Cooperativity in Cytochrome P450 3A4

LINKAGES IN SUBSTRATE BINDING, SPIN STATE, UNCOUPLING, AND PRODUCT FORMATION*

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Understanding the detailed metabolic mechanisms of membrane-associated cytochromes P450 is often hampered by heterogeneity, ill-defined oligomeric state of the enzyme, and variation in the stoichiometry of the functional P450-reductase complexes in various reconstituted systems. Here, we describe the detailed characterization of a functionally homogeneous 1:1 complex of cytochrome P450 3A4 (CYP3A4) and cytochrome P450 reductase solubilized via self-assembly in a nanoscale phospholipid bilayer. CYP3A4 in this complex showed a nearly complete conversion from the low- to high-spin state when saturated with testosterone (TS) and no noticeable modulation due to the presence of cytochrome P450 reductase. Global analysis of equilibrium substrate binding and steady-state NADPH consumption kinetics provided precise resolution of the fractional contributions to turnover of CYP3A4 intermediates with one, two, or three TS molecules bound. The first binding event accelerates NADPH consumption but does not result in significant product formation due to essentially complete uncoupling. Binding of the second substrate molecule is critically important for catalysis, as the product formation rate reaches a maximum value with two TS molecules bound, whereas the third binding event significantly improves the coupling efficiency of redox equivalent usage with no further increase in product formation rate. The resolution of the fractional contributions of binding intermediates of CYP3A4 into experimentally observed overall spin shift and the rates of steady-state NADPH oxidation and product formation provide new detailed insight into the mechanisms of cooperativity and allosteric regulation in this human cytochrome P450.

Cytochrome P450 3A4 (CYP3A4)3 is the most abundant P450 in the human liver (1). It is responsible for the metabolism of nearly half of the known drugs as well as many endogenous compounds (2). Like most mammalian cytochromes P450, CYP3A4 uses molecular dioxygen and reducing equivalents from NADPH to metabolize substrates, coupled through its redox partner cytochrome P450 reductase (CPR) (2). Numerous biophysical and biochemical studies of CYP3A4 have revealed an extremely rich and complex functional landscape, which includes homo- and heterotropic cooperativity, important roles of other protein effectors such as cytochrome b5, and a variety of inhibitors (3–6). Possible heterogeneity of detergent-solubilized membrane proteins, the known tendency of CYP3A4 to form oligomers, and the lack of control of protein stoichiometry also make the detailed characterization of reaction steps difficult. As a result, the main kinetic determinants of the CYP3A4 catalytic cycle are less well understood in comparison with its soluble counterparts (7).

Previous works elucidated the linkage of substrate binding and the thermodynamics of the CYP3A4 reaction cycle (8–12). Most of the studies of purified CYP3A4 have been hampered by the need for specific additives such as anionic phospholipids and salts (3, 13, 14). These complex reconstitution conditions and possible sample heterogeneity impede the detailed biophysical characterization of intermediates in the CYP3A4 catalytic cycle. CYP3A4 is known to have a large active site (15, 16) and is capable of binding multiple substrate molecules simultaneously. For instance, up to three testosterone molecules can associate with this enzyme. Overall, the experimentally observed functional properties depend on the unknown fractional contribution of the binding intermediates at different saturation levels. Continuous efforts are being made to deconvolute the kinetic and thermodynamic parameters of CYP3A4 binding and metabolism of multiple substrate molecules (11, 12, 17).

The use of a homogeneous population of CYP3A4 solubilized in a nanoscale phospholipid bilayer (Nanodiscs) has allowed the detailed characterization of purely monomeric CYP3A4 in a native membrane environment (18). Nanodiscs represent a discoidal phospholipid bilayer that is encompassed by two amphipathic helical proteins, termed membrane scaffold protein (MSP), and have been used to help stabilize and study several classes of integral membrane proteins (19–23). Nanodiscs allow for self-assembly of membrane-associated target proteins into the phospholipid bilayer with the ability to control the oligomerization state (20, 23, 24). Here, we document the first detailed characterization of a catalytically competent unit of CYP3A4 and CPR in a phospholipid bilayer Nanodisc of defined size. This provided the ability to investi-

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* The abbreviations used are: CYP3A4, cytochrome P450 3A4; CPR, cytochrome P450 reductase; MSP, membrane scaffold protein; POPC, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine; TS, testosterone; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid; Ni-NTA, nickel-nitritotriacetic acid.

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gate type I spin conversion induced by substrate binding, utilization of reducing equivalents, and coupled metabolism of testosterone. Simultaneous analysis of equilibrium substrate titration and steady-state kinetics of NADPH oxidation was used to characterize the binding intermediates of CYP3A4 and to delineate the functional properties of the enzyme in complex with one, two, and three testosterone molecules bound. From this resulted a clear definition of the cooperativity linkages between spin state, uncoupling, and substrate hydroxylation.

EXPERIMENTAL PROCEDURES

**Chemicals**—Imidazole, sodium cholate, NADPH, testosterone (TS), and androstenedione were purchased from Sigma. 6β-, 2β-, and 15β-OH-TS were from Steraloids, Inc. (Newport, RI). CHAPS was from Anatrace, Inc. (Maumee, OH); Emulgen 913 was from Karlan Research Products Corp. (Santa Rosa, CA); and POPC was from Avanti Polar Lipids, Inc. (Alabaster, AL). All other chemicals were purchased from Fisher, were at least ACS grade, and were used without further purification.

