Cytochrome P450cam (CYP101Fe^{3+}) regioselectively hydroxylates camphor. Possible hydroxylating intermediates in the catalytic cycle of this well-characterized enzyme have been proposed on the basis of experiments carried out at very low temperatures and shunt reactions, but their presence has not yet been validated at temperatures above 0 °C during a normal catalytic cycle. Here, we demonstrate that it is possible to mimic the natural catalytic cycle of CYP101Fe^{3+} by using pulse radiolysis to rapidly supply the second electron to the catalytic cycle to camphor-bound CYP101[FeO_2]^{2+}. Judging by the appearance of an absorbance maximum at 440 nm, we conclude that CYP101[FeOOH]^2+ (compound 0) accumulates within 5 μs and decays rapidly to CYP101Fe^{3+}, with a k_{440 nm} of 9.6 × 10^4 s^{-1}. All processes are complete within 40 μs at 4 °C. Importantly, no transient absorbance bands could be assigned to intermediates that have been ascribed to Cpd 1 (9–11) and Cpd 2 ([FeO]^2-). However, indirect evidence for the involvement of Cpd 1 was obtained from the kinetics of formation and decay of a tyrosyl radical. 5-Hydroxycamphor was formed quantitatively, and the catalytic activity of the enzyme was not impaired by exposure to radiation during the pulse radiolysis experiment. The rapid decay of compound 0 enabled calculation of the limits for the Gibbs activation energies for the conversions of compound 0 → compound 1 → compound 2 → CYP101Fe^{3+}, yielding a ΔG° of 45, 39, and 39 kJ/mol, respectively. At 37 °C, the steps from compound 0 to the iron(III) state would take only 4 μs. Our kinetics studies at 4 °C complement the canonical mechanism by adding the dimension of time.

Cytochrome P450 (CYP) enzymes are mono-oxygenases that contain heme as a prosthetic group. These enzymes catalyze a wide variety of oxidation reactions, including stereo- and regioselective hydroxylation of non-activated C–H bonds (1). Oxidation of C–H bonds by CYP enzymes with molecular dioxygen, per turnover, requires two electrons that originate from NADH or NADPH (reaction 1) that are transferred sequentially to cytochrome P450, in the case of CYP101 by putidaredoxin (2).

RH + O_2 + 2H^+ + 2e^- → ROH + H_2O

**REACTION 1**

The electrode potential of this half-reaction is estimated to be +1.5 V at pH 7 (3).

Convincing, detailed structural evidence for the existence of intermediates (Fe^{3+}, Fe^{3+}-camphor, Fe^{2+}, and [FeO_2]^2-) in the reaction cycle (Fig. 1) up to and including the Fe^{2+}-dioxygen complex has been presented (4–6). The addition of a second electron and a proton leads to the formation of “compound 0” (Cpd 0, [FeOOH]^2+), which has been observed after cryoreduction of [FeO_2]^2+ (7). Subsequent formation of “compound 1” (8) (Cpd 1, [FeO_2^+por^+]) and “compound 2” (Cpd 2, [FeO]^2-) has been invoked, but these intermediates have never been unequivocally observed during reactions that involve Cpd 0. Intermediates that have been ascribed to Cpd 1 (9–11) and that are capable of hydroxylation (12) have been produced by exposure of cytochromes P450 to peroxycids in reactions that bypass Cpd 0. More recently, rapid mixing of H_2O_2 with iron(III) cytochrome P450 allowed direct observation of Cpd 0, but Cpd 1 and 2 were not detected (13). However, an unusual cytochrome P450 (OleT) that decarboxylates fatty acids does form Cpd 0 by H_2O_2 addition, with compounds 1 and 2 both characterized in subsequent steps (14).

For the best characterized cytochrome P450 isozyme, CYP101, it has been shown that the supply of electrons from putidaredoxin to cytochrome P450 is rate-limiting (15). Hence, it is not possible to observe with standard spectroscopic methods reactive intermediates that occur after the formation of the [FeO_2]^2+ intermediate. However, the hydrated electron (eaq), which can be generated by the pulse radiolysis technique, reduces proteins at diffusion-controlled rates on the microsecond time scale. Under pseudo–first order conditions, with CYP101 in excess, CYP101Fe^{3+} reacts with eaq with a rate constant of 3 × 10^{10} M^{-1} s^{-1}; the yield of reduced heme is ~10% relative to eaq (16, 17). Kobayashi et al. (18) used pulse radiolysis to add an electron to the oxy-form of 2,4-diacytyleuetro-cytochrome P450cam, and the difference absorption spectrum.
Jumpstarting cytochrome P450

Figure 1. The cytochrome P450 catalytic cycle with the reduction step driven by pulse radiolysis highlighted in purple.

observed was assigned to Cpd 2; however, these authors were unable to analyze formation of hydroxylated camphor; thus, no conclusions could be drawn about the catalytic competence of Cpd 2.

