The Ferrous-Dioxygen Intermediate in Human Cytochrome P450 3A4: Substrate Dependence of Formation and Decay Kinetics *

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Running title: Oxy-complex of CYP3A4 in Nanodiscs

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The oxy-ferrous complex is the first of three branching intermediates in the catalytic cycle of cytochrome P450, at which the total efficiency of substrate turnover is curtailed by the side reaction of autoxidation. For human membrane bound cytochromes P450 the oxy-complex is believed to be the primary source of cytotoxic superoxide and peroxide, although information on the properties and stability of this intermediate is lacking. Here we document stopped-flow spectroscopic studies of the formation and decay of the oxy-ferrous complex in the most abundant human cytochrome P450 (CYP3A4) as a function of temperature in the substrate-free and substrate bound form. CYP3A4 solubilized in purified monomeric form in nanoscale POPC bilayers is functionally and kinetically homogeneous. In substrate-free CYP3A4 the oxy-complex is extremely unstable with a half-life ~30 ms at 5°C. Saturation with testosterone or bromocriptine stabilizes oxy-ferrous intermediate. Comparison of the autoxidation rates with the available data on CYP3A4 turnover kinetics suggests that the oxy-complex may be an important route for uncoupling.

Cytochrome P450 CYP3A4 is the most prevalent P450 monooxygenase in human liver and is responsible for the metabolism of almost half of xenobiotics encountered by man (1). This isozyme has a large and flexible active site, and is able to bind and catalytically convert multiple substrates, often displaying homotropic and heterotropic cooperativity in substrate binding and product formation. As a central player in human drug metabolism, CYP3A4 is one of the most intensely studied P450s, either in isolated human liver microsomes (2-5), detergent solubilized form (6) or purified soluble aggregates (7,8). Recently, two groups have reported the crystal structure of a truncated CYP3A4 (9,10). Despite this structural information, precise chemical and biophysical characterization of the human P450s has lagged that of the monomeric, soluble P450 isozymes isolated from bacteria. In particular, the critical intermediates in the reaction cycle of human P450s have not been precisely documented, due in part to the inability to form a robust, monomeric and soluble entity that is amendable to the rapid reaction and spectroscopic methodologies successfully applied to the bacterial P450s. This lacuna is significant, as CYP3A4 and other human P450s display complex aspects of substrate recognition and catalytic mechanism which are not present in the simpler P450s such as CYP101 from Pseudomonas.

The current version of the catalytic reaction cycle of cytochrome P450 monooxygenases involves intermediate reduction states of heme iron and atmospheric dioxygen and represents the result of over thirty years of intensive research (11,12). Traditionally, the cyclic process begins with the ferric low spin species and a water molecule occupying the sixth coordination site of the heme. In many cases the complementary fit of the native substrate into the pocket displaces this water ligand allowing the system to assume a predominantly high-spin electronic configuration, although in some instances substrates may induce only partial spin shift (13,14). Electron transfer from a redox
partner reduces the iron to the ferrous form which
then can bind atmospheric dioxygen. The resultant
ferrous-oxy complex in the P450 cytochromes is
analogous to that of oxygenated hemoglobin and
myoglobin. However, in the case of the P450s, the
axial thiolate ligand and the facile interaction with
redox partners allows a second electron input to
elicit dioxygen bond scission, the production of
water and generation of an active heme-oxidant
capable of substrate functionalization. Thus, the
ferrous dioxygen intermediate is the key juncture
between reversible dioxygen transport and
oxygenase metabolism.

