The One Electron Autoxidation of Human Cytochrome P450 3A4*

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Running title: Substrate Modulation of CYP3A4 Autoxidation

Abstract

Monomeric CYP3A4, the most prevalent cytochrome P450 in human liver, can simultaneously bind one, two, or three molecules of substrates and effectors. The difference in the functional properties of such binding intermediates gives rise to homotropic and heterotropic cooperative kinetics of this enzyme. In order to understand the overall kinetic processes operating in CYP3A4, we have documented the kinetics of autoxidation of the oxy-ferrous intermediate of CYP3A4 as a function of testosterone concentration. The rate of autoxidation in the presence of testosterone is significantly lower than that observed with no substrate present. Stability of the oxyferrous complex in CYP3A4 and the amplitude of the geminate CO rebinding increase significantly as a result of binding of just one testosterone molecule. In contrast, the slow phase in the kinetics of cyanide binding to the ferric CYP3A4 correlates with a shift of the heme iron spin state, which are only caused by the association of a second molecule of testosterone. Our results show that the first substrate binding event prevents the escape of diatomic ligands from the distal heme binding pocket, stabilizes the oxyferrous complex and thus serves as an important modulator of the uncoupling channel in the cytochromes P450.

Introduction

Cytochrome P450 3A4 (CYP3A4) is the most prevalent cytochrome P450 in human liver and is responsible for the breakdown of numerous xenobiotics, including more than 50% of the currently marketed pharmaceuticals (1,2). Most cytochromes P450 operate via a catalytic cycle wherein atmospheric dioxygen ligates to the ferrous heme of the P450 protein and is reduced through electron input from pyridine nucleotide oxidation as provided by an associated electron transfer chain. In the case of CYP3A4 this is an 80 kD reductase molecule containing both FAD and FMN prosthetic groups. A central intermediate in this reaction cycle is the ferrous-dioxygen adduct, generated through ferric-ferrous reduction of the heme center and dioxygen binding. This intermediate, the “oxy-complex” can either decay via release of superoxide and regeneration of the ferric heme (3) or be reduced with a second redox equivalent resulting in the cleavage of the oxygen-oxygen bond and generation of a higher valent metal-oxo complex that can initiate radical chemistry and subsequent substrate oxygenation. The former autoxidation pathway represents the first branching point between productive and unproductive pathways in P450 catalysis, wherein reducing equivalents leak into the production of unwanted reduced oxygen species (ROS) (4) (5-7). Subsequent uncoupling channels include the release of the two electron reduced dioxygen as peroxide and a further reduction of the higher valent metal-oxo complex to produce a second water molecule.

The oxy-ferrous intermediate in various cytochromes P450 was documented many decades ago (3,8-11). For the soluble bacterial systems, the autoxidation process was shown to be first order (3,8,9) but for the membrane associated isozymes
was often described by multiphasic processes (11) presumably due to the heterogeneous aggregated state of the protein preparations when placed in aqueous solution. The Nanodisc system (12,13), which consists of ~10 nm diameter discoidal phospholipid bilayers rendered soluble in aqueous solution via an encompassing membrane scaffold protein, provides an ideal system to provide a homogenous, soluble and monomeric P450 CYP3A4 for detailed kinetic investigations.

Recently we reported the influence of substrate binding on the stability of this oxy-ferrous complex in human CYP3A4, using the Nanodisc system to provide a homogeneous and soluble preparation of this integral membrane protein (14). We have shown that the autoxidation rate of the substrate free CYP3A4 is extremely rapid, notably much faster than the steady-state NADPH consumption rates that are measured under the same turnover conditions (14,15). Saturation with various substrates was found to reduce the rate of autoxidation by almost two orders of magnitude, thus inhibiting the unproductive decomposition of the oxy-ferrous intermediate and production of ROS. This work provided important information in terms of the overall efficiency of redox equivalent consumption, or coupling ratio determined as the ratio of the steady-state rates of product formation and NADPH consumption. Analysis of the functional properties of the stoichiometric complex of CYP3A4 with the cytochrome P450 reductase in these steady state experiments of testosterone hydroxylation revealed the distinct fractional contributions of CYP3A4 with different numbers of testosterone molecules bound to metabolism and the total of uncoupling pathways (15). In particular, the rates of NADPH consumption and product formation were shown to be very different for the CYP3A4 with one and two TS molecules bound.