We set out to study the kinetics of the reaction of $e_{aq}$ with the Fe$^{3+}$ and [FeO$_2$]$^{2+}$ forms of camphor-hydroxylating cytochrome P450, CYP101, by UV-visible spectroscopy, with the expectation to observe reactions 2 and 3.

$$\text{CYP101Fe}^{3+} + e_{aq} \rightarrow \text{CYP101Fe}^{2+}$$

**REACTION 2**

$$\text{CYP101[FeO}_2\text{]}^{2+} + e_{aq} + H^+ \rightarrow \text{CYP101[FeOOH]}^{2+}$$

**REACTION 3**

We studied reaction 3 at 4 °C in the presence of camphor and observed spectral changes that correspond to formation of Cpd 0. During the experiments, large concentrations of O$_2$ and, thus, of H$_2$O$_2$ and O$_2$, were produced. CYP101Fe$^{3+}$ does not react with O$_2$ (16) and reacts only slowly, on the second time scale, with H$_2$O$_2$. Because the absorbance changes we observed continued only up to 1 ms, reactions with H$_2$O$_2$ can be ignored. Compared with the cryo-irradiations of cytochromes P450 described by Davydov et al. (7), the radiation dose from the pulse radiolysis technique is much lower, which mitigates the risk of damage to the protein. Detection of 5-hydroxycamphor as the primary product from Cpd 0 provides evidence that this intermediate is a relevant oxidant in the CYP101 catalytic cycle. We show that the pulse radiolysis technique can be used to “jump-start” the catalytic cycle of cytochrome P450 by providing the second electron to reduce the oxy-form of the enzyme. Although we find no spectral evidence for accumulation of Cpd 1, our evidence indicates that it must be formed.

**Results**

**Half-life of the hydrated electron**

The half-life, $t_{1/2}$, of $e_{aq}$ decay, measured after irradiation (100 Gy) of a corresponding N$_2$-saturated 10 mM potassium phosphate buffer at pH 7.4 in the presence of 10 μM camphor but without CYP101, is 0.65 μs (Fig. 2A).

**Reduction of CYP101Fe$^{3+}$ by hydrated electrons**

The rate constant for the reaction of the $e_{aq}$ with the heme of CYP101Fe$^{3+}$ was determined by competition kinetics, i.e. via the yield of this reaction. The yield of reduction was determined by irradiating a N$_2$-saturated solution of 2.0 μM CYP101Fe$^{3+}$ containing 2 mM t-BuOH at a dose of 80 Gy, which results in the formation of 22 μM $e_{aq}$. The observed difference absorbance spectrum (Fig. 2B) is consistent with conversion of CYP101Fe$^{3+}$ to CYP101Fe$^{2+}$. The absorbance at 390 nm, which was used to follow the reduction of CYP101Fe$^{3+}$ (Fig. 2C), decreased by a total of ~65 mAbs in 50 μs. However, over the course of 5 half-lives of $e_{aq}$ decay (~3 μs), the corresponding loss of absorbance at 390 nm was ~40 μAbs, which, given the extinction coefficients of the oxidized and reduced proteins at that wavelength (2) and the 6-cm path length, corresponds to a concentration of CYP101Fe$^{2+}$ of 0.18 μM. This first phase of absorbance change reflects reduction of ~9% of the heme iron(III) relative to protein and a yield of reduction of the heme relative to $e_{aq}$ of 0.8%. The same result was found when traces recorded at 410, 415, 420, and 425 nm were analyzed. The second stage of heme reduction, the loss of the remaining 25 μAbs at 390 nm, occurred after essentially all of the $e_{aq}$ decayed, proceeding for ~50 μs, with $k_{obs, reduction} = 5.4 \times 10^9 \text{ M}^{-1} \text{s}^{-1}$ (Fig. 2C). This stage of heme reduction is likely caused by adducts of $e_{aq}$ and H with amino acids (21, 22) formed during the first 3 μs, with reducing equivalents then transferred to the heme by a tunneling or hopping mechanism.

**Rate constant for the formation of CYP101[FeO$_2$]$^{2+}$**

To validate pulse radiolysis as the method of choice to provide reducing equivalents, we followed the formation of CYP101[FeO$_2$]$^{2+}$, as shown in the following reaction,

$$\text{CYP101Fe}^{2+} + O_2 \rightarrow \text{CYP101[FeO}_2\text{]}^{2+}$$

**REACTION 4**

by reducing CYP101Fe$^{3+}$ with $e_{aq}$ in the presence of excess O$_2$. The rate constant measured, $k_4 = 2.1 \times 10^9 \text{ M}^{-1} \text{s}^{-1}$ (Fig. S1a), agrees with that determined by stopped flow, $2.4 \times 10^9 \text{ M}^{-1} \text{s}^{-1}$ (Fig. S1b), and is the same order of magnitude as the literature value, 7.7 $\times 10^8 \text{ M}^{-1} \text{s}^{-1}$ (19). Moreover, these experiments demonstrate that, at 4 °C, CYP101[FeO$_2$]$^{2+}$ does not autoxidize significantly for at least 1 min (Fig. S1c).

**The reaction of hydrated electrons with CYP101[FeO$_2$]$^{2+}$**

To observe intermediates of the catalytic cycle, we formed substrate-bound CYP101[FeO$_2$]$^{2+}$ at 4 °C in the irradiation cell by mixing an anaerobic solution of CYP101Fe$^{2+}$, produced by reducing CYP101Fe$^{3+}$ in the presence of camphor with a slight excess of S$_2$O$_4^{2-}$ inside a N$_2$-filled glove box, with a buffered solution containing O$_2$. The CYP101[FeO$_2$]$^{2+}$ formed can autoxidize; thus, the mixture was irradiated as soon as possible, generally within 30 s. Two distinct absorption bands with maxima at 410 and 440 nm were detected upon exposure to $e_{aq}$ decay at different rates (Fig. 3A), which indicates that two processes take place simultaneously. Both appear within 5 μs, i.e. “instantaneously.” The absorbance at 440 nm decays by a first-order reaction, $k_{440 \text{ nm}} = 9.6 \times 10^9 \text{ s}^{-1}$ (Fig. 3B), whereas that at 410 nm decays by two consecutive processes with rate constants $k_{410 \text{ nm-1}} = 4.2 \times 10^9 \text{ s}^{-1}$ and $k_{410 \text{ nm-II}} = 2.5 \times 10^8 \text{ s}^{-1}$ (Fig. 3C). All processes are complete within <1 ms. All rate data have been collected in Table 1.
Importantly, no transient absorbance bands that could correspond to Cpd 1 are detected, and the spectrum recorded after irradiation indicates that all iron has returned to the Fe$^{3+}$ state (Fig. 3D), which leads us to conclude that the heme did not suffer radiation damage. We also determined that the rate of NADH oxidation, which has been shown to reflect the rate of substrate hydroxylation by CYP101 (20), was similar before and after irradiation; the observed rate constant of $k_{\text{obs, activity after PR}} = (3.7 \pm 0.3) \times 10^2$ min$^{-1}$ demonstrates that the enzyme activity is not significantly impaired by radiolysis (Fig. S3).

