termed a branching ratio. Some P450 species, such as CYP19A1 (aromatase), have been shown to be highly sequential enzymes, in that metabolic intermediates (19-hydroxyandrostenedione and 19-oxoandrostenedione) rarely dissociate and the substrate undergoes multiple rounds of P450 catalysis before the final product, estrone, is ultimately released into solution.[13] A separate study using rat CYP2C11 indicated that the sequential metabolism of testosterone to 16α-hydroxyandrostenedione was predominantly dissociative.[27] It does not appear possible to predict a priori the level of sequential metabolism for a given enzyme−substrate pair, and the determinants for highly sequential metabolism in P450s are, as of yet, undefined. It is likely that some enzymes evolved this capability, while others, such as CYP3A4, display this kinetic behavior in the apparent absence of a specific evolved function or particular metabolic advantage.

Incubations of deuterium-labeled NR and unlabeled M1 were performed using the same procedures used for the experiments with unlabeled NR. Yields of labeled and unlabeled NR metabolites were quantified using LC−MS/MS and analyzed according to the methods outlined by Sugiyama et al.[27] Results from the kinetic experiments suggested that ANF acts by increasing the amount of sequential metabolism, and this was corroborated by the isotope dilution results. In the absence of ANF, the branching ratio, k_{off}/k_{cat}, is 4.38 ± 0.62 and represents a ratio where the off rate of the [CYP·M1] complex is 4.38 times the rate of catalysis to form the [CYP·M2] complex. In the presence of ANF, the ratio decreases to 2.42 ± 0.31, which indicates that either the off rate is attenuated, the catalysis rate is augmented, or a combination of the two.

Simulations of Branch Point Modulation. Simulations of NR sequential metabolism were conducted using COPASI with the enzyme concentration set to 30 nM. Individual rate constants were manipulated as shown in Figure 4. The metabolite ratio can be manipulated by changing the off rate of M1. Importantly, however, the K_d need not change for the [M1·CYP3A4] complex for this off rate effect to manifest as a change in the metabolite ratio. This effect would not be observed under conditions where substrates and enzyme were in rapid equilibrium as postulated in the derivation of the Michaelis−Menten equation. We show two simulations in which only the on rate and off rate of M1 are changed (k_{-3} and k_3, respectively). Because the off rate and the catalysis rate branch from a single species, changing the off rate relative to the catalysis rate, even when the enzyme−substrate binding affinity is not changed and the catalysis rate k_4 is unchanged, still results in a large effect on the metabolite ratio.

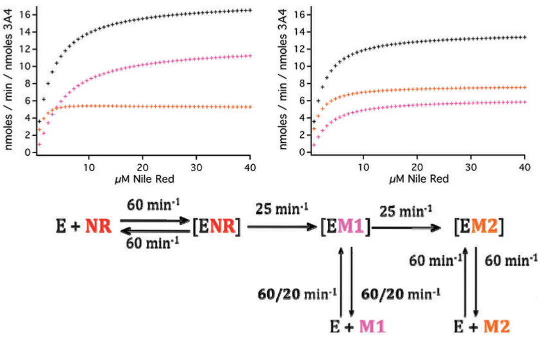


Figure 4. Simulated NR metabolism. Data for M1 formation are colored pink, those for M2 formation orange, and those for total metabolite black. The kinetic constants ($\min^{-1}$) for the simulation on the left are in reference to Scheme 2: k_1 = 60, k_{-1} = 60, k_2 = 25, $\boldsymbol{k_3}$ = **60**, $\boldsymbol{k_{-3}}$ = **60**, k_4 = 25, k_5 = 60, and k_{-5} = 60. Bimolecular rate constants have units of $\mu M^{-1} \min^{-1}$. Values for the graph on the right are as follows: k_1 = 60, k_{-1} = 60, k_2 = 25, $\boldsymbol{k_3}$ = **20**, $\boldsymbol{k_{-3}}$ = **20**, k_4 = 25, k_5 = 60, and k_{-5} = 60. The values that change are in bold for emphasis. Importantly, this simulation shows that the M1:M2 metabolite ratio can change via modulation of only the off rate of M1, even if the M1 K_d does not change. In this scenario, however, the total level of metabolite production does not increase, so we conclude that ANF must be modulating catalysis rates in addition to M1 dissociation rates.