**MSP1E3D1 Expression, Purification, and Cleavage**—MSP1E3D1 was designed using two previously described proteins, MSP1E3 and MSP1D1 (25, 26). MSP1E3 is an MSP that has been extended by three 22-amino acid α-helices to form Nanodiscs with a larger diameter (25). MSP1D1 has a deletion of amino acids 1–11 in the first helix of the MSP, which has been shown not to be a part of the Nanodisc protein/lipid interface (25). These two progenitor proteins have been characterized extensively (25, 26) and led to MSP1E3D1, an extended MSP with a deletion of amino acids 1–11 in the first α-helix. MSP1E3D1 has an incorporated tobacco etch virus protease site to cleave the histidine affinity tag used for MSP purification. MSP1E3D1 was cloned into the pET-28 plasmid (Novagen), expressed in E. coli BL21-Gold (DE3) cells (Stratagene), and purified as described previously (25, 27). Cleavage of the histidine affinity tag and further purification have been described previously (18, 25). MSP with the histidine affinity tag removed is termed MSP1E3D1(−).

**CYP3A4 and CPR Expression and Purification**—CYP3A4 with a histidine affinity tag was expressed from the NF-14 construct in the pCWOrI+ vector and purified without any changes as described previously (8, 9, 28). The CYP3A4/NF-14/pCWOrI+ vector was a generous gift from Dr. F. P. Guengerich (Vanderbilt University, Nashville, TN). CYP3A4 was deemed pure by a single band when analyzed by SDS-PAGE with an Rz ratio (A_{417 nm}/A_{280 nm}) of 1.3 or higher. CYP3A4 concentration was measured by carbon monoxide difference spectra as described previously (29) using an extinction coefficient of 91 mM⁻¹ cm⁻¹. CPR from rat was expressed in the pOR262 vector and purified as described (30). The rat CPR/pOR262 plasmid was a generous gift from Dr. Todd D. Porter (University of Kentucky, Lexington, KY). CPR purity was ascertained by a single band when analyzed by SDS-PAGE with an Rz ratio (A_{576 nm}/A_{456 nm}) of 8.7 (31). The specific activity of CPR was measured using the cytochrome c activity assay (32), and concentration was determined using an extinction coefficient of 24.1 mM⁻¹ cm⁻¹ at 456 nm (33).

Self-Assembly and Purification of CYP3A4 and CPR Nanodiscs—To self-assemble Nanodiscs containing both CYP3A4 and CPR, the proteins at a molar ratio of 1:2 were incubated with 0.1% (v/v) Emulgen 913 for 1 h at room temperature. This mixture was then added to Nanodisc components consisting of cholate-solubilized POPC and MSP1E3D1(−). The phospholipids had been quantitated by inorganic phosphate analysis (34, 35), dried under a stream of nitrogen, and stored overnight in a vacuum desiccator. The final reconstitution molar ratio of all components was 0.1:0.2:240:120:1 CYP3A4/CPR/cholate/POPC/MSP1E3D1(−). One Nanodisc was formed by two MSP1E3D1(−) molecules and 240 POPC molecules. Thus, on average, the assembly mixture produced an excess of bare Nanodiscs statistically favoring the formation of a single CYP3A4-CPR complex in the Nanodisc. Once the total reconstitution mixture was added at the appropriate ratios, it was incubated for 45 min at room temperature and then placed on ice for an additional 1 h. To remove the detergents and concomitantly initiate the self-assembly process, a 60-fold excess of Bio-Beads SM-2 (Bio-Rad) was added. The Bio-Bead SM-2 excess was calculated based on the known adsorption rate and capacity of the Bio-Beads SM-2 (18, 36–38). This complex mixture was then incubated on ice for an additional 2 h. The formed Nanodisc assemblies were removed from the Bio-Beads SM-2 and filtered through a 33-mm 0.22 μM syringe filter (Millipore Corp.). As a result, several populations of Nanodiscs were formed, viz. Nanodiscs that contained no proteins except MSP, Nanodiscs that contained a single protein (either CYP3A4 or CPR), and Nanodiscs that contained both target proteins. The Nanodisc mixture was then injected onto a calibrated Superdex 200 HR 10/30 size exclusion column (GE Healthcare) equilibrated with 10 mM Tris-HCl (pH 7.4) and 100 mM NaCl and connected to a Waters HPLC system with a photodiode array detector at a flow rate of 0.5 ml/min. The Nanodisc fraction was pooled before further purification. The whole Nanodisc fractions that contained proteins and the empty Nanodiscs were then applied to a nickel-nitrilotriacetic acid (Ni-NTA) column (0.6 × 3.5 cm; Qiagen Inc.) equilibrated with 10 mM Tris-HCl (pH 7.4) and 100 mM NaCl. The nickel affinity column selectively bound CYP3A4 incorporated into Nanodiscs and induced separation from empty Nanodiscs and Nanodiscs containing CPR alone. The column was then washed with 5 column volumes of 10 mM Tris-HCl (pH 7.4), 100 mM NaCl, and 15 mM imidazole. This step was necessary to remove any Nanodiscs with nonspecific interactions with the Ni-NTA resin. The nickel affinity column was then eluted with 5 column volumes of 10 mM Tris-HCl (pH 7.4), 100 mM NaCl, and 250 mM imidazole, and the colored fractions were pooled. The pooled fractions were directly loaded onto a 2',5'-ADP-agarose column (0.6 × 2.5 cm; Sigma) equilibrated with 10 mM Tris-HCl (pH 7.4) and 100 mM NaCl. CPR bound to the 2',5'-ADP column and Nanodiscs containing only CYP3A4, but not CPR flow-through fractions. This column was then washed with the equilibration buffer and eluted with the same buffer containing 2.5 mM 2'-AMP. The samples were fully purified and dialyzed against 1000-fold volume of 100 mM HEPES (pH 7.4), 7.5 mM MgCl₂, and 0.1 mM dithiothreitol with three buffer exchanges every 6–8 h. All purification samples were then re-
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injected onto a calibrated Superdex 200 HR 10/30 column with proteins of known size and retention time and also analyzed by SDS-PAGE to verify the fidelity of the purification techniques. In theory, assembling two proteins into a Nanodisc could result in both proteins being on the same side of the 2-fold symmetric bilayer, or, on the other hand, the two proteins could occupy opposite sides. It has been suggested that, for integral membrane proteins that have a measurable affinity for each other, the self-assembly dynamics favor pre-association prior to collection of phospholipids and the final structure of the encircling MSP belt (23). Although it is experimentally very difficult to rule out a small amount of Nanodiscs with CPR and CYP3A4 on opposite sides of the bilayer, the homogeneous nature of the final analytical size exclusion chromatogram suggests a single hydrodynamic radius of the particle.