Analysis of kinetics experiments carried out in D$_2$O followed at 440 nm give a result for $k_{\text{obs,0, nm}}$ (D$_2$O) = $3.9 \times 10^4$ s$^{-1}$ (Fig. S4a). The difference relative to the rate constant for the reaction in water (Fig. 3B) amounts to a kinetic isotope effect of $\sim$2. In contrast, the kinetics at 410 nm are not affected (Fig. S4b).

Product analysis after pulse radiolysis

To confirm that the transient species observed occur along the pathway of camphor hydroxylation, two samples of $\sim$1 ml each were collected for product analysis. These samples, containing 50 $\mu$M of the oxy-enzyme, had each been exposed to a dose of 200 Gy. The substrate and products were extracted with chloroform and analyzed by GC-MS. The GC-MS elution profile shown in Fig. 4A shows, in addition to the peak corresponding to the camphor substrate at 4.9 min, two additional peaks with elution times of 5.9 and 6.9 min. The peak at 6.9 min corresponds to 5-hydroxycamphor (167 Da) with $m/z = 168$ (after single protonation by electrospray ionization; Fig. 4B), as follows from a comparison with the mass spectrum of authentic 5-hydroxycamphor (Fig. 4C). The yields, quantified by GC with flame ionization detection (FID), were, from two separate experiments, 2.7 and 3.7 $\mu$M. The elution profile for $m/z = 166$ with the peak at 5.9 min shown in Fig. 4D, together with the mass spectrum (Fig. 4E), indicates that a small amount ($\sim 0.9 \pm 0.3$ $\mu$M, quantified by GC-FID) of 5-keto-camphor, a secondary oxidation product, was also formed. No oxidation products of camphor were observed in control experiments performed without CYP101 present or in the presence of lysozyme, a non-heme protein, in place of CYP101.

Simulation of pulse radiolysis reaction conditions and theoretical product yield

The theoretical yield of 5-hydroxycamphor was calculated with a chemical kinetics simulator (IBM Research) program by simulating the reactions shown in Table S1. To provide a value for $k_3$ for use in this simulation, we assume it to be equal to $k_2$, which we calculate according to Equation 1,

$$ \text{yield} = \frac{k_3[CYP101Fe^{3+}]}{k_2[CYP101Fe^{3+}] + k_{\text{control}}} \quad (\text{Eq. 1}) $$

where $k_{\text{control}} = 1.0 \times 10^6$ s$^{-1}$ is the rate at which $e_{\text{aq}}^-$ disappears in the absence of protein (Fig. 2A). We found the yield of reduc-
Jumpstarting cytochrome P450

Figure 3. Reaction of $e_{aq}$ with CYP101[FeO$_2$]$^{2+}$ at 4.0 °C. 6 μM CYP101Fe$^{2+}$ with 12 μM camphor was mixed 1:1 with 100 μM O$_2$, 10 μM camphor, and 2 mM t-BuOH in 10 mM potassium phosphate buffer, at pH 7.4, from gas-tight syringes and irradiated at 80 Gy. [e$_{aq}$] = 22 μM) within 30 s after mixing. A, difference spectra for CYP101Fe$^{3+}$-CYP101[FeO$_2$]$^{2+}$ at 5 (red circle), 7 (blue triangle), 20 (inverted purple triangle), 50 (green diamond), and 100 (left-facing dark blue triangle) μs, and a difference spectrum of CYP101Fe$^{3+}$-CYP101[FeO$_2$]$^{2+}$ calculated from typical UV-visible recordings (gray line). B, the decay of the 440 nm absorbance (red line), fit to a rate constant of $k_{440\,\text{nm}} = 9.4 \times 10^8 \text{s}^{-1}$ (black line), and the residual (green line). C, the decay of the 410 nm absorbance (red line), fit to two rate constants: $k_{410\,\text{nm-I}} = 4.2 \times 10^8 \text{s}^{-1}$ and $k_{410\,\text{nm-II}} = 2.5 \times 10^8 \text{s}^{-1}$ (black line), and the residual (green line). D, UV-visible spectra of CYP101Fe$^{3+}$ (blue line) and CYP101Fe$^{2+}$ (red line) taken before and the spectrum of CYP101Fe$^{3+}$ (dotted bright green line) taken after pulse radiolysis (PR). Note that the blue and green lines are superimposable.