DISCUSSION

These studies are aimed at improving our understanding of sequential metabolism and heterotropic allosteric activation, which are two well-appreciated aspects of CYP3A4 enzymology. Although sequential metabolism and allosteric effects are well-appreciated, the two aspects have not been considered in combination. Many drug molecules containing tertiary amines are dealkylated, and importantly, this process can occur iteratively, sometimes with the generation of tight binding inhibitory complexes.[35] Circulating levels of these drug metabolites are of increasing interest to CYP inhibition and drug−drug interactions.[36] Predicting metabolite concentrations of sequential systems will depend on an improved mechanistic understanding of CYP-catalyzed sequential metabolism in general. Models that probe allosteric effects in sequential metabolism are likely to be valuable.

The model we develop here highlights the need for non-Michaelis−Menten analyses with sequential allosteric systems. Before we introduce our kinetic model, some qualitative analysis of NR sequential metabolism with an emphasis on observed phenomenological trends is valuable. In the rapid equilibrium (M−M) assumption, the product off rate is assumed to be fast relative to the catalytic rate, but the sequential nature of NR metabolism suggests that the NR sequential system is not in rapid equilibrium. If the M1 off rate is very fast relative to M2 formation, all M2 will be formed dissociatively (i.e., nonsequentially). Conversely, if M2 formation was predominantly sequential, with very little M1 release followed by rebinding to the enzyme to be metabolized to M2, then M2 formation would depend on NR concentration and M2 formation plotted versus NR concentration would display saturable hyperbolic behavior. We observe, in Figure 2, that M2 formation can indeed be saturated by NR and is reasonably described by a hyperbola, and the K_M for M2 production is not statistically distinguishable from the K_M for M1 production. This indicates that M2 production is dependent on NR concentration and likely proceeds largely from the [CYP·NR] complex and is "significantly" sequential. The presence of the intermediate primary metabolite (M1) in solution indicates that the M1 off rate and M2 catalysis rates are similar and is important evidence that the process is not completely sequential. If none or very little M1 was detected, one might conclude that the M2 catalysis rate is much faster than the M1 off rate, but this is not found to be the case. These qualitative observations indicate that M2 production is at least partially sequential, and that the M2 catalysis rate and M1 off rate are similar.

To allow a more quantitative assessment of NR sequential metabolism, steady-state rate equations were derived on the basis of the microscopic rate constants as shown in Scheme 2a. These equations allow the approximation of the catalytic rates because the B−H equations explicitly include the sequential nature of NR metabolism and the competition of substrate and primary metabolite for free enzyme. Because most terms are shared between equations, an added benefit of this analysis is the possibility of analyzing M1 and M2 formation rates globally, linking all shared terms. One disadvantage of this approach is that the complexity of the equations makes the explicit treatment of allosterism intractable. Instead, we have performed experiments at several ANF concentrations and examined the trends in affinity and velocity parameters phenomenologically. A full ANF concentration analysis would be attractive, but because of the inability of rapid equilibrium assumptions to describe NR sequential metabolism, we instead interpret the velocity and branching ratio data as a representation of concentration-weighted averages of homotropic [NR·E·S] species and heterotropic [ANF·E·S] species (S is NR or M1) without specific information about binding stoichiometry. Still, the experiments are highly informative with regard to the ANF effects, particularly when considering the cases of minimal and maximal ANF effects, which report on the homotropic and heterotropic species.

ANF causes an increase in the total level of metabolite formation (green traces, Figure 2) from NR but has a stronger effect on the relative amount of M2 produced versus M1 (orange and pink traces, Figure 2). This occurs at ANF concentrations that do not contribute significant competitive inhibition to NR turnover. The K_s of ANF has been determined to be 10.7 μM when fit to the Hill equation (data not shown), but the amount of ANF product 5,6-oxide produced at that concentration is ~1000-fold smaller than the total amount of NR metabolite formed. The ANF metabolism data are intriguing to compare to the ANF stimulation of NR metabolism. These data suggest that while the first ANF binding event does not contribute to ANF turnover, it significantly augments NR metabolism. This observation is interesting when compared to those of previous fluorescence experiments,[15] which were interpreted as evidence of ANF binding to a peripheral binding site, where ANF and NR compete for this "allosteric" site. Thus, the two results are consistent with one another inasmuch as they suggest that a high-affinity binding site for ANF exists distal from the immediate heme environment, and this binding site modulates NR metabolism.