A major step in the understanding of P450
mechanism came in the early 1970s when two
groups observed the ferrous-oxy state in the P450
CYP101 camphor hydroxylase from Pseudomonas
putida (15,16). Similar experiments carried out in
the Coon laboratory with P450LM2 and P450LM4
were less successful, due to the relative instability
of this state and the aggregate nature of these
purified preparations (17). In the ensuing thirty
years, despite the importance of this intermediate
in P450 catalysis, the observation and
characterization of oxy-ferrous intermediate in
mammalian cytochromes P450 has been
problematic. In several cases the oxy-complex was
reported with heterogeneous kinetic properties,
which was variously attributed to an essential
complex behavior of the enzyme, to the presence
of multiple populations of aggregated forms
of P450, or both. This ambiguity leaves unanswered
many mechanistic questions about the specific
roles of different substrates as determinants of
cytochrome P450 kinetics and in the degree of
coupling of redox equivalent consumption to
product formation. Guengerich and co-workers
have published several important works in the
initial characterization of the rate limiting steps of
CYP3A4 catalysis, together with detailed kinetic
modeling (2,18).

In this communication, we make use of a
self-assembled nanoscale phospholipid bilayer to
stabilize human CYP3A4 as a soluble, robust and
monomeric entity. In this system, the cytochrome
is present in its native membrane bound
configuration, and the robustness of the
monodisperse preparation allows rapid mix
stopped flow measurements. We report here the
isolation and detailed spectral characterization of
the oxy-ferrous state of CYP3A4, document the
dependence of the autoxidation rate on the
substrate presence and analyze these kinetics in
terms of a model of redox coupled dioxygen and
superoxide interaction with the ferric/ferrous
hemoprotein.

**EXPERIMENTAL PROCEDURES**

*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 (19). The presence of His-tag
does not perturb turnover parameters of CYP3A4
(20). Heterologous expression and purification
from *E. coli* was carried out using a modified
procedure (21) as described in the Supplemental
Data. CYP3A4-Nanodiscs were prepared in
substrate free (SF) form and kept at 4°C.

The application of Nanodisc system for
solubilization of integral membrane proteins
incorporated into nanoscale bilayers has been
described in detail in several publications (21-25).
Assembly of human CYP3A4 in Nanodiscs was
accomplished using the scaffold protein MSP1D1
with the poly(histidine) tag (26) removed as
described previously (21). Briefly, purified
CYP3A4 from the *E. coli* expression system was
solubilized by 0.1 % Emulgen 913 and mixed with
disc reconstitution mixture containing MSP1D1,
POPC and sodium cholate present in 1:65:130
molar ratios. Detergents were removed by
treatment with Biobeads (BioRad, Hercules, CA),
which initiates self-assembly. 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 (see Supplemental
Data).

*Substrate binding* - Formation and decay of the
oxy-ferrous complex was studied for CYP3A4
saturated with testosterone (TS, 200 µM) or
bromocriptine (BC, 3 µM) and in the substrate free
form. Substrates were added using stock solutions
in methanol with the final concentration of
methanol always less than 1%. Representative
eamples of spectral titration of CYP3A4
Nanodiscs with TS was published earlier (21) and
with BC is shown in Fig. 2S (Supplemental Data).
For both substrates, a spin conversion higher than
90% was reached at saturating concentrations,
confirming the structural and functional homogeneity of CYP3A4 in Nanodiscs (21), and in agreement with the recent study of reduction kinetics of CYP3A4 (27).

Solutions of CYP3A4 in Nanodiscs were deaerated under the flow of argon gas and reduced by addition of small excess of anaerobically prepared dithionite solution with the concentration determined using a molar absorption coefficient $\varepsilon_{315} = 8.05 \text{ mM}^{-1}\text{cm}^{-1}$ (28). Complete reduction was confirmed by anaerobic absorption spectroscopy.

**Stopped-flow experiments.** All stopped flow 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. 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. Replicate experiments were completed at different temperatures from 5°C to 37°C. In each 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 1 sec). The integrity of CYP3A4 Nanodisc after stopped-flow mixing was tested in separate experiments by size-exclusion HPLC, and the samples appeared indistinguishable from those before the stopped-flow experiments.