Spectral Characterization of CYP3A4 and CPR Nanodiscs—The absorption spectra of the fully purified CYP3A4–CPR Nanodiscs were measured from 250 to 700 nm using a Cary 300 Bio UV-visible spectrophotometer (Varian, Inc., Palo Alto, CA) in a self-masking cuvette (2 × 10 mm; NSG Precision Cells, Inc., Farmingdale, NY) with a sample volume of 400 μl. The spectra of CYP3A4–CPR Nanodiscs were then fitted to the basis set of the known molar spectra of each of the protein components using the left division subroutine in MATLAB (The MathWorks, Inc., Natick, MA) to determine the concentrations of the protein components. The results reported were averaged over seven experimental preparations.

TS Type I Binding to CYP3A4-Reductase Nanodiscs—TS binding titrations used CYP3A4–CPR Nanodiscs in the Cary Bio 300 UV-visible spectrophotometer with a Peltier thermostat maintaining the temperature at 37 °C. Titrations were carried out in a self-masking cuvette (2 × 10 mm) containing 400 μl of 0.5 μM CYP3A4–CPR Nanodiscs in 100 mM HEPES (pH 7.4), 7.5 mM MgCl2, and 0.1 mM dithiothreitol. TS was added from a 20 mM stock solution in methanol at 0.25–μl increments from 0 to 75 μM TS with a 0.5-μl syringe, in 0.5-μl increments from 75 to 150 μM, and in 1.0-μl increments from 150 to 247 μM with a 1-μl syringe. The final methanol concentration was 1.2% at the highest TS concentration measured. The CYP3A4–CPR Nanodiscs were then assayed using carbon monoxide difference spectra with the addition of sodium dithionate- and carbon monoxide-saturated buffer, and the sample showed no measurable signs of conversion to cytochrome P420.

NADPH Oxidation with CYP3A4–CPR Nanodiscs—The NADPH oxidation rate was measured by monitoring absorption at 340 nm using the Cary Bio 300 UV-visible spectrophotometer at 37 °C. NADPH oxidation measurements were performed with 400 μl of sample containing 30 pmol of CYP3A4–CPR Nanodiscs in 100 mM HEPES (pH 7.4), 7.5 mM MgCl2, and 0.1 mM dithiothreitol. Samples were equilibrated at 37 °C for 2 min with different concentrations of TS from 0 to 200 μM, and then 60 nmol of NADPH was added from a 20 mM stock solution. The rate of NADPH consumption was calculated from the absorption changes at 340 nm monitored for 6 min using an extinction coefficient of 6.22 mM cm⁻¹. The data are an average of three or more experimental data sets.

TS Metabolism with CYP3A4–CPR Nanodiscs—TS metabolism was measured using 30 pmol of CYP3A4–CPR Nanodiscs with different concentrations of TS (30–240 μM) from a 20 mM stock solution in methanol. The resultant small concentrations of methanol in the final assay mixture had no noticeable effect. The buffer contained 100 mM HEPES (pH 7.4), 7.5 mM MgCl2, and 0.1 mM dithiothreitol. The 500-μl samples were equilibrated at 37 °C for 2 min, and then 700 nmol of NADPH was added to each reaction. After different time points (1, 2, 4, 6, and 8 min), the reactions were quenched with 2 ml of dichloromethane. The reaction progress was linear over this time-frame. All samples had 20 nmol of androstenedione added as an internal standard. The quenched samples were extracted and dried under a stream of nitrogen. The dried samples were resuspended in 200 μl of methanol (Optima, Fisher) and loaded onto a Waters Nova-Pak C18 column (3.9 × 150 mm) connected to a Waters HPLC system with a photodiode array detector at a flow rate 0.75 ml/min. The TS and metabolites were monitored at 240 nm with a gradient of methanol/water/acetonitrile (39:60:1 to 80:18:2) over 55 min (39). The amount of products was calculated using authentic standards of 2β-, 6β-, and 15β-OH-TS and androstenedione as a reference. The identification of 15β-OH-TS was surmised from published elution profiles of TS metabolism and relative elution times (39, 40), and quantitation was calculated using the integration areas of 2β-OH-TS because 1β-OH-TS was not commercially available. All chromatographic base-line correction and integration calculations were done using GRAMS/AI (Thermo Fisher Scientific, Inc., Waltham, MA). The data were an average of three or more data sets.