Table 1
Summary of kinetics data

| Rate constant, $k$ | Value | Corresponding process |
|------------------|-------|----------------------|
| $k_{\text{obs, reduction}}$ | $5.4 \times 10^8 \text{s}^{-1}$ | Reduction of the heme by $e_{aq}$, likely caused by adducts of $e_{aq}$ and H$^+$ with amino acids formed during the first 3 μs (Fig. 2C) |
| $k_{440\,\text{nm}}$ | $9.6 \times 10^8 \text{s}^{-1}$ | Decay at 440 nm, presumably of Cpd 0 after reaction of $e_{aq}$ with CYP101[FeO$_2$]$^{2+}$ (Fig. 3B) |
| $k_{410\,\text{nm-I}}$ | $4.2 \times 10^8 \text{s}^{-1}$ | First phase of the decay at 410 nm after reaction of $e_{aq}$ with CYP101[FeO$_2$]$^{2+}$ (Fig. 3C) |
| $k_{410\,\text{nm-II}}$ | $2.5 \times 10^8 \text{s}^{-1}$ | Second phase of the decay at 410 nm after reaction of $e_{aq}$ with CYP101[FeO$_2$]$^{2+}$ (Fig. 3C) |
| $k_{440\,\text{nm-(D$_2$O)}}$ | $3.9 \times 10^8 \text{s}^{-1}$ | Decay at 440 nm of Cpd 0 after reaction of $e_{aq}$ with CYP101[FeO$_2$]$^{2+}$ in D$_2$O (Fig. 5a) |
| $k_{410\,\text{nm-(D$_2$O)}}$ | $4.0 \times 10^8 \text{s}^{-1}$ | First phase of the decay at 410 nm after reaction of $e_{aq}$ with CYP101[FeO$_2$]$^{2+}$ in D$_2$O (Fig. 5b) |
| $k_{410\,\text{nm-II-(D$_2$O)}}$ | $2.3 \times 10^8 \text{s}^{-1}$ | Second phase of the decay at 410 nm after reaction of $e_{aq}$ with CYP101[FeO$_2$]$^{2+}$ in D$_2$O (Fig. 5b) |
| $k_{\text{contact}}$ | $1.0 \times 10^8 \text{s}^{-1}$ | Disappearance of $e_{aq}$ in the absence of protein (Fig. 2A) |
| $k_3$ | $(3.8 \pm 0.7) \times 10^8 \text{m}^{-1} \text{s}^{-1}$ | The reaction of CYP101Fe$^{3+}$ with $e_{aq}$, $k$ derived from $\text{[CYP101Fe}^{2+}]\text{[e}_{aq}]$, the yield of reduction of the heme relative to $e_{aq}$ is 0.8% (Eq. 5) |
| $k_4$ | $2.1 \times 10^8 \text{m}^{-1} \text{s}^{-1}$ | Formation of CYP101[FeO$_2$]$^{2+}$ measured by pulse-radiolysis (reaction 4, Fig. 5a) |
| $k_{\text{obs, activity before PR}}$ | $(3.8 \pm 0.2) \times 10^7 \text{min}^{-1}$ | Formation of CYP101[FeO$_2$]$^{2+}$ measured by stopped-flow (reaction 4, Fig. 5b) |
| $k_{\text{obs, activity after PR}}$ | $(3.7 \pm 0.3) \times 10^7 \text{min}^{-1}$ | NADH oxidation before and after pulse radiolysis (Fig. 5c) |

From a simulation of substrate oxidation by the primary oxidant generated via reduction of the heme of 50 μM CYP101[FeO$_2$]$^{2+}$ at 200 Gy, we estimate the product yield to be $3.1 \pm 0.6 \mu$M (Fig. S5). We found experimental yields of 2.7 and 3.7 μM hydroxycamphor product, from which we conclude that the reaction is, within the error, quantitative. Because there is no indication that pulse radiolysis impairs enzyme function or its spectral properties, we assume that reaction of $e_{aq}$ with the protein causes no damage to the protein, which allows us to ignore protein damaging reactions in the simulations.

In addition to 5-hydroxycamphor, formation of a further oxidation product, 5-ketocamphor, was observed. To test the hypothesis that the alcohol is converted to the ketone by reaction with H$_2$O$_2$ in the presence of Fe$^{3+}$, we incubated 5-hydroxycamphor with CYP101Fe$^{3+}$ in the presence of H$_2$O$_2$ at concentrations of up to 1 mM, a concentration more than 1 order of magnitude higher than can be achieved by the radia-
tion dose used, and found no evidence for the formation of 5-ketocamphor, even after overnight incubation. Hydrogen peroxide clearly does not interfere with our measurements. Formation of 5-ketocamphor was observed only with H\textsubscript{2}O\textsubscript{2} present at concentrations of /H\textsubscript{11350} 2mM (Fig. S6 a). To test the hypothesis that 5-hydroxycamphor remaining in the active site could be oxidized during a second reaction cycle, we irradiated a solution of 50 \textsubscript{9262} M CYP101\textsubscript{Fe\textsuperscript{3}+} with 100 \textsubscript{9262} M of 5-hydroxycamphor as substrate in the presence of 50 \textsubscript{9262} M O\textsubscript{2}; analysis of a sample by GC-MS shows formation of 0.7 \textsubscript{9262} M 5-ketocamphor (Fig. S6 c). As a negative control, a solution of 75 \textsubscript{9262} M of CYP101\textsubscript{Fe\textsuperscript{2}+} was irradiated in the presence of 100 \textsubscript{9262} M O\textsubscript{2} without substrate present, after which the sample was mixed with a solution of 100 \textsubscript{9262} M 5-hydroxycamphor before analysis by GC-MS. Surprisingly, formation of 0.7 \textsubscript{9262} M 5-ketocamphor was found as well in this negative control (Fig. S6 b).

**Discussion**

Our main findings are that the pulse radiolysis technique can be used to jump-start the catalytic cycle of cytochrome P450, providing the electrons to reduce the enzyme, and that Cpd 0 so formed is converted to Cpd 1 very rapidly. The latter reacts either with bound substrate or with a protein moiety even faster, such that Cpd 1 does not accumulate. Additionally, we demonstrate that reduction of CYP101 by pulse radiolysis is productive, *i.e.* that 5-hydroxycamphor is formed.