**Data analysis** - The resulting data were analyzed according to the following reaction scheme:

$$\text{Fe}^{2+} + \text{O}_2 \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \text{Fe}^{3+} + \text{O}_2^-$$

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. In most experiments oxygen binding was complete within the dead time of the instrument and $k_1$ could only be approximated. In case of CYP3A4 saturated with BC at low temperatures, however, formation of the oxy-complex was slow enough to resolve as a separate process completed during the first 30 – 40 ms after mixing.

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) (29). For substrate free CYP3A4 as well as for the experiments conducted in the presence of TS, only two spectral components corresponding to the spectra of oxy-ferrous and the final ferric P450 were derived from the spectral data sets. In the case of CYP3A4 saturated with BC, the initial oxygen binding was slower than in other samples, and the third component, the initial spectrum of deoxy ferrous CYP3A4 was also possible to discern. The spectra of pure species and rate constants were also calculated using the program SPECFIT (Spectrum Software Associates, Marlborough, MA).

**RESULTS AND DISCUSSION**

The ferrous-oxy complex of the cytochromes P450 is at the critical bifurcation point between a commitment to oxygenase catalysis and simple reversible dissociation of atmospheric dioxygen. This transient intermediate was isolated and characterized in the microbial P450 CYP101 from *Pseudomonas* by spectroscopic methods (30-33) and X-ray crystallography (34). Similar structural and functional characterization of the ferrous-oxy complex in mammalian cytochromes P450 has met with significant frustrations due to the membrane bound nature of these monooxygenases and the difficulty in generating a monomeric and monodisperse solution preparation for biophysical investigations.

Using the Nanodisc self-assembled phospholipid bilayer system (22), we incorporated human CYP3A4 into a homogeneous preparation and characterized the fundamental substrate binding isotherms. With both substrates used in this work the spin conversion upon saturation was
higher than 90%, indicating functional homogeneity in equilibrium titration experiments.

Figure 1A shows spectra and decay kinetics of the oxy-complex of CYP3A4 in Nanodiscs without substrate present. The spectrum of the oxy-complex shows a Soret band at 418 nm and a single broad maximum at 552 nm in the visible region. This spectrum looks very similar to the spectra of bacterial oxygenated cytochromes P450 (15,35,36). The rate of autoxidation of substrate free CYP3A4 in Nanodiscs is very high, $k=20 \text{ s}^{-1}$ at $5^\circ \text{C}$ and approximately $140 \text{ s}^{-1}$ at $29^\circ \text{C}$. Because of extremely fast decomposition of the oxy-complex it was impossible to measure the rate of this reaction at physiological temperature $37^\circ \text{C}$. This means that, even if reduction of the substrate free CYP3A4 is possible in vivo, the oxy-complex, once formed, would decompose much faster than the subsequent steps of enzymatic catalysis. Such fast autoxidation reactions have not been reported previously for other cytochromes P450 (Table 1).

The results of stopped-flow experiments with CYP3A4 saturated with TS are shown in Figure 1B. The oxy-complex is formed within several milliseconds after mixing a deoxygenated solution of reduced CYP3A4 with oxygen. Even at low temperatures, e.g. $6^\circ \text{C}$, the apparent first order rate of oxygen binding could only be estimated as $350 - 400 \text{ s}^{-1}$, corresponding to a second-order rate of $5 \times 10^5 \text{ M}^{-1} \text{s}^{-1}$. At higher temperatures, oxygen binding is completed even faster and could not be measured with the current experimental methodologies. The spectra of the oxy-complex of CYP3A4 saturated with TS shows a broad Soret band with a Soret maximum at 424 - 425 nm, which is red-shifted as compared to the 418 – 420 nm values observed for the oxy-complex of other cytochromes P450 and resembles the spectra of oxygenated nitric oxide synthase (NOS) and chloroperoxidase, which also have thiolate as a proximal ligand to the heme iron. This shift may be caused by the specific perturbation of the heme and/or dioxygen ligand by substrate molecule bound in the active site of CYP3A4, as was suggested recently for NOS (37-39). The shape of absorption spectra of the CYP3A4 oxy-complex with TS bound and the positions of the main absorption bands do not depend on temperature in the range from $6^\circ \text{C}$ to $37^\circ \text{C}$.