The rapid rate of autoxidation of the ferrous-oxy complex suggested that this pathway was a predominate means for uncoupling in this human hepatic enzyme. In order to better understand the mechanism of the allostERIC regulation of CYP3A4 catalysis and the significance of simultaneous binding of multiple substrate molecules, we have studied the autoxidation of CYP3A4 as a function of substrate concentration. This allowed determining the fundamental one-electron autoxidation rate for each of the functional states of the enzyme with one, two and three molecules of substrate bound. These results demonstrate that although the state with a single testosterone bound is not able to effectively catalyze substrate hydroxylation, it is responsible for a dramatic slowing of the one electron autoxidation rate, thus improving the overall coupling of pyridine nucleotide reducing equivalents. This suggests that the first substrate binding event induces a critical conformational change in the protein that stabilizes the ferrous-oxy complex and could block a superoxide exit pathway from the heme center. In order to explore the possibility of a steric block to ligand egress, we also measured we measured the amplitude of CO geminate rebinding from the carbonmonoxo ferrous complex in CYP3A4, which is a measure of ligand escape after photolysis, as well as the kinetics of cyanide binding to ferric CYP3A4. These experiments provide additional information on the role of the first and second substrate molecule in the modulation of diatomic ligand interactions with the heme iron and provide a consistent picture for the role of substrate binding in the efficient channeling of reducing equivalents to monooxygenation chemistry versus unproductive autoxidation.

Methods

Expression and purification of CYP3A4. Cytochrome P450 3A4 was expressed from the NF-14 construct in the PCWori+ vector with a C-terminal pentahistidine tag generously provided by Dr. F. P. Guengerich as previously described (16). The presence of the histidine-tag has been shown to not perturb the measured turnover parameters of CYP3A4 (17). Heterologous expression and purification from E. coli was carried out using a modified procedure (18) as described in the Supplemental Data contained in (14).

CYP3A4 in Nanodiscs. The application of Nanodisc system for solubilization of integral membrane proteins incorporated into nanoscale bilayers has been described in detail in several publications (18-23). Assembly of human CYP3A4 in Nanodiscs was accomplished using the scaffold protein MSP1D1 with the poly(histidine) tag (24) removed as described previously (18). Briefly, purified CYP3A4 from the E. coli expression system was solubilized by 0.1 % Emulgen 913 and mixed
with the disc reconstitution mixture containing MSP1D1, POPC\(^{1}\) and sodium cholate. A molar mixing ratio of 0.1:1:65:130 (CYP3A4:MSP1D1:POPC:cholate) was chosen to favor formation of monomeric CYP3A4 incorporated into Nanodiscs with the proper stoichiometry of scaffold protein and lipid. Detergents were removed by treatment with Biobeads (BioRad, Hercules, CA), which initiates self-assembly. Purification of the fraction of Nanodiscs with incorporated CYP3A4 was achieved using Ni-NTA affinity column followed by size exclusion chromatography as described in (14). The result of this self-assembly reaction is a monomer of CYP3A4 contained in a discoidal POPC bilayer ~10 nm in diameter stabilized by the encircling amphipathic membrane scaffold protein belt. CYP3A4-Nanodiscs were prepared in substrate free form and kept at 4° C.

Substrate binding. Formation and decay of the oxy-ferrous complex, geminate rebinding of carbon monoxide, and binding of cyanide was studied with CYP3A4 in the presence of different testosterone concentrations as well as in the substrate free form. Testosterone was added using stock solutions in methanol with the final concentration of methanol always less than 1%. Spectral titration data were analyzed using Hill equation \( F = A\cdot S^n/(S^n + S^b) \), where \( A \) is the spectroscopic amplitude, \( S \) is the substrate concentration, and \( S^b \) is the spectral dissociation constant. For autoxidation and geminate rebinding experiments solutions of CYP3A4 in Nanodiscs were deoxygenated under the flow of argon gas and reduced by addition of small excess of anaeroically prepared dithionite solution with the known concentration determined using a molar absorption coefficient \( \varepsilon_{315} = 8.05 \text{ mM}^{-1} \text{ cm}^{-1} \) (25).

Stopped-flow experiments. All kinetic experiments utilized an Applied Photophysics SX.18MV stopped-flow spectrophotometer using typical mixing volumes of 150 µl in each syringe with a dead-time of 1.5 ms at the temperature 6° C. For autoxidation studies, anaerobic solutions of reduced CYP3A4 Nanodiscs (with or without substrate) in one syringe were mixed in 1:1 ratio with buffer saturated with pure oxygen gas containing the same concentration of substrate, if present. The resulting high oxygen concentration (~690 µM after mixing) was chosen in order to accelerate formation of the oxy-complex. Kinetics of cyanide binding was studied by mixing the solution of ferric CYP3A4 in Nanodiscs with solution of KCN (final concentration 20 mM after mixing) with the same concentrations of testosterone present in both syringes. High concentration of KCN was chosen based on the results of equilibrium titration experiments to ensure the pseudo-first order binding. In each kinetic experiment from 400 to 800 spectra were collected on a logarithmic time scale for the slow reactions (total collection time 50 sec) or linear time scale for faster processes (collection time up to 2.3 sec).