Data Analysis and Fitting—To compare the experimental data obtained for CYP3A4 in 1:1 complex with CPR in Nanodiscs with previous results, the data were initially analyzed using the Hill equation. Analysis of the spectral titration of CYP3A4 in Nanodiscs with and without CPR was performed using singular value decomposition as described previously (18). For each experiment, all absorption spectra of CYP3A4 measured at 37 °C between 350 and 700 nm in the presence of different TS concentrations were corrected for the dilution factor and combined into the matrix. Singular value decomposition of this spectral matrix revealed that the data are described by two spectral components, with the second one corresponding to the difference spectrum of low and high ferric CYP3A4. The third singular value was always <3%. The resulting singular concentration vector corresponding to the transition of CYP3A4 from low to high spin as a function of TS concentration was fitted to the Hill equation: \( F = S^n/(K^n_s + S^n) \), where \( F \) is the normalized fraction of high-spin shift caused by TS binding, \( S \) is the substrate concentration, \( K_s \) is the spectral dissociation constant, and \( n \) is the Hill coefficient. The parameters obtained for TS binding did not depend on the presence of CPR in Nanodiscs.

The same Hill equation was used for the analysis of concentration dependence of the TS turnover rate in CYP3A4–CPR Nanodiscs. The turnover numbers (expressed as nanomoles of total product formed per min/nmol of CYP3A4 and measured in multiple experiments) were fitted to the following equation: \( v = V_{max} S^n/(S_{50}^n + S^n) \), where \( S_{50} \) is the substrate concentration at which the turnover rate is half of the maximum rate observed at the highest TS concentration.
Because of the limitations of Hill analysis and the lack of mechanistic information provided by the Hill coefficient, the experimental data were also analyzed globally using the reaction shown in Scheme 1. The experimentally observed dependence of different properties of CYP3A4 on the substrate concentration is represented as a linear combination of corresponding signals from substrate-free cytochrome as well as enzyme molecules with one, two, or three TS molecules bound, distributed over multiple possible microscopic configurations and averaged over time. In total, three enzyme-substrate complexes must be used in this global analysis because it was impossible to adequately describe all available data with only one or two TS molecules bound to CYP3A4. Each of these binding intermediates is characterized by a specific fraction of high-spin state, the steady-state rate of NADPH oxidation, and the rate of product formation. The overall signal measured at the given TS concentration is determined by the fractions of these enzyme-substrate complexes, which are in turn defined by the stepwise dissociation constants ($K_i, i = 1, 2, 3$) corresponding to the partial equilibria between species with different numbers of bound substrate molecules (Scheme 1).

\[
E + S \rightarrow ES + S \rightarrow ES_2 + S \rightarrow ES_3
\]

**SCHEME 1**

where $E$ is the concentration of substrate-free CYP3A4, $S$ is the substrate concentration, and $ES_i$ is the concentration of binding intermediates, *i.e.* complexes of CYP3A4 with $i$ molecules of substrate TS bound, where $i = 1, 2, 3$.

Simultaneous analysis of CYP3A4 titration curves and NADPH consumption rates as a function of TS concentration was performed using internally consistent fitting parameters for both data sets, viz. the $K_i$ (41). Data sets were normalized with respect to their maximum values to ensure equal statistical weight in the fitting procedure; S.E. values were estimated from the multiple experiments (from three to seven independent preparations). Fractions of the enzyme-substrate complexes containing different number of TS molecules bound to one molecule of CYP3A4 were expressed using standard binding polynomials (Equation 1) (18, 42),

\[
Y = \frac{S \left( \frac{S}{K_1} + \frac{S^2}{K_1K_2} + \frac{S^3}{K_1K_2K_3} \right)}{1 + \left( \frac{S}{K_1} + \frac{S^2}{K_1K_2} + \frac{S^3}{K_1K_2K_3} \right)} \quad (Eq. 1)
\]

where $Y$ is the average substrate saturation of the enzyme with substrate $S$, assuming that up to three molecules of substrate can bind to one protein molecule ($0 \leq Y \leq 1$). The fraction of high-spin CYP3A4 ($Y_S$) in a type I titration experiment is then given by Equation 2,

\[
Y_S = \frac{a_0 + a_1S + a_2S^2 + a_3S^3}{1 + \left( \frac{S}{K_1} + \frac{S^2}{K_1K_2} + \frac{S^3}{K_1K_2K_3} \right)} \quad (Eq. 2)
\]

where $a_0, a_1, a_2$, and $a_3$ are fractions of the high-spin state corresponding to the intermediates with zero, one, two, or three TS molecules bound to CYP3A4, respectively (18). Comparison of Equations 1 and 2 shows that, if more than one substrate molecule can bind to cytochrome P450, the experimentally measured signal shift ($Y_S$) caused by substrate binding is no longer equal to $Y$, the average substrate saturation, because of the difference in spectroscopic amplitudes ($a_i$). This also means that, if P450 binds more than one substrate molecule simultaneously, the true values of dissociation constants ($K_i$) cannot be derived from a type I spectral titration curve if the corresponding spectral amplitudes ($a_i$) are not known (18).