Previous experiments support a model in which NR binds to CYP3A4 at multiple sites, and ANF also binds to CYP3A4 and competes with NR for one, but not both, binding sites at low ANF concentrations.[15] Subsequent single-molecule fluorescence experiments showed that the NR off rate was attenuated in the presence of ANF.[16] Although NR sequential metabolism and ANF heteroactivation give complex kinetics, incorporating previous biophysical experiments with kinetic data allows for the generation of a mechanistic model of allosteric activation in CYP3A4. Analysis of NR sequential metabolism suggests that ANF acts by modulating the branching at sensitive points in the P450 cycle. The data in Table 2 are estimated from the global fitting parameters derived from data in Figure 2. Scheme 2b summarizes the global fitting parameters in the context of the NR sequential kinetic model. Because of the complexity of the sequential system, with the additional homotropic and heterotropic allosterism, we sought an experimental technique that used some of the inherent complexities of NR sequential metabolism to our advantage.

Isotope dilution, analogous to traditional pulse−chase experiments, has been used to study CYP-mediated sequential metabolism.[27] Rather than pulsing with isotopically labeled substrate and chasing with "cold" substrate, the experimental design calls for co-incubation of isotopically labeled substrate (d-NR) and nonlabeled primary metabolite (M1). The amounts of labeled and unlabeled secondary metabolite (M2) can then be used to approximate the amount of sequential (d-M2) versus dissociative secondary metabolite (M2). The unlabeled M1 acts as a competitive inhibitor of labeled d-M1 released from the enzyme and corrects for "dissociatively" produced d-M2. We have conducted isotope dilution experiments on NR sequential metabolism with deuterated NR, prepared as described in Materials and Methods. The specific output of these experiments, termed here the branching ratio (k_{cat}/k_{off}), refers to the microscopic rate constants k_4 and k_3 in Scheme 2a. We offer two interpretations of this ratio. The first interpretation disregards coupling effects. In the absence of uncoupling, which would present an additional branching pathway from the active [E·M1] complex, the ratios of labeled and unlabeled NR metabolites simplify to the k_{off}/k_{cat} branching ratio. Because we can approximate k_{cat} (k_4) from kinetic experiments, we can estimate the k_{off} term, and as shown in Figure 5, ANF causes a large shift in this ratio. The concentration dependence of this shift is also important to the model, because it shows no sigmoidal behavior. However, this ratio does decrease at high ANF concentrations. The simple interpretation of these data agrees with the original single-molecule fluorescence work[16] in that ANF appears to increase the residence time of the substrate, or metabolite, in the CYP3A4 active site. The observation of effects of ANF on NR binding in CYP3A4 nanodisks is consistent with the isotope dilution results in the turnover system utilized here, which includes not only lipid and detergent but also the redox partner proteins CPR and Cyt b5.

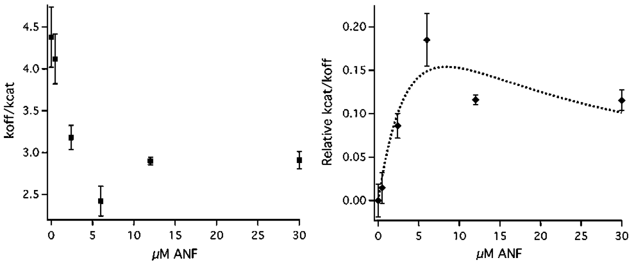


Figure 5. Branching ratio (left) and inverse branching ratio (right; for ease of fitting, the lowest ratio was set to zero) of NR sequential metabolism as determined by isotope dilution. ANF increases the sequential production of M2 vs dissociative production. The inverse branching ratio (right) is fit to a sequential binding substrate inhibition equation to give two affinity constants.