Data analysis. The data on autoxidation kinetics were analyzed according to the following reaction scheme:

\[
\text{Fe}^{2+} + O_2 \rightarrow \text{Fe}^{2+} \text{O}_2 \rightarrow \text{Fe}^{3+} + O_2^- (1)
\]

Here \( \text{Fe}^{2+} \) and \( \text{Fe}^{3+} \) denote the reduced (ferrous) and oxidized (ferric) state of CYP3A4 heme iron, \( k_1 \) is the apparent pseudo-first order rate constant of oxygen binding to ferrous cytochrome P450 and \( k_2 \) is the rate constant for autoxidation, i.e. formation of ferric CYP3A4 and release of superoxide. Spectra collected in each kinetic experiment were arranged into a matrix with each column vector representing the spectra, and rows representing the absorption at each wavelength as a function of time. The number of independent spectrally distinguishable components and their time dependent concentrations were determined using singular value decomposition (SVD) (26). The data analysis for substrate free and substrate saturated CYP3A4 was accomplished as described (14). For intermediate concentrations of TS the method was modified due to the presence of two spectral components originating from the high spin (HS) and low spin (LS) fractions of the final oxidized CYP3A4. Because of this, two spectral processes were observed simultaneously, namely the transition of the oxyferrous CYP3A4 to the HS CYP3A4 and the same oxyferrous CYP3A4 to the LS CYP3A4, each with the characteristic difference spectrum and total amplitude corresponding to the fractions of HS and LS states under experimental conditions and at the given TS concentration. After fast formation within 10 – 20 ms, the oxyferrous CYP3A4 in the
presence of intermediate TS concentrations autoxidized through a biphasic process with the
typical apparent first-order rates of ~20 s\(^{-1}\) and ~0.5
s\(^{-1}\). In most cases the slower phase could be better
represented as a bi-exponential decay. Because of
the large difference between the rates of the fast and
slow phase, these two processes could be analyzed
separately with the goal to minimize the number of
fitted parameters and improve the precision.
Calculations of the total fraction of CYP3A4
autoxidized at each kinetic phase was done based on
the absolute difference spectra obtained by
subtraction of the spectra of pure LS and HS
CYP3A4 from the corresponding spectra of
oxyferrous CYP3A4 in the absence of substrate and
in the presence of 200 µM TS (14). Amplitudes of
fast and slow phases of cyanide binding to CYP3A4
at different TS concentrations were analyzed in a
similar way, using molar absorption spectra of
substrate free, substrate bound, and cyanide bound
CYP3A4 measured under the same conditions. For
the analysis of the fast phase with the rate 200 s\(^{-1}\),
the first 16 spectra collected at 2 – 24 ms were used
to calculate the fraction of CYP3A4 which binds
cyanide within this time frame. The slow phase
amplitude was obtained from the spectra collected
between 25 ms and 6 seconds. Total concentration of
CYP3A4 was measured from the end point of each
run using the known spectrum of cyanide complex
of CYP3A4. Fraction of the slow phase was
determined as the ratio of the total amplitude of the
slow phase and the total CYP3A4 concentration.

**Carbon monoxide photolysis and rebinding.**
Flash photolysis of the CO-bound form of P450
CYP3A4 in Nanodiscs was accomplished using the
system previously described (27) with the indicated
modifications. Photolysis was initiated by a 200 mJ
pulse of 532 nm light from a tripled Nd:YAG laser
(Spectra Physics Model GCR 150). The probe beam
is from a Cermax 500W xenon light source (ILC
Technologies) which is heat filtered. For these
experiments a monochromator is placed in front of
the sample to produce a monochromatic probe light
source at the probe wavelength indicated in the text.
A second monochromator between the sample and
the photomultiplier tube reduces the noise due to
scattered laser light. Signals from the Oriel model
77341 photomultiplier were amplified using a
Stanford Research Systems SR445 fast preamplifier
(300 MHz bandwidth) and digitized using a LeCroy
model 9350 oscilloscope (1 Gs/s). Signals were
continuously averaged at a flash repetition rate of
0.5 Hz until an acceptable signal to noise was
obtained, usually 60 to 75 transients. Samples of 1 –
2 µM ferrous CYP3A4 were prepared in 0.8 ml
deoxygenated buffer containing 200 µM sodium
dithionite. The ferrous-CO complex was generated
by the addition of 0.1 ml CO saturated buffer.
Testosterone was added from stock solutions made
in methanol. Absorbance values were typically
measured at 450 nm and normalized to the expected
change in absorbance obtained from the CO
difference spectra from an individually run ferrous-
CO minus ferrous CYP3A4 spectrum. The
normalization results in a plot of fraction dissociated
verses time. Samples were maintained at 6°C for all
experiments and the observed transients fit to a sum
of two exponentials. The resultant geminate yields
were obtained from the value of the fit at times > 4
µs. The dependence of geminate yield on substrate
concentration was fit to the following quadratic
equation (28) (pp. 72-74):

\[
Y = Y_o + \frac{(Y_{max} - Y_o) \times (b - \sqrt{b^2 - 4E_iS_i})}{2E_i}
\]

where:

\[
b = (S_i + E_i + K_d)
\]

and we assume:

\[
\frac{ES}{E_i} = \frac{Y - Y_o}{Y_{max} - Y_o}
\]

Here Y is geminate yield, Y\(_o\) is geminate yield
of substrate free CYP3A4, Y\(_{max}\) is the geminate yield
of substrate saturated enzyme, ES is concentration of
enzyme substrate complex, E\(_i\) is the total enzyme
concentration and S\(_i\) is the total substrate
concentration.