**Validation of the pulse radiolysis technique**

We demonstrate that it is possible to use the pulse radiolysis technique, which allows electrons to be added to the heme one at a time and permits reactions that occur within a few microseconds to be followed spectroscopically, to probe the natural catalytic cycle of CYP101. Pulse radiolysis is an inherently quantitative technique in which the dose is used to calculate \([e_{aq}]\), and the yields of all reaction products can be estimated relative to \([e_{aq}].\)

Although reactions after formation of Cpd 0 are too rapid to be accessible by stopped-flow methods, the third reaction in the CYP101 cycle—formation of CYP101\textsubscript{Fe\textsuperscript{2}+} from CYP101\textsubscript{Fe\textsuperscript{2}+} and O\textsubscript{2}, Fig. 1—is considerably slower. We could analyze this relatively slow reaction by both the stopped-flow and pulse radiolysis techniques to confirm that pulse radiolysis is suitable.
Jumpstarting cytochrome P450

for analysis of the more rapid reactions in the cycle. The rate constant measured by pulse radiolysis, \( k_\gamma = 2.1 \times 10^{7} \text{ M}^{-1} \text{s}^{-1} \), is essentially identical to that measured by stopped flow, \( 2.4 \times 10^{5} \text{ M}^{-1} \text{s}^{-1} \), and is in agreement with the literature (19).

**Intermediates in the CYP101 reaction cycle identified by one-electron reduction steps**

The one-electron reduction of CYP101[FeO\(_2\)]\(^{2+}\) initially gives rise to an absorbance spectrum that closely resembles that published for Cpd 0. There are minor differences at higher wavelengths that may originate from the 100-fold higher dose of radiation used by Denisov et al. (23). We assign the maximum at 440 nm (Fig. 3A) to Cpd 0. The decay kinetics at 440 nm are monophasic, with \( k_{440 \text{ nm}} = 9.6 \times 10^{5} \text{ s}^{-1} \) (Fig. 3B). The initially increased absorbance observed at 410-nm decays in a biphasic first-order fashion, but with different rate constants, \( k_{410 \text{ nm-I}} = 4.2 \times 10^{4} \text{ s}^{-1} \) and \( k_{410 \text{ nm-II}} = 2.5 \times 10^{3} \text{ s}^{-1} \) (Fig. 3C). Given that the absorbance bands at both 410 and 440 nm are present within \( \mu \text{s} \) after the pulse, we conclude that the absorbance decays at these wavelengths reflect not **serial**, but **parallel** processes. The transient absorbance at 410 nm may indicate the presence of a tyrosyl radical (TyrO\(^{\bullet} \)), which is unlikely to result from reaction with O\(_2\)\(^{\bullet} \), present in high concentrations directly after the pulse, because this radical does not react with amino acids (24). Instead, the absorbance at 410 nm is probably due to camphor-free CYP101, which has been shown to form Cpd ES upon reaction with a peracid (25, 26). Cpd ES, first reported by Schüneemann et al. (27), contains an oxidoiron(IV) and TyrO\(^{\bullet} \), with an absorbance maximum at 406 nm. Using the \( K_p \) for camphor of 1.46 \( \mu \text{M} \) at 4 °C (28), we calculate that more than 85% of 3 \( \mu \text{M} \) CYP101[Fe\(^{3+}\)] binds to camphor under the experimental conditions. Therefore, the remaining <15% may well form Cpd ES and account for the observed transient absorbance at 410 nm. The short lifetime of the 410-nm bands makes further characterization very difficult, but in all likelihood the decay corresponds to the reduction of TyrO\(^{\bullet} \).

The presence of this small amount of CYP101[FeO\(_2\)]\(^{2+}\) without camphor bound allows us to appreciate the time scale of the events after formation of Cpd 0. The formation and decay of the camphor radical, which has no absorbance maxima in the UV-visible range, cannot be observed, but if the absorbance at 410 nm is indeed caused by formation of TyrO\(^{\bullet} \), this may be taken as a “reporter” for formation of Cpd 1. Thus, although we do not directly observe formation of Cpd 1, we conclude that any Cpd 1 formed immediately oxidizes camphor, or, in the absence of camphor, oxidizes Tyr and does not accumulate. In agreement with this interpretation, we observe that the decay of TyrO\(^{\bullet} \) “trails” that of Cpd 0; the first rate constant for the decay of TyrO\(^{\bullet} \) is about half that of Cpd 0 (Table 1).

Within the error, the yield of product formation approaches 100%, that is, for every molecule of Cpd 0 produced, one of 5-hydroxycamphor was formed. This finding agrees with the cryoreduction study (7), with one difference. By pulse radiolysis, we find quantitative conversion of CYP101 to Cpd 0, whereas the reported yield of Cpd 0 by cryoreduction was ~50% (7), which may reflect changes in conformation of CYP101[FeO\(_2\)]\(^{2+}\) upon reduction at 4 °C that would not be possible at the much lower temperatures used in the cryoreduction studies.

**Isotope effect**

The observed kinetic deuterium isotope effect of 2 corroborates our assignment of the observed absorption band of 440 nm to Cpd 0, which becomes protonated after reduction according to the canonical cycle shown in Fig. 1. In contrast, the rate of decay of absorbance at 410 nm is essentially the same in D\(_2\)O and H\(_2\)O (Fig. 3C and S4b); the absence of an isotope effect fits with the hypothesis that any TyrO\(^{\bullet} \) formed would be reduced by other amino acids, with no isotope effect.