A similar equation was used for the analysis of steady-state NADPH oxidation rate dependence on TS concentration (Equation 3),

\[
V = \frac{v_0 + \frac{v_1S}{K_1} + \frac{v_2S^2}{K_1K_2} + \frac{v_3S^3}{K_1K_2K_3}}{1 + \left( \frac{S}{K_1} + \frac{S^2}{K_1K_2} + \frac{S^3}{K_1K_2K_3} \right)} \quad (Eq. 3)
\]

where $v_i (i = 0, 1, 2, 3)$ is the relative rate of steady-state NADPH consumption by enzyme-substrate complexes with $i$ molecules of TS bound to one molecule of CYP3A4, calculated as $v_i = V_i/V_{max}$, where $V_i$ is the absolute rate for intermediates with $i$ TS molecules bound to one CYP3A4 molecule. $v_0 = 25$ nmol/min/nmol is the rate of NADPH oxidation measured in the absence of substrate, and $V_{max}$ is the maximum rate obtained experimentally (272 nmol/min/nmol). Again, it is impossible to derive independently $K_i$ and $v_i$ from kinetic measurements at different substrate concentrations because of the high correlation between these parameters. However, if the relative values of spectral amplitudes ($a_i$) in Equation 2 and rates ($v_i$) in Equation 3 are not directly proportional to each other, the global analysis of these two data sets can resolve binding constants ($K_i$) as well as fractional spectral amplitudes ($a_i$) and rates ($v_i$) due to the fact that binding intermediates of CYP3A4 provide different fractions of the total signal measures in each type of experiment.

Equations 2 and 3 were used for simultaneous fitting of equilibrium TS titration and NADPH consumption data shown in Fig. 5. The same dissociation constants ($K_i$) were used for both data sets; thus, a total of nine parameters were used in fitting. The fitting program was written in MATLAB using the Nelder-Mead simplex minimization algorithm implemented in subroutine “fminsearch.m.” The fractional populations of binding intermediates of CYP3A4 were calculated using the recovered set of dissociation constants ($K_i$) and used to determine the rates of catalytic substrate turnover for CYP3A4 with different numbers of bound TS molecules. The total turnover rate ($k$) was expressed as the linear combination of the fractional rates of substrate hydroxylation ($k_i$, where $i$ is the number of TS molecules bound to one molecule of CYP3A4 ($i = 1, 2, 3$), and the fractions of binding intermediates were calculated using dissociation constants ($K_i$) derived from the global fit as described above (Equation 4).
RESULTS

To resolve the cooperativity linkages for productive and uncoupled metabolism in CYP3A4, we assembled a catalytically competent complex of both CYP3A4 and CPR at a 1:1 stoichiometric ratio into a Nanodisc phospholipid bilayer environment (18–21, 23). Self-assembly of Nanodiscs with CYP3A4 and CPR generated empty Nanodiscs, Nanodiscs containing only one of the two target proteins, and those with both CYP3A4 and CPR co-incorporated. Further purification of a single population of CYP3A4-CPR Nanodiscs was accomplished as described under “Experimental Procedures” using three separate chromatographic steps shown schematically in Fig. 1. First, size exclusion chromatography was used to separate out any large aggregates of protein or lipid not incorporated into Nanodiscs. The Nanodisc fractions with average Stokes diameters ranging from 12.3 to 14.1 nm were pooled from the size exclusion chromatographic elution. Nanodiscs containing His-tagged CYP3A4 were further purified using a Ni-NTA column and a low imidazole wash step to remove any nonspecifically bound Nanodiscs that did not contain the His-tagged enzyme. After removal of the target complex with a high concentration of imidazole, a final purification was accomplished with a 2',5'-ADP column, which bound the CPR and allowed the removal of those Nanodiscs containing only CYP3A4. The results of SDS-PAGE analysis of representative samples from these three purification steps are shown at Fig. 2. The fully purified CYP3A4-CPR Nanodiscs were re-injected onto a size exclusion column, which showed a single homogeneous peak with a retention time corresponding to a Stokes diameter of 14.0 ± 0.2 nm (Fig. 3). The protein stoichiometry of CYP3A4-CPR Nanodiscs was determined by UV-visible spectral analysis and fitted to the molar absorption spectra of the individual protein components. Least-squares regression analysis demonstrated a CYP3A4/CPR ratio of 0.94 ± 0.14 to 1 in the isolated fraction used for the experiments described.

The TS type I binding spectra of CYP3A4 in Nanodiscs containing CPR revealed an almost complete conversion of the enzyme from the predominantly low-spin form with no substrate present to an ~95% high-spin state at the highest concentrations of TS. This is consistent with a homogeneous population of monomeric CYP3A4 that has been characterized.
The rate of steady-state NADPH consumption measured as a function of TS concentration is shown in Fig. 5. The observed increase in this rate from 25 nmol/min/nmol in the absence of substrate to 272 nmol/min/nmol in the presence of 100 μM TS confirmed that the consumption of reducing equivalents by CYP3A4 is regulated by the presence of substrate. The value of the steady-state NADPH oxidation rate measured without substrate was slightly higher, but consistent with a previous result obtained for substrate-free CYP3A4 (12). The steep increase in
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NADPH consumption rate upon the gradual addition of TS suggests that the rate of the first electron transfer and reduction of ferric CYP3A4 is the rate-limiting step at low substrate concentrations. A strong dependence of the steady-state NADPH oxidation rate and first electron transfer rate on the substrate concentration has been well characterized by Guengerich and co-workers in several studies (4, 12, 14, 46). In the nanometer scale lipid domain of the Nanodisc system, the NADPH oxidation rate reaches a maximum of 270 nmol/min/nmol at 100 μM TS, and the observed rate is several times faster than in other CYP3A4 systems (3, 5, 47). As will be discussed, the direct comparison with other published values is complicated by the fact that the rates of NADPH oxidation with CYP3A4 and CPR are rarely reported for a simple 1:1 P450/CPR molar ratio. The NADPH oxidation rates with TS and excess CPR are between 20 and 90 nmol/min/nmol (3, 5, 47). With the Nanodisc system, the ability of CPR to transfer electrons from NADPH to CYP3A4 seems to be enhanced due to the close proximity of CPR to CYP3A4. This may also be due to the almost complete conversion of CYP3A4 to the high-spin state obtained in these monodisperse systems, allowing for an increased rate of reduction by CPR.