**Results**

**Testosterone binding.** Testosterone binding to
CYP3A4 in Nanodiscs was monitored at a
temperature of 279 K (6°C) using absorption
spectroscopy (300 nm – 700 nm). The resulting
optical spectra of CYP3A4 in the presence of
different testosterone concentrations are shown in
Figure 1 and indicate the gradual shift of the heme iron spin state from the predominantly low spin in the absence of the substrate to approximately 90% high spin at the highest TS concentration 270 μM. Analysis of the fraction of the spin shift as a function of TS concentration using the Hill equation returns a spectral dissociation constant $S_D = 56 \mu M$ and a Hill coefficient of $n = 1.9$, consistent with earlier reported data (29). The high apparent cooperativity of the spin shift is manifested through the relatively minor spectral changes initiated by the first TS binding event while exhibiting an almost complete spin shift following binding of the second TS molecule as was discussed previously (15,29).

**Autoxidation rates.** Our previous results (14) indicate that the rate of autoxidation of CYP3A4 strongly depends on the presence of substrates. For substrate free CYP3A4 the autoxidation is very fast with an apparent first order rate of $\sim 20 \text{ s}^{-1}$ at 278 K. At near saturating concentration of testosterone, the autoxidation rate is slowed to $\sim 0.37 \text{ s}^{-1}$, i.e. approximately 50 times lower (14). In order to better understand the substrate dependent stability of the oxy-ferrous intermediate in the CYP3A4 catalytic cycle we measured the autoxidation rate at different TS concentrations at low temperatures. Rapid mixing of an anaerobic solution of the reduced CYP3A4 Nanodiscs with oxygen saturated buffer with identical concentrations of TS present in both syringes results in fast (within several ms) formation of oxy ferrous complex of CYP3A4 with a broad absorption band at 424 nm, as described (14). The kinetics of subsequent decomposition of this complex was monitored using UV-Vis absorption spectroscopy at 279 K.

Experimental results showing the autoxidation kinetics in the presence of different TS concentrations are shown in Figure 2. Even in the presence of 7 μM TS, the kinetics is clearly biphasic, with an apparent first order rate of the slow phase 1.5 s$^{-1}$ and amplitude of approximately 20%. Further increase of TS concentration results in gradual elimination of the fast phase, which even at lower TS concentrations (60 μM), is resolved only as a minor component (<5%) and is not observed at all at higher concentrations of this substrate. These data are summarized and compared to the equilibrium spectral titration of the ferric CYP3A4 with testosterone under identical conditions at 279 K in Figure 3.

CYP3A4 can bind up to three molecules of testosterone, and the first binding event produces only a minor spin shift (15). Thus, the total high spin fraction of CYP3A4 is due to the contributions from the enzyme with two and three molecules bound. This is reflected in the spectral dissociation constant $S_D = 56 \mu M$ obtained by the Hill analysis. However, from the data in Figure 3 it is clear that the rate of autoxidation becomes significantly slower even at low TS concentrations. Thus, the presence of the first TS molecule bound to the enzyme has a profound effect on the stability of the oxy-ferrous complex, dramatically decreasing the rate of its irreversible decomposition into the ferric CYP3A4 and superoxide. The effect of substrate binding on the autoxidation of heme enzymes has been observed previously ((14) and references therein), and can be attributed to the structural and/or dynamic restrictions on the escape of superoxide from the distal binding pocket of the protein caused by the presence of the substrate and/or an increased stability due to conformational change or alteration of the local dielectric around the heme. In some cases this effect is correlated with the spin shift and redox potential of the heme iron, as described in (30,31), which can also be rationalized through a destabilization of the water ligand to the ferric heme iron (32-34). However, in case of CYP3A4 binding testosterone, such significant effect could not be predicted from the substrate dependent spin shift and reveals the presence of a distinct mechanism controlling the overall stability of the oxy-ferrous complex. The origin of this increased stability could be due to the aforementioned alteration of local dielectric or a direct occlusion of the superoxide escape channel, thereby increasing the “geminate” rebinding of superoxide. Either of these effects could be manifested through a substrate induced conformational change of the enzyme that does not alter the water molecule bound as an axial heme ligand.

**Diatomic Ligand Binding.** In order to explore other measures of active site accessibility and obtain more detailed information we measured the access and escape of other diatomic ligands to CYP3A4. In a first set of experiments to probe the structure of the ferrous enzyme, we studied the amplitude of CO
geminate rebinding from the ferrous carbon-monoxy adduct. Extension to the ferric state of CYP3A4 was realized by quantitating the on-rate for cyanide binding. In both cases, measurements were made as a function of TS concentration in order to probe the reactivity profiles of each state with stoichiometric substrates bound.