Similar results were obtained with CYP3A4 saturated with BC. Representative spectra of the CYP3A4 oxy-complex with BC bound are shown in Figure 1C. For these samples oxygen binding was slower by an order of magnitude, with the apparent first-order binding rates ranging from $24 \text{ s}^{-1}$ at $6^\circ \text{C}$ to $300 \text{ s}^{-1}$ at $37^\circ \text{C}$ (data not shown). At all temperatures the spectra of the oxy-complex look very similar, with the Soret maximum at 420 nm.

Once formed, the oxygenated CYP3A4 quickly autoxidizes via a single exponential process with no spectrally distinguishable intermediates. SVD analysis of experimental data shows that all data sets can be described with high precision with only two basis spectra corresponding to oxygenated and high-spin ferric CYP3A4. At the lowest temperatures, several spectra measured at first 10 ms with TS, and within first 20 – 120 ms with BC were omitted from such analysis, because they revealed the presence of ferrous CYP3A4 before oxygenation was completed.

Kinetics of autoxidation of substrate free CYP3A4 and of CYP3A4 in the presence of substrates at different temperatures is shown in Figure 2 together with the corresponding fits to the single-exponential decay. Careful analysis of the data obtained for CYP3A4 in the presence of 3 µM BC reveals the presence of the second process with typical amplitude of $15 - 20\%$ at all temperatures. This second process can be tentatively attributed to the presence of several conformers of CYP3A4 and/or multiple BC binding modes which are in slow equilibrium on the time scale of our experiments. Since the major fraction of autoxidation kinetics could be approximated reasonably well with a single-exponential decay, we used the same simple model to fit the data obtained with BC to compare the influence of two different substrates on the autoxidation rates and to calculate the activation parameters of this reaction.

Both substrates were found to significantly stabilize the oxy-complex against autoxidation. With BC this effect is more pronounced, the rates of spontaneous decomposition of oxygenated CYP3A4 vary from $0.12 \text{ s}^{-1}$ at $6^\circ \text{C}$ to $2.6 \text{ s}^{-1}$ at $37^\circ \text{C}$. With TS the rates at the same temperatures are $0.37 \text{ s}^{-1}$ and $20 \text{ s}^{-1}$, i.e. 3 – 8 times higher. The stabilizing effect of the substrate on oxy-complex in CYP3A4 is slightly more pronounced with BC and is the same with
TS as compared to that in CYP101 with camphor (16). The kinetics of autoxidation of heme proteins at high oxygen concentration is determined mostly by the rate of direct escape of superoxide or hydroperoxyl radical from the binding pocket (40-42), although the experimentally observed rate of decay of the oxy-ferrous complex may also depend on possible nucleophilic ligand displacement (42-44). The differences observed between the autoxidation rates measured in CYP3A4 saturated with BC versus TS imply that the presence of substrates could modulate the escape of superoxide from the distal binding pocket of the protein. The rates of autoxidation measured at 24°C, 3.6 s⁻¹ for TS and 0.84 s⁻¹ for BC, are of the same order as the dissociation rates of these substrates, 20 s⁻¹ and 0.25 s⁻¹ at 23°C (45). For substrate free protein, autoxidation at 6°C is 60 times faster than with TS, and more than 150 times faster than with BC. However, autoxidation is very fast, even in case of substrate saturated CYP3A4, and is much faster than in other cytochromes P450. This suggests that autoxidation may be a key factor which determines the uncoupling behavior and production of toxic superoxide by cytochromes P450 in humans.