Comparison of the fraction of high-spin cytochrome and the NADPH oxidation rate shows a significant difference between these two parameters as a function of TS concentration (Fig. 5). Thus, the properties of individual CYP3A4 binding intermediates, i.e., cytochromes P450 with one, two, or three bound TS molecules (denoted $E_{S_1}$, $E_{S_2}$, and $E_{S_3}$, respectively), are quite distinct and can be resolved using a global analysis of all observables. The results of simultaneous analysis of substrate binding and the rate of NADPH consumption as a function of TS concentration are shown in Fig. 5 and Table 1. Both data sets could be successfully fitted assuming that up to three TS molecules can simultaneously bind to one CYP3A4 molecule, as described under “Experimental Procedures.” Attempts to fit these data using only one or two substrates/enzyme were unsuccessful and could not reproduce the observed maximum in NADPH oxidation rate dependence on TS concentration.

As a result of the global curve fitting shown in Fig. 5, TS binding to CYP3A4 co-incorporated with CPR into a Nanodisc can be described by three dissociation constants of 19, 37, and 56 μM, corresponding to the first, second, and third TS molecule binding events. The fractional populations of CYP3A4 binding intermediates are shown in Fig. 5B as dashed curves. The fractions of high-spin shift caused by the first, second, and third TS molecules, i.e., the spectroscopic amplitudes $a_i$ in Equation 2, are 24, 98, and 96%, respectively. Within error, both the second and third substrate molecules are able to shift the spin state of CYP3A4 almost completely to the high-spin form of the enzyme. Hence, binding of the third TS molecule is spectrally silent, although the presence of the third substrate molecule changes the overall functional dependence of the spin shift on TS concentration. Interestingly, binding of the first TS molecule also gives rise to non-negligible spin shift (~25%).

The partial rates of steady-state NADPH consumption recovered from the global fit of CYP3A4 binding intermediates are 110, 440, and 155 nmol/min/nmol for CYP3A4 with one, two, and three TS molecules bound, respectively (Table 1). As expected from the non-monotonic dependence of the overall NADPH oxidation rate on TS concentration, this rate turns out to be almost three times higher for CYP3A4 with two TS molecules bound than for CYP3A4 with three TS molecules. For substrate-free CYP3A4, the background rate of NADPH consumption is 25 nmol/min/nmol. Comparison of these parameters shows that binding of the first and second TS molecules gives rise to ~4-fold acceleration of NADPH oxidation per each TS bound, increasing the consumption of NADPH from 25 to 440 nmol/min/nmol. Interestingly, the third binding event results in an almost 3-fold decrease in NADPH consumption. This behavior of the NADPH consumption rate as a function of TS concentration in steady-state kinetics indicates that substrate binding plays an important regulatory role. Multiple TS molecules interact with CYP3A4 both as substrates, which are catalytically metabolized at the active site, and as allosteric regulators, changing the rate of enzymatic catalysis as well as consumption of redox equivalents.

To further understand the overall catalytic mechanism of TS metabolism, the rate of product formation was measured as a function of TS concentration. The total hydroxy-TS production rate versus substrate concentration is shown in Fig. 6A. The sum of the different TS metabolites (1B-, 2B-, 6B-, and 15β-OH) is shown as the total product metabolism rate. 6β-OH-TS is the main metabolite and constitutes >95% of the total product. Other isomers (2B-, 15β-, and 1β-OH) are responsible for the remaining 5% or less in decreasing order over all concentration ranges. These minor metabolites do not show any statistical variation in percentage yield across the concentration ranges used (data not shown). To compare these results with the literature, the data were fitted to the Hill equation, which gave an $S_{50}$ value of 34 μM and a Hill coefficient of 1.75 (data not shown). These Hill values are consistent with values that have been measured previously (e.g., $n = 1.6$) (48, 49). The range of $S_{50}$ values for TS metabolism is from 38 to 160 μM as reported in various systems (9, 47–49), but our results are in a good agreement with a study by Kim et al. (47), who also used phospholipid bilayer-incorporated CYP3A4 and CPR to study TS binding and metabolism. The maximum rate of TS hydroxylation, 21.5 nmol/min/nmol (Fig. 6), is faster than that previously seen at a 1:1 ratio of CYP3A4 to CPR (e.g., 0–14 nmol/min/nmol for reconstituted fusion protein or E. coli membranes) (3, 46). The comparison is limited, however, as there are only a few studies that used a 1:1 CYP3A4/CPR ratio (3, 46). In reconstituted systems, excess CPR is usually used to acceler-

| State* | Spin shift | NADPH rate | Turnover rate | Coupling* |
|--------|------------|------------|--------------|-----------|
|        | %          | min$^{-1}$ | min$^{-1}$   | %         |
| $E$    | 7          | 25         |              |           |
| $E_{S_1}$ | 24        | 110        | 0            | 0         |
| $E_{S_2}$ | 98        | 440        | 23.5         | 5         |
| $E_{S_3}$ | 96        | 155        | 21.5         | 14        |

* States $E$, $E_{S_1}$, $E_{S_2}$, and $E_{S_3}$ refer to substrate-free CYP3A4 and complexes of CYP3A4 with one, two, and three molecules of testosterone bound, respectively.