**Cyanide association.** Results of the studies of equilibrium binding of cyanide to CYP3A4 at 6°C in the presence of different testosterone concentrations are shown in Figure 4. With no substrate present, CYP3A4 in Nanodiscs binds cyanide with dissociation constant $K_d = 0.4$ mM, while in the presence of 200 μM TS TS cyanide the overall affinity is slightly weaker, with a dissociation constant of 1.4 mM. Thus, binding of testosterone does not dramatically hinder cyanide binding to CYP3A4, certainly much less than that observed with either camphor binding to CYP101 (35) or androstenedione ligating to aromatase (36). The kinetics of cyanide binding to the substrate free CYP3A4 is well described by a single exponential process with observed pseudo-first order rate $k_{obs} = 200$ s$^{-1}$ at a cyanide ion concentration of 20 mM. The second order rate of cyanide binding, $k_{on}$, as well as the dissociation rate from the cyanide complex of the substrate free CYP3A4, $k_{off}$, can be calculated using $k_{obs} = k_{on}[CN^-] + k_{off}$; $K_d = k_{off}/k_{on}$. The binding of cyanide to the substrate free CYP3A4 is well described by the single exponential process with a spectral amplitude more than 90% and the apparent first order rate of ~200 s$^{-1}$, corresponding to a second-order binding rate of 10 mM$^{-1}$s$^{-1}$. A minor slow component with amplitude between five and eight percent of the major peak and a rate approximately 80 fold slower was also usually detected. Both pseudo-first order rates were proportional to the concentration of cyanide, as shown in separate control experiments, confirming the true second-order binding mechanism. From this analysis $k_{on} = 10$ mM$^{-1}$s$^{-1}$ and $k_{off} = 4$ s$^{-1}$ for CYP3A4 in Nanodiscs with no substrate present in the active site. These data are shown in the Table 1 together with the rates of cyanide binding to different heme proteins including several other cytochromes P450.

Addition of substrate results in the gradual decrease in amplitude of the fast phase and an increase in the contribution of the slow process to the overall cyanide binding kinetics. The large difference between the rates of fast and slow phase makes possible separate analysis of the data obtained within the first 25 ms following stopped flow mixing of cyanide with the ferric enzyme. The fast phase process is completed during this time period and at times between 0.03 and 10 seconds only a slow phase is observed. Separate singular value decomposition analysis (SVD) of the time-resolved spectra obtained within indicated time intervals revealed sufficient spectral differences between the fast and slow phases. The difference spectra of the fast phase correspond exclusively to cyanide binding to the low-spin fraction of CYP3A4, while the spectral changes during the slow phase represent the transition from the predominantly high-spin CYP3A4 with addition of a small fraction of the low-spin to the cyanide complex. This distinction between fast and slow processes could be also monitored at the isosbestic points for the corresponding difference spectra shown in Figure 4C, i.e. 420 nm and 426 nm for the cyanide binding to high spin CYP3A4 and low-spin CYP3A4 respectively. Analysis of the kinetic traces extracted at these wavelengths is in complete agreement with the results of SVD analysis of the full spectral matrix. At 420 nm, where the signal is due exclusively to the cyanide binding to a low-spin fraction of CYP3A4, the kinetics is described by a single fast process (apparent first order rate ~200 s$^{-1}$), and almost no absorption changes at time longer than 30 ms. In contrast, at 426 nm the fast process cannot be seen at all, and the kinetics is described by a bi-exponential decay with rate constants of 3 s$^{-1}$ and 0.6 s$^{-1}$.

The kinetics of cyanide binding to the ferric CYP3A4 in the presence of different TS concentrations monitored at 446 nm is depicted at Figure 5 and Table 2. The amplitude of the slow phase was determined from bi-exponential fits of single wavelength kinetic traces extracted at the isosbestic point of the difference spectra shown in Figure 4C, as well as from the fits of the time dependent spectra collected from 370 nm to 500 nm for the difference basis spectra. The latter fits were used to estimate independently the spectra of the fast and slow fractions of CYP3A4, and to confirm that the fast phase is represented by exclusively low spin CYP3A4. Total concentrations of CYP3A4 were determined from the final spectra of cyanide
saturated enzyme in the end of each run, and the fraction of the slow phase was obtained as the ratio of the slow phase amplitude to the total concentration. The results of kinetic experiments of cyanide binding to CYP3A4 at different TS concentrations are summarized in Figure 6. Comparison with the fraction of the high spin fraction of CYP3A4 reveals the similar dependence of the slow phase kinetic amplitude on TS concentration with only minor differences at low TS concentrations. This means that the kinetics of cyanide binding is determined by the spin state of the heme iron, while the diffusional constraints induced by the binding of the first TS molecule to CYP3A4 has almost no effect on the rates of formation of cyano-ferric heme complex. The rate of cyanide binding, however, is much lower for the high spin CYP3A4. The same is true for the dissociation rate of cyanide, which can be calculated from the equilibrium dissociation constant and the binding rate. This result can be attributed to the steric constraints caused by the presence of the TS molecules in the immediate vicinity of the heme iron. As indicated by only a three fold difference between the dissociation constants for cyanide binding to CYP3A4 in the absence of substrate and in the presence of 200 µM testosterone, the TS molecules bound at the active center of ferric CYP3A4 are sufficiently mobile and do not perturb significantly the stability of the distal cyanide ligand. At the same time, for the high spin fraction of CYP3A4, where the substrate destabilizes the distal water ligand, the binding and dissociation kinetics of the stronger ligand cyanide is significantly slower.