The temperature dependencies of autoxidation rates were analyzed according to the Eyring equation in order to derive the activation enthalpies and entropies of this reaction in the absence and in the presence of substrates (46). For CYP3A4 we obtained similar free energies of activation (Figure 3) to those measured for CYP101 (16) and CYP11A1 (47,48). The mechanism of autoxidation in CYP3A4 is likely the same, but the rates are faster by two to three orders of magnitude both with and without substrate. Analysis shows that the activation entropy provides a more significant contribution to the temperature dependence of autoxidation rate measured for TS saturated CYP3A4 than for the BC-CYP3A4 complex. This may indicate the difference in the active site volume available to the superoxide prior to its escape from the interior of the protein molecule.

In well characterized systems such as CYP101 (16,35) and CYP102 (49-51), the overall stability of the oxy-complex is known to depend on presence of the substrate. However, in these bacterial cytochromes P450, the consumption of pyridine nucleotid e is effectively coupled with product formation, and this first branch point does not contribute significantly to the overall uncoupling because the overall turnover rates are typically 10 – 40 s⁻¹, i.e. much faster than the autoxidation rates of the oxy-complex, which are in the range of 0.002 – 0.05 s⁻¹ in these enzymes. On the contrary, product formation in human cytochromes P450 is usually much slower, 0.02 – 0.25 s⁻¹, and significantly lower than the rates of spontaneous decomposition of oxy-complex in CYP3A4 measured in this work. The essential feature of these P450s is the overall high degree of uncoupling, which though well established in general, but usually is not assigned to any specific step of P450 cycle (see (52) for recent review).

Our results provide new information on the properties of oxygenated CYP3A4 solubilized in monomeric form in a POPC bilayer. We show that CYP3A4 in Nanodiscs is monodisperse and kinetically homogeneous with respect to formation and oxidative decomposition of oxy-ferrous complex. Without substrate, the oxy-complex in CYP3A4 autoxidizes very quickly and returns to the ferric low spin state. Saturation of CYP3A4 with TS or BC significantly stabilizes this oxy-ferrous state. This effect is similar to the pronounced stabilization of other cytochromes P450 by their substrates against autoxidation, and together with the dramatic acceleration of reduction rate by the same substrates, it may be attributed to a positive shift of redox potential caused by substrate binding to cytochromes P450.

The possibility of essential uncoupling of NADPH consumption by microsomal cytochrome P450 through the direct decomposition of oxy-ferrous complex and dependence of this process on the enzyme saturation by substrate has been suggested previously (53). For most of the well characterized cytochromes P450, the autoxidation rates are lower than the total rate of pyridine nucleotide consumption and the rate of catalytic turnover. For the case of CYP3A4 in the absence of substrate, however, the oxy-complex decomposes extremely quickly and likely is not stable enough to be effectively reduced to the p peroxy-level as required for monoxygenase catalysis. The latter kinetic selection may serve as an additional mechanism of regulation of NADPH consumption through coupling with the substrate binding, together with the known thermodynamic
trigger, spin shift and acceleration of the first reduction rate (54).

Further experimental studies focused on other reaction steps are needed to complete the kinetic characterization of the full enzymatic cycle of CYP3A4 and to elucidate the relative roles in total uncoupling at three branching intermediates, namely the oxy-ferrous, peroxo- and hydroperoxo-ferric, and ferryl-oxo heme complexes. The kinetic partitioning between the productive pathway and abortive decay of these intermediates critically depends on their stability as well as on the efficiency of the electron transfer from cytochrome P450 reductase and possibly other components, for example, cytochrome b5. The overall results of such in vitro studies depend on the stoichiometry of all proteins in the reconstituted system as well as on other factors. To our knowledge there is no available data on the rate of the reduction of oxy-ferrous intermediate for CYP3A4 or any other mammalian membrane bound cytochrome P450. However, the rate of spontaneous oxidative decomposition documented in this study for the oxygenated CYP3A4 saturated with TS, 20 s\(^{-1}\) at the physiological temperature 37\(^{\circ}\)C, can be used as the reference value for the estimation of the efficiency of the second electron transfer and for the uncoupling ratio at the first branch point. If the reduction of the oxygenated CYP3A4 is also 20 s\(^{-1}\) at the same conditions, the uncoupling due to autoxidation will be at least 50%. If it is slower, the major fraction of redox equivalents from reductase will be consumed through this autoxidation pathway with concomitant release of superoxide. This latter option is in agreement with the direct comparison of NADPH oxidation rate and superoxide production by human microsomes enriched in CYP3A4 (4).