* Coupling is defined as the ratio of the steady-state rates of testosterone hydroxylation and NADPH oxidation shown in the preceding columns.
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One factor complicating the understanding of atypical kinetics observed with CYP3A4 is due to the population heterogeneity of CYP3A4 oligomers (18, 43, 50). Analysis of non-Michaelis kinetics and of homotropic and heterotropic cooperativity in cytochromes P450 in these systems is hampered by the presence of multiple kinetic processes often attributed to the multiple conformationally constrained populations of CYP3A4 (43, 50). With the use of Nanodisc technology, the ability to study a monomeric and functionally homogeneous population provides a detailed insight into the functional properties of isolated complexes of CYP3A4 and CPR without detergents that can lead to other biophysical anomalies (44). We have described the self-assembly of a single catalytic complex of CYP3A4 and CPR incorporated into a nanoscale phospholipid bilayer and stabilized by a genetically engineered MSP. In previous work, Nanodisc systems with only CYP3A4 incorporated were found to be homogeneous and showed almost complete spin conversion upon TS binding as well as monophasic dithionite-dependent reduction kinetics (18, 43). The CYP3A4 Nanodiscs have also proved to be an advantageous system for a stopped-flow study of the oxyferrous intermediate of CYP3A4 and its stabilization upon substrate binding (51).

In this study, we have demonstrated how the concentration of the substrate TS plays an important role in the overall cytochrome P450 catalytic cycle through the fractional population of CYP3A4 substrate-binding intermediates, each of which has functionally different properties. We quantitated TS binding by monitoring the heme iron spin shift, overall utilization of NADPH, and formation of hydroxylated product in a 1:1 CYP3A4/CPR bilayer system. Global analysis of these functional observables, measured over a broad range of substrate concentrations, allowed us to simultaneously resolve the fractional contributions of each CYP3A4 TS-binding intermediate (Table 1) and illuminate the cooperative linkage relationships in this human cytochrome P450.

With CYP3A4-CPR Nanodiscs, we observed an almost complete conversion from low to high spin with TS at 37 °C, indicating a homogeneous population with no apparent change induced by the presence of a CPR molecule in close proximity. It was important to establish this fact because the possible influence of CPR on the substrate binding properties of CYP3A4 could not be excluded a priori, and TS binding and high-spin conversion have not been studied previously in a functional CYP3A4-CPR complex. Comparison of the titration curves shown in Fig. 4 reveals an almost identical high-spin shift caused by TS binding by CYP3A4 in the presence or absence of CPR.

The steady-state NADPH oxidation rates measured over a broad range of TS concentrations are faster than those previously measured for CYP3A4 (3, 5, 47). The nature of this acceleration of NADPH consumption in CYP3A4-CPR Nanodiscs may be attributed to the close proximity of CPR to cytochrome P450 when both proteins are confined in a 12-nm Nanodisc with an effective phospholipid bilayer diameter of ~9.0 nm. An
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important factor in the interaction between CYP3A4 and CPR is their height relative to the bilayer. This has been measured in Nanodiscs by atomic force microscopy and shown to be compatible with a productive P450/CPR interface (52, 53). It has been noted that the phospholipid bilayer may facilitate the specific orientation of these two proteins that favors the optimum electron transfer (47, 54). As noted by several laboratories, phospholipids are needed for optimum activity of CYP3A4 (3, 13, 14, 47). The overall CYP3A4/lipid molar ratio used in previous studies (55, 56) is of the same magnitude as the protein/lipid stoichiometry in the Nanodisc experiments reported herein (~1:100).

Control experiments with CPR but no incorporated CYP3A4 showed an NADPH consumption rate of 4 nmol/min/nmol, which increased to 25 nmol/min/nmol when CYP3A4 was co-incorporated (Table 1), indicating an uncoupling at the heme protein active site. The addition of substrate up to 100 μM dramatically increased the NADPH consumption rate to 272 nmol/min/nmol, as shown at Fig. 5A. However, further addition of TS slowed down the observed steady-state rate of NADPH oxidation by ~20%. The fractional rates obtained from the global analysis for the binding intermediates reported herein reveal the allosteric regulation of CYP3A4 kinetics by TS binding. Whereas association of the first and second substrate molecules each results in an ~4-fold increase in NADPH consumption rates (110 and 440 nmol/min/nmol), the third binding event slows the overall rate of NADPH oxidation to 155 nmol/min/nmol. However, CYP3A4 with only one substrate molecule bound (ES1) is completely uncoupled and does not show any detectable TS hydroxylation, whereas the rates of product formation are very similar for the other binding intermediates with two (ES2) and three (ES3) TS molecules bound per CYP3A4 molecule. This means that all three binding intermediates have a distinct overall uncoupling ratio defined as the quotient of the steady-state NADPH oxidation rate and the rate of product formation. For ES2, this ratio is ~19, and for ES3 it is 7.2. These values can be interpreted as the mean number of oxidized NADPH molecules/product molecule formed by the corresponding CYP3A4 binding intermediate.

An important result of the global analysis of the data shown in Fig. 5 is the fact that three TS molecules can bind to CYP3A4. Whereas each of the data sets shown in Fig. 5 (A and B) could be separately fit using only two binding events, the Adair constants recovered from these fits are quite different and cannot be brought into mutual agreement. Simultaneous analysis of both data sets with two binding events was unsuccessful and could not reproduce the experimentally obtained dependence of NADPH oxidation rate on TS concentration shown in Fig. 5A. The ability of CYP3A4 to bind as many as three TS molecules was suggested before (9, 57, 58), and our data are consistent with these observations.