**Discussion**

Efficient metabolism by cytochrome P450 systems requires tight coupling between the provision of needed reducing equivalents via pyridine nucleotide oxidation and the utilization of atmospheric dioxygen together with product formation. Alternate non-productive pathways include autoxidation of the one-electron oxygen bound ferrous protein to produce superoxide which is subsequently converted to hydrogen peroxide, as well as the direct release of hydrogen peroxide from a two-electron reduced peroxo intermediate. Previous investigations have suggested that in the case of P450 CYP3A4, a major pathway for uncoupling is from the oxy-ferrous state (14).

In order to better understand the regulation of stability of oxygenated CYP3A4 by substrate binding, we have studied the autoxidation kinetics of this human cytochrome P450 solubilized in monomeric form in Nanodiscs as a function of testosterone concentration. As a result, we have discovered that autoxidation is dramatically slowed, even at low testosterone concentrations, below that required for complete saturation of the enzyme. This is the first experimentally observed indication of the important functional changes of CYP3A4 caused by binding of one testosterone molecule. In addition we have measured the amplitude of CO geminate rebinding following flash dissociation from the ferrous heme as well as the association kinetics of
cyanide binding to ferric CYP3A4 in order to compare the changes caused by TS binding with the results obtained in the autoxidation studies. Both methods are commonly used as sensitive probes of the structural and dynamic effects caused by mutations and by interactions with substrates and cofactors in various heme enzymes (37-48) (35,49-59).

As we recently described (15,60), the dependence of overall functional properties of CYP3A4 on testosterone concentration can be represented as a sum of contributions of binding intermediates, i.e. the populations of binary, ternary, and quaternary complexes of CYP3A4 with testosterone, [CYP3A4 – TS], [CYP3A4 – (TS)₂], and [CYP3A4 – (TS)₃]. We have found that binding of the first TS molecule leads to relatively small spin shift and a four fold acceleration of NADPH consumption, but no product formation, the latter result explaining the relatively high overall cooperativity of substrate turnover in this system. The binding of the second TS molecule is crucial for the formation of fully active enzyme – substrate complex, while the third binding event significantly improves efficiency of NADPH consumption with no further increase of the rate of substrate turnover. Clearly, multiple TS molecules play substrate and effector roles at the same time, modulating the functional properties of ternary and quaternary enzyme-substrate complexes. It is important to document which reaction steps in the catalytic cycle are affected by sequential substrate binding events, and what chemical mechanism is responsible for these functional differences between the binding intermediates.

The autoxidation of CYP3A4 is very fast, compared to the overall steady state product forming kinetics (14) and thus may be a major branching point for the productive and unproductive paths in the catalytic cycle of this cytochrome P450. We describe in this work the kinetics of autoxidation as a function of TS concentration and show that the oxy-ferrous intermediate in CYP3A4 is significantly stabilized by the binding of the first substrate molecule. The magnitude of this effect clearly does not correlate with the minor high spin fraction measured under the same conditions but rather reflects an increased stability of the oxy-ferrous complex due to alteration of the local dielectric environment, formation of a significant steric and/or dynamic restriction for the escape of superoxide anion from the heme distal pocket caused by the bound TS molecule or a protein conformational change linked to either of these processes. The formation of a steric block following the binding of a single TS molecule is confirmed by measurement of the amplitude of CO geminate rebinding, which is also dramatically increased at low TS concentrations. Both results indicate that not only the equilibrium properties of CYP3A4, but also the kinetics of interaction with diatomic ligands are modulated by the presence of TS in a complicated and non-trivial way.

The goal of the current study was to probe the kinetic properties of CYP3A4 at different redox and ligation states in the presence of different concentrations of testosterone. The changes of autoxidation kinetics provide information about the influence of substrate binding to CYP3A4 on the stability of the oxy-ferrous complex and the rate of superoxide escape from the distal pocket in the resultant oxidized ferric protein. Clearly the first TS binding event stabilizes the oxy-ferrous complex. Though via water displacement upon substrate binding, the a more non-polar environment near the heme iron may hinder the separation of charge occurring in the autoxidation reaction. In addition, substrate binding may increase the effective “geminate yield” of superoxide release by blocking an escape pathway. In order to probe the active site accessibility in the ferric state, the kinetics of cyanide binding was also measured as a function of TS concentration and compared to the geminate rebinding of carbon monoxide as a characteristic of the ferrous CYP3A4 conformation. We note a strong similarity between the changes of the fraction of the slow phase in autoxidation kinetics and the amplitude of geminate CO rebinding. A sharp increase of the geminate rebinding amplitude and of the fraction of the slow phase in autoxidation kinetics at low TS concentrations suggests that the escape of diatomic heme ligands, superoxide and carbon monoxide, is significantly hindered by the first testosterone molecule bound to CYP3A4. This conclusion is based on the large difference between these properties and the high spin fraction measured under the same conditions, Figures 6 and 7. As has been documented, the shift of CYP3A4 spin state is caused by binding of the second TS molecule (15)
and hence this is an effect occurring with only a single TS molecule bound at the active site.