In summary, we report the formation and decay of the oxy-complex in CYP3A4 with and without bound substrates. The use of Nanodiscs for solubilization and isolation of monomeric functional CYP3A4 incorporated in POPC bilayer provides a stable and highly homogeneous enzyme preparation. Previous work (21) noted that CYP3A4 in Nanodiscs undergoes almost 100% conversion upon saturation with TS at 293 K. In this work the same complete conversion to the high spin state was obtained upon saturation with TS and BC at the physiological temperature range. Oxygen binding to the reduced CYP3A4 in stopped-flow experiments generates a transient oxy-complex, also with 100% yield, and autoxidation of CYP3A4 with and without substrate is well described by single exponential decay. In all cases the final reoxidized protein remains fully functional.

These results suggest that the autoxidation is a very important process for the overall uncoupling reactions of human liver cytochromes P450. The life-time of the oxy-complex in the absence of substrate is negligible as compared with the NADPH consumption rate measured for substrate free CYP3A4 (2,6,45) and with the total turnover rate. For the substrate bound CYP3A4 autoxidation is also fast, and with TS bound at 310 K, it is much faster than turnover measured in vitro (55-58). Our results suggest that the oxy-ferrous intermediate of the P450 cycle may be the most important natural determinant of CYP3A4 activity and human drug metabolism. It represents the first branching point between the productive pathway of the substrate turnover and the abortive decay with the formation of superoxide. The coupling of redox equivalent consumption with the substrate turnover at this step of P450 catalytic cycle is determined by the lifetime of the oxy-complex which must be sufficiently stable to permit the second electron transfer from P450 reductase. The presence of substrate at this step is critically important for stabilization of oxygenated CYP3A4, for the efficient use of redox equivalents and for minimization of the side reaction generating toxic superoxide and peroxide. These observations help to understand the multiple regulatory roles of substrates in the physiological functioning of CYP3A4.

**Supplemental Data:** Preparation and characterization of monomeric CYP3A4 Nanodisc, representative BC spectral titration of CYP3A4, and a brief description of the Eyring analysis are provided.

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**FOOTNOTES**

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1 The abbreviations used are: BC, bromocriptine; membrane scaffold protein D1; POPC, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine; SF, substrate free; SVD, singular value decomposition; TS, testosterone.

**FIGURE LEGENDS**

Fig. 1. Time-dependent spectra of oxygenated CYP3A4 showing gradual autoxidation and formation of ferric cytochrome P450 at T= 6° C. Arrows show the direction of spectral changes from ferrous-oxy to ferric CYP3A4. (A) Autoxidation of CYP3A4 (Soret maximum at 420 nm) and formation of low-spin ferric CYP3A4 (Soret maximum at 417 nm) in the absence of substrate. The indicated spectra were acquired at 4.5 ms, 17 ms, 34 ms, 54 ms, 74 ms, 102 ms, 153 ms, and 255 ms after mixing ferrous CYP3A4 (1.0 µM final concentration) with oxygen saturated buffer (oxygen concentration is 690 µM after mixing). (B) Autoxidation of CYP3A4 (Soret maximum at 425 nm) and formation of high-spin ferric CYP3A4 (Soret maximum at 392
nm) in the presence of TS (200 µM). The concentration of CYP3A4 is 1.1 µM after mixing. The individual spectra shown were acquired at 19 ms, 27 ms, 53 ms, 79 ms, 153 ms, 255 ms, 357 ms, 460 ms, 565 ms, and 1023 ms after mixing. (C) The autoxidation of oxy-ferrous CYP3A4 (Soret maximum at 420 nm) and formation of high-spin ferric CYP3A4 (Soret maximum at 392 nm) obtained in the presence of the substrate BC (3 µM). The concentration of CYP3A4 is 1.1 µM. The individual spectra illustrated were acquired at 130 ms, 230 ms, 330 ms, 470 ms, 855 ms, 1.53 s, 2.7 s, 4.7 s, and 8.2 s after mixing.