**Cpd 1 does not accumulate**

It is intriguing that, with camphor bound to CYP101, we find no indication for formation of Cpd 1, i.e., no absorbance increase near 370 or 690 nm, whereas Rittle and Green (12) found convincing support for production of such a species in the absence of substrate, after the reaction of CYP119 with chloroperbenzoate. Given the small absorptivity, 4 \( \times 10^{5} \text{ M}^{-1} \text{cm}^{-1} \) at 690 nm (29), it is unlikely that under our experimental conditions, we would observe any increase in absorbance at that wavelength, but an intermediate giving rise to a band at a wavelength lower than 400 nm should have been observable. Formation of Cpd 1 was also not observed in cryoexperiments (7). Without substrate bound, CYP101[FeO\(_2\)]\(^{2+}\) is too unstable to be effectivaly reduced by pulse radiolysis.

Although we did not observe any accumulation of Cpd 1, we must infer from isotope studies at low temperature that Cpd 1 is involved in the mechanism (30): the hydrogen attached to camphor is retained in the hydroxylated form when the reaction is carried out with deuterated camphor (31). Furthermore, it would seem unlikely that Cpd 0, with electrode potential \( E^\circ = +0.93 \text{ V} \) (3), could rapidly oxidize Tyr, which would require a redox partner with \( E^\circ = +0.97 \text{ V} \) (32). Indeed, Cpd 1, with \( E^\circ = +1.35 \text{ V} \) (3), is a far more likely candidate.

Our results suggest that hydroxylation is rather fast, as inferred from the observation that at 4 °C, the conversion of Cpd 0 to CYP101Fe\(^{3+}\) takes 37 \( \mu \text{s} \), calculated from the half-life of the decay at 440 nm, 7.4 \( \mu \text{s} \), based on \( k_{440 \text{ nm}} = 9.6 \times 10^{4} \text{ s}^{-1} \), and multiplying the half-life by 5. Given this rate constant for the disappearance of Cpd 0 and under the assumption that Cpd 1 is formed, the Gibbs activation energy is about 45 kJ/mol (Fig. 5), as calculated via Equation 2,

\[
    k = \frac{k_B T}{h} e^{-\frac{\Delta G^\circ}{R T}}
\]

in which \( k \) is the rate constant, \( k_B \) is Boltzmann’s constant, \( h \) is Planck’s constant, \( T \) is the temperature, \( \Delta G^\circ \) is the Gibbs energy of activation, and \( R \) is the gas constant. Because the next two steps, abstraction of a hydrogen from camphor and the rebound (33) step, are not observable, these reactions must have rates of \( \approx 10^{9} \text{ s}^{-1} \) and thus activation energies of \( \approx 39 \text{ kJ/mol} \). With radical clock experiments, it has been established that the rebound step is extremely fast, with rate constants on the order of \( 10^{10} \text{ – } 10^{11} \text{ s}^{-1} \) (34), values that imply activation energies much smaller than 39 kJ/mol, indeed, close to 0 kJ/mol. Taking
into consideration that the experiments were carried out at 4 °C and using the value of 45 kJ/mol in Equation 2, we calculate that, at a physiological temperature of 37 °C, conversion of Cpd 0 to Cpd 1 is faster by a factor of 11. This factor implies that, at physiological temperatures, this process requires only 3.5 μs.

**Further oxidation of 5-hydroxycamphor**

In addition to the 5-hydroxycamphor, we observed formation of the further oxidized product 5-ketocamphor (Fig. 4A), production of which has also been reported from multiple turnovers of the enzyme (35, 36). The ketone product could also be formed by reaction with H2O2, which is a direct product of pulse radiolysis that can oxidize alcohols in the presence of Fe3+ (37). We concluded that the ≲20 μM of H2O2 generated during pulse radiolysis is unlikely to cause further oxidation of 5-hydroxycamphor, because even bulk addition of 1 mM H2O2 to a mixture of CYP101-Fe3+ and hydroxycamphor did not produce any 5-ketocamphor (Fig. S6a). The organic hydroperoxides (CH3)2C(OH)CH2OOH formed in small amounts from the scavenger molecule t-BuOH directly after irradiation could possibly react with 5-hydroxycamphor to form 5-ketocamphor via a yet undocumented reaction mechanism. Conversion of the primary alcohol product to a ketone may also be catalyzed during multiple turnovers of CYP101 (36); 5-hydroxycamphor remaining in the active site could be oxidized during a second reaction cycle initiated by transfer of electrons to the heme from aromatic residues, followed by binding of O2, etc. We observed the formation of very small amounts of 5-ketocamphor when 5-hydroxycamphor was added as a substrate to CYP101Fe2+, believed to have been stripped of camphor, either before irradiation in the presence of O2 or after irradiation as a negative control.

**Energetics**

The abstraction of H from camphor by Cpd 1 is, within the error of the estimates, thermoneutral (reaction S1), whereas the rebound step is very favorable, −241 kJ/mol, (Reaction S4). The energetics of the hydroxylation of camphor by CYP101 are shown in Fig. 5.

The value of $E^\circ(C^+\text{H}^+/\text{CH}) = +1.7 \text{ V}$ (see supporting materials), or +1.3 V at pH 7, in which CH represents camphor and C+ represents the camphor radical, corresponds to a bond dissociation energy ΔBDEH° = 397 kJ/mol, which agrees very well with that calculated by in silico methods (38). Oxidation of methane or other small alkanes would require dissociation of a stronger C–H bond. For CH4, ΔBDEH° = 435 kJ/mol, which corresponds to an electrode potential of +2.0 V at pH 0, and +1.6 V at pH 7; slightly smaller numbers are obtained for other small alkanes. Compared with the $E^\circ$ of +1.3 V for the one-electron oxidation of camphor, the value of +1.6 V required for CH4 implies that the activation barrier for hydrogen abstraction is higher by ≈24 kJ/mol, making the reaction much slower, with an important consequence. A small alkane bound to the active site of cytochrome P450 would diffuse readily away, and hydroxylation is possible only when the substrate cannot escape, as has been achieved by the addition of perfluorocarboxylic acids that block the entrance of the active site of cytochrome P450 (39).