The substrate access to and escape from the active center of cytochromes P450 was studied by analysis of available X-ray crystal structures and molecular dynamics, as reviewed in (61,62). Molecular dynamics has also been used to probe the pathways to and from the heme distal pocket of cytochromes P450 which can be accessed by small molecules, water for several human enzymes (63), and CO for CYP101 (64). All of these studies identify several pathways which can be either found in the X-ray structures, or open and close transiently. Thus, the ability of the first TS molecule to hinder the escape of superoxide and carbon monoxide from CYP3A4 is in part unexpected, because it is difficult to visualize a single substrate molecule blocking multiple paths at the same time in the absence of a major conformational change in the enzyme induced by the first TS binding event. Nevertheless, it is still possible that pathway may dominate for the escape of CO and superoxide and this is blocked by the first TS bound. If the escape rates of these ligands through other pathways are much lower, the overall observed kinetics may mostly depend on the positioning of the substrate molecule at the main escape pathway. In addition, the dynamics of the whole CYP3A4 molecule may change as a result of the first TS binding, and the escape through other pathways, even if not blocked directly, may become much slower in the presence of substrate. A third possibility, which can be eliminated, is an unusual linkage between the sequential binding of the three testosterone molecules and the redox state of the CYP3A4 heme. Recent results in our laboratory suggest that testosterone binding significantly alters the observed redox potential of CYP3A4 and that this shift is directly correlated with the ferric spin state of the heme iron (Das, Grinkova and Sligar, unpublished results). Hence, the difference observed in the effect of testosterone concentration on the autooxidation of the oxy-ferrous intermediate as compared to the modulation of the ferric spin state equilibrium suggests that the tightest binding TS molecule can alter the flux through this uncoupling pathway through direct steric effects or a change in the global conformational dynamics of the enzyme.

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Footnotes

1 The abbreviations used are: HS, high spin; LS, low spin; POPC, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine; SVD, singular value decomposition; TS, testosterone.

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Figure 1. Absorption spectra of CYP3A4 in Nanodiscs in the presence of increasing testosterone (TS) concentration. All measurements were made at 6°C to slow the autoxidation process. Concentrations of TS are 0, 15, 29, 44, 59, 88, 117, 146, and 204 µM. The Soret band at 417 nm indicates low spin CYP3A4 in the absence of TS, and at 393 nm the predominantly high spin CYP3A4 observed at higher substrate concentrations. Every other spectrum is shown for clarity. Inset: The fraction of ferric spin shift induced upon TS binding (dots) and the analysis of this data using the Hill equation (dashed line) with the following parameters: SD = 56 µM, n = 1.9, and a spectral amplitude of 1.087. See (7) for complete discussion of this analysis.

Figure 2. Autoxidation kinetics of CYP3A4 in the presence of increasing testosterone concentrations. Experimental points are shown as the solid circles, with lines illustrating the results of the analysis according to a bi-exponential decay process (See text). Concentrations of TS are: (1) 7 µM; (2) 20 µM; (3), 35 µM; (4), 60 µM; (5), 100 µM; (6) 200 µM. The analysis parameters are summarized in Table 2.

Figure 3. Fraction of the slow phase observed in the autoxidation kinetics (circles) and the high spin fraction of CYP3A4 (triangles) as a function of testosterone concentration.

Figure 4. Titration of CYP3A4 with cyanide in the absence of substrate (A) and in the presence (B) of 200 µM testosterone. Concentrations of cyanide are: (A) 0, 0.17, 0.33, 0.5, 1, 2, 3, 5, 21, and 36 µM; (B) 0, 0.17, 0.33, 0.5, 1, 2, 3, 4.5, 7.8, 16, and 40 µM. Panel (C) shows difference spectra for cyanide binding by CYP3A4 in the absence of substrate (predominantly low spin CYP3A4) and in the presence of 200 µM testosterone (predominantly high spin CYP3A4).

Figure 5. Kinetics of cyanide binding to CYP3A4 at different testosterone concentrations as monitored at the isosbestic point of the difference spectra shown in Figure 4C (446 nm). Closed circles are the experimental data normalized to the fraction of the slow phase using the
differential extinction of 47 mM$^{-1}$cm$^{-1}$ while the lines illustrate the corresponding biexponential fits (parameters in Table 2). Concentrations of TS are: (1) 15 μM; (2) 35 μM; (3) 50 μM; (4) 70 μM; (5) 100 μM; (6) 200 μM.