Fig. 2  Kinetics of oxygenated CYP3A4 decay at different temperatures. The fraction of oxy CYP3A4 as a function of time was calculated from SVD decomposition of the data shown in Fig. 1 as the normalized second concentration vector. (A) Autoxidation of CYP3A4 in the absence of substrate at T= 6° C (squares), T= 10° C (triangles), and T= 15° C (circles). (B) Autoxidation of CYP3A4 in the presence of 200 µM testosterone at T= 6° C (squares), T= 15° C (triangles), and T= 24° C (circles). (C) Autoxidation of CYP3A4 in the presence of 3 µM bromocriptine at T= 6° C (squares), T= 15° C (triangles), and T= 32° C (circles). Lines show the single exponential fits of the data to the equation F = A*exp(-k*t)+B, where F is the second concentration vector determined by SVD.

Fig. 3  Eyring plot of the temperature dependencies of autoxidation rate constants for CYP3A4. Experimental data (symbols) and linear fits (solid lines) are shown for substrate free CYP3A4 (diamonds) and for CYP3A4 saturated with TS (triangles) and BC (circles). Activation free energies, enthalpies and entropies are 14.7 kcal/mol, 12.4 kcal/mol, and -7.7 cal/(mol K) for substrate free CYP3A4; 16.6 kcal/mol, 21.9 kcal/mol, and 18 cal/(mol K) for CYP3A4 with TS; 17.5 kcal/mol, 16.1 kcal/mol, and -4.8 cal/(mol K) for CYP3A4 with BC.
Table 1. Observed autoxidation rates for cytochromes P450

| P450   | Substrate | Conditions  | Rate, s\(^{-1}\) | Source |
|--------|-----------|-------------|-----------------|--------|
| CYP101 | +sub      | 278 – 293 K | 0.0003 – 0.0043 | (49)   |
|        | +cam      | 293 K       | 0.005 - 0.01    | (16)   |
| Mutants|           |             |                 |        |
| T252A,V| +cam      | 283 K       | 0.003 – 0.005   | (59)   |
| CYP102 | -sub      | 288 K       | 0.02 – 0.46     | (51)   |
|        | +sub      | 288 K       | 0.013 – 0.37    |        |
|        | +sub      | 277 – 293 K | 0.025 – 0.22    | (49)   |
| CYP108 | +sub      | 277 – 293 K | 0.0007 – 0.017  | (49)   |
| CYP119 | -sub      | 278 K       | 0.08            | (60)   |
| CYP158 | +sub      | 296 K       | 0.042 – 0.09    | (61)   |
| CYP1A2 | ?         | 277 K       | 0.2 – 0.8       | (62)   |
| CYP2A6 | +sub      | 296 K       | 0.3             | (63)   |
| CYP2B4 | +sub      | 288 K       | 0.09            | (64)   |
| CYP3A4 | No substrate | 278 – 302 K | 20 - 140        | This work |
|        | Testosterone | 279 – 310 K | 0.37 - 20       |        |
|        | Bromocriptine | 279 – 310 K | 0.12 – 2.5      |        |
| CYP11A1| +sub      | 277 K       | 0.01            | (47)   |
Figure 1
Figure 2
The ferrous-dioxygen intermediate in human cytochrome P450 3a4: substrate dependence of formation and decay kinetics
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