The mass spectral isotope dilution data unambiguously demonstrate that the branching ratio k_4/k_3 increases in the presence of ANF. This could be due to an increase in apparent k_4, a decrease in k_3, or both. Therefore, simulations of NR sequential metabolism were conducted to distinguish possible mechanisms for ANF heteroactivation. The results of the simulations and the experimental results are consistent with only the latter. The best fit of the simulated kinetic scheme to the experimental data (Scheme 2) demonstrates that k_2 and k_4 values for both M1 formation and M2 formation increase to a nearly identical extent when ANF is added. There is no differential effect of ANF on k_2 and k_4, which increase by 2.1- and 2.3-fold, respectively. However, with the constraint that k_2 and k_4 increase by nearly equal amounts, to observe the inversion of the product ratio, which favors M1 in the absence of ANF, to favor M2 in the presence of ANF, the off rate for M1 must decrease in its presence, as indicated by the recovered parameters from the simulations, wherein the off rate k_3 decreases by 1.8-fold. In other words, given that the recovered k_2 and k_4 parameters increase for both M1 and M2 production to the same extent but the product ratio changes, it is clear that an internal branching ratio must change, as proven by the isotope dilution data. Thus, the simulations that reproduce the observed experimental kinetic behavior are consistent with the isotope dilution results. In addition, the results (Table 2) indicate that there is an increase in the relative fraction of the enzyme as [CYP·M1] versus [CYP·NR] in the presence of ANF. This occurs despite the fact that both k_2 and k_4 rate constants increase to the same extent. The relative increase in the level of the [CYP·M1] complex with the nearly identical increase in k_2 and k_4 demands that k_3 decrease. The molecular basis for the increases in k_2 and k_4 is not addressed here. One possibility is that the actual chemical step for hydrogen atom abstraction by the perferryl iron−oxo intermediate is faster. Alternatively, a faster k_2 or k_4 could be obtained if uncoupling to reduced oxygen species was slower in the presence of ANF. This would effectively increase the steady-state concentrations of the [CYP·M1] and [CYP·NR] complexes and increase the rate of M2 or M1 formation. Together, the isotope dilution results and the best fit of the kinetic data to the model suggest that there is either an increase in coupling or an increase in the rate of hydrogen abstraction and rebound, and a decrease in the off rate for M1. While this conclusion is consistent with our previously published data concerning the effects of ANF on the off rate of NR, it raises the additional hypothesis that the level of uncoupling is decreased in the presence of ANF. Future work will focus on the quantitative effects on these isolated parameters. We speculate that the level of uncoupling is decreased by ANF.

Regardless of the complexity of the sequential metabolic scheme, the data presented herein unambiguously demonstrate branch point modulation by the allosteric effector where substrate off rates are attenuated (or k_{cat} is increased) and the apparent k_{cat} for M1 increases. This does not necessarily require an ANF-induced change in substrate affinity as shown by kinetic simulation. Although we cannot infer the location of ANF binding, it is clear that ANF, at low concentrations, binds to CYP3A4 and activates rather than inhibits NR metabolism. This activation causes small changes in the branching ratios of different products from a crucial intermediate during the P450 cycle, the oxygen-associated [E·S] complex, and this small change in branching ratio causes rather significant changes in metabolite production.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Derivation of the equations used in the global analysis of Nile Red sequential metabolism with a discussion of the assumptions used in the analysis. This material is available free of charge via the Internet at http://pubs.acs.org.

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■ ABBREVIATIONS

ACN, acetonitrile; δ-ALA, δ-aminolevulinic acid; ANF, α-naphthoflavone (7,8-benzoflavone); B−H, Briggs−Haldane; BME, β-mercaptoethanol; CHAPS, 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate; CYP3A4, cytochrome P450 3A4; *d*-NR, deuterated Nile Red; DMSO, dimethyl sulfoxide; EDTA, ethylenediaminetetraacetic acid; EPR, electron paramagnetic resonance; FA, formic acid; FMN, flavin mononucleotide; GSH, glutathione; HA, hydroxyapatite; IPTG, isopropyl β-D-1-thiopyranoside; KP_i, potassium phosphate; LB, Luria-Bertani medium; M1, monodesethyl-Nile Red; M2, didesethyl-Nile Red; M−M, Michaelis−Menten; NR, Nile Red [7-(diethylamino)-3,4-benzophenoxizine-2-one]; PCR, polymerase chain reaction; rCPR, rat cytochrome P450 reductase; rCYT b5, rat cytochrome b_5; R_f, retention factor; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; TB, Terrific Broth; TCEP, tris(2-carboxyethyl)phosphine; TLC, thin layer chromatography; TOF, time-of-flight; TST, testosterone.

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