**Figure 6.** Fraction of the slow phase process observed in the stopped flow cyanide binding (triangles) and high spin fraction of CYP3A4 (circles) as a function of testosterone concentration.

**Figure 7.** CO geminate yield as a function of TS concentration. The data are fit to a simple one site binding isotherm. Inset: representative geminate rebinding traces: (A) substrate free, (B) 110 μM TS (See Methods).
Figure 1
Figure 2
Figure 3
Figure 4

![Graph showing absorption spectra with wavelength in nanometers on the x-axis and absorption on the y-axis. The graph is divided into three sections labeled A, B, and C, each showing different absorption patterns.](http://www.jbc.org/Downloaded from)
Figure 5

[Graph showing the relationship between time and slow phase fraction]
Figure 6
Table 1. Kinetic parameters of cyanide binding and dissociation from CYP3A4 and other heme proteins

| Heme protein       | Conditions         | $k_{on}$, mM$^{-1}$s$^{-1}$ | $k_{off}$, s$^{-1}$ | $K_d$, mM | Reference |
|--------------------|--------------------|-----------------------------|---------------------|----------|----------|
| CYP3A4, -sub       | 279 K, pH 7.4      | 10                          | 4                   | 0.4      | This work |
| CYP3A4 + TS        | same               | 0.13                        | 0.18                |          | (35)     |
| CYP101, -sub       | 293 K, pH 7.9      | -                           | -                   | 1.4      |          |
| CYP101+sub         | 298 K, pH 7.3      | -                           | -                   | 0.12     |          |
| CYP102A2, -sub     | 303 K, pH 7.4      | -                           | -                   | 4.7      |          |
| CYP102A3, -sub     | 292 K, pH 7.0      | -                           | -                   | 3.1      | (49)     |
| P450$\text{RR1}$  |                    |                             |                     | 2.8      |          |
| Chloroperoxidase   | 298 K, pH 3 – 7    | 52                          | 5                   | 3.6      | (50)     |
| eNOS, -BH$_4$, -sub| 297 K              | 1.8                         | 22                  | 5.6      | (54)     |
| eNOS, -BH$_4$, +sub|                   | 0.005                       | 0.2                 | 40       |          |
| PGHS               | 277 K              | 2.8                         | -                   | 0.065    | (53)     |
| TXAS               | 290 K, pH 7.4      | 2.8                         | -                   | 0.1      | (52)     |
| HRP                | 298 K, pH 6 – 8    | 0.0024                      | 0.11                | 7.3      | (58)     |
| LPO                | 298 K, pH 7.5      | 100                         | -                   | 0.029    | (55)     |
| CCP                | 298 K, pH 7.5      | 110                         | 0.46                | 0.004    | (57)     |
| KatG               |                    | 480                         | -                   | 0.016    | (59)     |

Abbreviations used in the Table: eNOS, endothelial nitric oxide synthase; BH$_4$, tetrahydrobiopterin; PGHS, prostaglandin H synthase; TXAS, Thromboxane synthase; HRP, horseradish peroxidase; LPO, lactoperoxidase; CCP, cytochrome c peroxidase; KatG, catalase-peroxidase.
Table 2. Kinetic parameters of autoxidation of and cyanide binding to CYP3A4 at different testosterone concentrations

| Testosterone, µM | Fast phase Rate, s\(^{-1}\) (fraction) | Slow phase Rate, s\(^{-1}\) (fraction) |
|------------------|-------------------------------------|-------------------------------------|
|                  |                                      |                                      |
|                  | **Autoxidation**                     |                                      |
| 7                | 28 (0.79)                            | 1.5 (0.21)                          |
| 20               | 19 (0.42)                            | 1.0 (0.58)                          |
| 35               | 11 (0.25)                            | 0.86 (0.75)                         |
| 60               | -                                   | 0.90 (0.51)                         |
|                  |                                      | 0.35 (0.49)                         |
| 100              | -                                   | 0.60 (0.68)                         |
| 200              | -                                   | 0.27 (0.32)                         |
|                  |                                      | 0.5 (1.00)                          |
|                  |                                      |                                      |
|                  | **Cyanide binding**                  |                                      |
| 0                | 220 (0.96)                           | 2.5 (0.04)                          |
| 15               | 210 (0.83)                           | 4.0 (0.07)                          |
|                  |                                      | 0.30 (0.10)                         |
| 35               | 220 (0.70)                           | 3.9 (0.15)                          |
|                  |                                      | 0.40 (0.15)                         |
| 50               | 230 (0.55)                           | 3.6 (0.20)                          |
|                  |                                      | 0.46 (0.25)                         |
| 70               | 215 (0.40)                           | 3.1 (0.28)                          |
|                  |                                      | 0.45 (0.32)                         |
| 100              | 238 (0.30)                           | 2.9 (0.30)                          |
|                  |                                      | 0.44 (0.40)                         |
| 200              | 250 (0.10)                           | 2.30 (0.40)                         |
|                  |                                      | 0.42 (0.50)                         |
The one electron autoxidation of human cytochrome P450 3A4
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