**Tethering in silico to reality**

In silico calculations yield energies larger than those calculated by means of thermodynamic cycles. For example, the reaction Cpd 0 + H+ → Cpd 1 + H2O, is “either almost thermoneutral or slightly exothermic, with a best estimated reaction energy of −8 kcal/mol” (40). However, the value of −8
Jumpstarting cytochrome P450

kcal/mol cannot be correct, because either Cpd 1 would not be sufficiently oxidizing to abstract H, or \( \text{H}_2\text{O}_2 \) would dissociate from Cpd 0, or both (3). Thus, “almost thermoneutral” must be correct. The activation enthalpy of the transition between Cpd 0 and Cpd 1 has been reported as \( \sim 15 \text{ kcal/mol or 63 kJ/mol} \) (38); the Gibbs energy would be higher. For this reaction, we estimate a Gibbs activation energy of only 45 kJ/mol (Fig. 5). If, at a minimum, the barrier were 63 kJ/mol, \( t_{1/2} \) would be 9 ns, and Cpd 0 should have been observed in stopped-flow experiments but was not. Thus, as no formation of Cpd 0 was detected, we question that the enthalpy could be as high as 63 kJ/mol. The third example is the energy for the release of \( \text{O}_2 \) from CYP101[FeO\(_2\)]\(^{2+}\), which has been reported as 19.5 kcal/mol (41). The value from a simple thermodynamic cycle, for which experimental values are used, is 34 kJ/mol (3) or 8.1 kcal/mol.

In silico calculations applied to complicated reactions have been questioned (42). It is possible and essential to make in silico calculations more quantitative by “tethering” them to semiempirical values and considerations of relatively simple thermodynamic cycles.

Conclusions

We demonstrate that it is possible to mimic the natural catalytic cycle of CYP101 by using pulse radiolysis to rapidly supply electrons to the protein. This rapid electron delivery allowed us to visualize the steps following the reduction of substrate bound CYP101[FeO\(_2\)]\(^{2+}\) on the microsecond timescale. We found that, as soon as Cpd 0 is formed, the rest of the cycle is completed within 40 \( \mu \text{s} \) at 4 °C. We found no spectral evidence that Cpd 1 accumulates. We demonstrate that product formation is quantitative once CYP101[FeO\(_2\)]\(^{2+}\) is reduced by one electron. Further, reduction of the enzyme by this method does not damage the enzyme, as is demonstrated by the complete recovery of enzyme activity and of \( \text{Fe}^{3+} \) after pulse radiolysis.

Experimental procedures

Protein preparation

CYP101 was expressed and purified as previously described, with slight modifications (41) (see supporting text and Fig. S7 for details). The ratio \( A_{392}/A_{280} \) was 1.62.

Pulse radiolysis

Pulse radiolysis produces ionizing radiation that leads to the formation of primary species from water with the following yields: \( G(\text{HO}^\bullet) = 2.65 \), \( G(e_{\text{aq}}) = 2.65 \), \( G(\text{H}^\bullet) = 0.54 \), \( G(\text{H}_2\text{O}) = 0.54 \), where \( G = 1.0 \) refers to 0.1036 \( \mu \text{mol} \) species generated per 1 kJ/kg energy absorbed. Experiments were performed with a 2 MeV Febetron 705 accelerator (Titan Systems Corp., presently L-3 Communications, San Leandro, CA), with \(<50-\text{ns} \) pulses of 80 Gy and a custom-built 6-cm quartz irradiation cell (Hellma, Mülhausen, Germany) with a volume of 180 \( \mu \text{l} \). Dosimetry was carried out with KSCN (43). Absorbance maxima of 380 – 450 nm were measured by means of a 300 nm Czerny–Turner type monochromater equipped with a grating of 150 grooves/mm with 300 nm blazing and a photomultiplier for detection; for wavelengths \( \geq 450 \text{ nm} \), a grating of 150 grooves/mm with 500 nm blazing was used. The monochromator slit width was set to 0.4 mm.

Sample preparation for reaction of hydrated electrons with [FeO\(_2\)]\(^{2+}\) CYP101

All experiments were carried out at 4 °C. CYP101Fe\(^{3+}\) in the presence of camphor was reduced with a slight excess of \( \text{S}_2\text{O}_4^{2-} \) inside a N\(_2\)-filled glove box and loaded into a 10-ml gas-tight syringe (Hamilton Sample Lock). A second gas-tight syringe contained a solution of 100 \( \mu \text{M} \) \( \text{O}_2 \) in 10 mM pH 7.4 potassium phosphate buffer, 10 \( \mu \text{M} \) camphor with 2 \( \mu \text{M} \) t-BuOH. t-BuOH was used to scavenge \( \text{HO}^\cdot \). The two air-tight syringes were placed in a syringe pump prior to pulse irradiation. To maintain the temperature at 4 °C before mixing, the capillaries from the syringes to the mixer were kept on ice. Irradiation took place within 30 s after mixing, such that CYP101[FeO\(_2\)]\(^{2+}\) did not decay significantly before irradiation. The buffer used in pulse radiolysis, UV-visible, GC-MS, and stopped-flow experiments was 10 mM potassium phosphate buffer, pH 7.4, containing 2 equivalents of camphor relative to CYP101, or as otherwise specified.