Resolution of the functional properties of CYP3A4 with various numbers of bound TS molecules depicted in Figs. 5 and 6 provides a detailed understanding of cooperativity in this human cytochrome P450. In the absence of substrate, CYP3A4 is predominantly in a low-spin state, but still can be slowly reduced by CPR, as shown by the background rate of NADPH consumption. The first TS binding event forms an enzyme-substrate complex (ES1) with a dissociation constant of 19 μM. CYP3A4 with one substrate molecule is reduced four times faster than in the substrate-free form, but there is no detectable product formation from this state. Instead, the utilization of reducing equivalents by this binding intermediate is fully uncoupled, and all redox equivalents from NADPH are lost through formation of superoxide and/or hydrogen peroxide. The absence of product formation from ES1 indicates that the putative ferrylloxo active intermediate involved in hydroxylation may not be formed before the second TS molecule binds to CYP3A4. This may serve as part of a protective mechanism in CYP3A4 catalysis by minimizing heme destruction and inactivation of P450 at low substrate concentrations.

The stoichiometric dissociation constant of 37 μM for formation of the second binding intermediate (ES2) is two times higher than that for the first binding event. Thus, TS binding to CYP3A4 does not reveal the features of a positive cooperativity. Hence, the experimentally observed sigmoidal behavior of type I substrate binding isotherms and the product turnover as a function of substrate concentration are due to fundamental differences in the reactivity profiles of the binding intermediates (ESi) and not to strongly cooperative binding. Fig. 5B shows that the fractional populations of binding intermediates ES1 and ES2 both reach ~30% at maximum, again indicating a very weak binding cooperativity. The high rates of steady-state NADPH oxidation and product turnover rates recovered for ES2 reveal that the second binding event plays a definitive role in CYP3A4 catalysis. With the second TS molecule bound, CYP3A4 undergoes a complete high-spin shift and a 4-fold increase in NADPH consumption rate (to ~440 nmol/min/nmol) and reaches the maximum product metabolism rate (23.5 nmol/min/nmol). The complete spin shift and high TS metabolism rate of ES2 indicate that the high-valent ferrylloxo catalytically active intermediate can be formed efficiently only after the second TS molecule is bound. Oxygen activation in CYP3A4 also requires the presence of the substrate molecule in the immediate vicinity of the heme iron, in agreement with the commonly accepted general mechanism of cytochrome P450 catalysis (59, 60). At the same time, ES2 is still highly uncoupled, and almost 95% of redox equivalents from NADPH are wasted through unproductive pathways.

The third binding step to form ES3 is characterized by a dissociation constant of 56 μM and exhibits almost the same spin shift and TS turnover rate as the step to form ES2. The important distinction between ES2 and ES3 is an overall decrease in the rate of NADPH consumption to 155 nmol/min/nmol when the third TS molecule binds to CYP3A4. The net result of this deceleration is an almost 3-fold better coupling in ES3 (Table 1). Thus, the role of the third binding event is to block unproductive pathways rather than to further activate TS turnover. This observation, which has implications for the heterotropic cooperative mechanisms in CYP3A4, represents yet another interesting aspect of allosteric behavior in xenobiotic metabolism. The mechanism of such unusual allosteric regulation may involve both structural and dynamic factors such as a tighter packing of more than one substrate molecule in the vicinity of the heme iron, conformational changes in CYP3A4 induced by TS binding, inhibition of uncoupling pathways through diffu-
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To our knowledge, there has been no previous attempt to resolve the functional properties of individual binding intermediates in CYP3A4-catalyzed TS hydroxylation. However, our results can be compared with kinetic analysis of CYP3A4 metabolism of several other substrates based on multisite models (57, 61). These models assume the presence of two or three distinct sites for substrate and effector binding and can fit the experimental turnover kinetics using up to eight parameters (61). Although our approach is formally similar to a multisite analysis, we do not follow the assumption of structurally distinct binding sites with different properties and consider the experimentally observed functional properties of each binding intermediate as an average over the multiple possible microscopic configurations of corresponding enzyme-substrate complexes with the same stoichiometry. By focusing simply on the number of bound substrate molecules without reference to a structurally defined “site,” we are able to deconvolute the critical properties of individual intermediate states.

In summary, we have used the Nanodisc system to form a functionally homogeneous and soluble 1:1 complex of CYP3A4 and CPR incorporated in a nanoscale POPC lipid bilayer. We have shown that the type I spectral changes observed upon TS binding by CYP3A4 are not perturbed by the presence of CPR in the Nanodisc and that CYP3A4 is converted almost completely to the high-spin state when saturated with this substrate. Measurements of the fraction of high-spin shift, the steady-state rate of NADPH consumption, and TS hydroxylation kinetics as a function of substrate concentration under the same conditions provide a basis for a global analysis of these multiple experiments. As a result, we are able to resolve the individual properties of CYP3A4 molecules with one, two, and three TS molecules bound. Binding of the first substrate molecule significantly increases the rate of NADPH oxidation, but virtually no product is formed from this intermediate, with all reducing equivalents shunted to an uncoupled pathway. Binding of the second substrate molecule is critically important for product formation because the rate of TS metabolism reaches maximum value with this state. Even so, almost 95% of NADPH consumption is wasted through unproductive pathways. Binding of the third TS molecule does not further improve the specific rate of product formation, but rather increases the efficiency of redox equivalent usage. Thus, we are able to define the metabolic channels open to each individual CYP3A4 binding intermediate and to document the cooperativity linkages operating in drug metabolism. This methodology can be directly extended to the rich functional landscapes of this and other mammalian cytochromes P450 to help define the precise impact of homotropic and heterotropic aspects of allosteric regulation on clinically important drug/drug interactions.

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