Product analysis

A solution of 100 \( \mu \text{M} \) reduced CYP101 containing 100 \( \mu \text{M} \) of camphor was mixed with air-saturated buffer prior to irradiation. The applied dose was 200 Gy in a single pulse. A total of \( \sim 1 \) ml of irradiated solution was collected. Substrate and products were extracted with chloroform (1:1 \( v/v \)) containing 1 mM decane as an external standard; the organic layer was concentrated by evaporation under a stream of \( \text{N}_2 \) and analyzed by GC-MS or GC-FID. Peaks were assigned by GC-MS and quantified by peak integration of the GC-FID traces and comparison with standard curves of the substrate and product. The product 5-hydroxycamphor was synthesized enzymatically as described previously (41).

Acknowledgments—We thank Dr. I. Schlichting (Max Planck Institute for Medical Research, Heidelberg, Germany), Prof. M. Green (Pennsylvania State University, University Park, Pennsylvania), and Dr. R. Kissner (ETH Zürich, Zurich Switzerland) for helpful discussions.

References

1. Denisov, I. G., and Sligar, S. G. (2015) Activation of molecular oxygen in cytochromes P450. In Cytochrome P450: Structure, Mechanism and Biochemistry (Ortiz de Montellano, P. R., ed) pp. 69–109, Springer, Berlin, Germany
2. Gunsalus, I. C., and Wagner, G. C. (1978) Bacterial P-450\(_\text{cam}\) methylene monooxygenase components: cytochrome \( m \), putidaredoxin, and putidaredoxin reductase. In Methods Enzymol. Biomembranes: Part C. Biolog-
1. George, P. (1952) Chemical nature of the secondary hydrogen peroxide compound formed by cytochrome-c peroxidase and horseradish peroxidase. Nature 169, 612–613
2. Nagano, S., and Poulos, T. L. (2005) Crystallographic study on the dioxygen complex of wild-type and mutant cytochrome P450cam. Implications for the dioxygen activation mechanism. J. Biol. Chem. 280, 31659–31663
3. Davydov, R., Makris, T. M., Kuznetsov, V. Y., Poulos, T. L., and Hoffman, B. M. (2001) Hydroxylation of camphor by reduced oxy-cytochrome P450cam: mechanistic implications of EPR and ENDOR studies of catalytic intermediates in native and mutant enzymes. J. Am. Chem. Soc. 123, 1403–1415
4. Lipscomb, J. D. (1980) Electron paramagnetic resonance detectable states. Amino Acids 2, 195–214
5. Denisov, I. G., Makris, T. M., and Sligar, S. G. (2001) Cryotrapped reaction intermediates of cytochrome P450 studied by radiolytic reduction with phosphorus-32. J. Biol. Chem. 276, 11468–114652
6. Bielski, B. H. J., Cabelli, D. E., Arudi, R. L., and Ross, A. B. (1985) Reactivity of HO·/O2− radicals in aqueous solution. J. Phys. Chem. Ref. Data 14, 1041–1100
7. Spolitak, T., Dawson, J. H., and Ballou, D. P. (2006) Rapid kinetics investigations of peracid oxidation of ferric cytochrome P450cam: nature and possible function of compound E. J. Inorg. Biochem. 100, 2034–2044
8. Denysov, I. G., Makris, T. M., and Sligar, S. G. (2001) Electrode potentials of L-tryptophan, L-tyrosine, 3-nitro-L-tyrosine, L-phenylalanine, and L-leucine. J. Phys. Chem. 105, 932–948
9. Ortiz de Montellano, P. R. (2010) Hydrocarbon hydroxylation by cytochrome P450 enzymes. Chem. Rev. 110, 932–948
10. Harada, K., Sakurai, K., Ikemura, K., Ogura, T., Hirota, S., Shimada, H., and Hayashi, T. (2008) Evaluation of the functional role of the heme-6-propionate side chain in cytochrome P450cam. J. Am. Chem. Soc. 130, 432–433
11. Prasad, B., Mah, D. J., Lewis, A. R., and Plettner, E. (2013) Water oxidation by the cytochrome P450: mechanism and function of the reaction. PLoS One 8, e61897
12. de Visser, S. P., Kumar, D., Cohen, S., Shacham, R., and Shaik, S. (2004) A predictive pattern of computed barriers for C-H hydrogenation by compound I of cytochrome P450. J. Am. Chem. Soc. 126, 8362–8363
13. Kawakami, N., Cong, Z., Shoji, O., and Watanabe, Y. (2015) Highly efficient hydrogenation of gaseous alkenes at reduced temperature catalyzed by cytochrome P450BM3 assisted by decoy molecules. J. Porphyrins Phthalocyanines 19, 329–334
14. Shaik, S., Cohen, S., Wang, Y., Chen, H., Kumar, D., and Thiel, W. (2010) P450 enzymes: their structure, reactivity, and selectivity—modeled by QM/MM calculations. Chem. Rev. 110, 949–1017
15. Vandermeulebroucke, A., Aldag, C., Siebrhte, M. T., Reider, M., and Hilvert, D. (2015) Kinetic consequences of introducing a proximal selenocysteine ligand into cytochrome P450cam. Biochemistry 54, 6692–6703
16. Plata, R. E., and Singleton, D. A. (2015) A case study of the mechanism of alcohol-mediated Morita Baylis-Hillman reactions: the importance of experimental observations. J. Am. Chem. Soc. 137, 3811–3826
17. Schuler, R. H., Patterson, L. K., and Janata, E. (1980) Yield for the scavenging of hydroxyl radicals in the radiolysis of nitrous oxide-saturated aqueous solutions. J. Phys. Chem. 84, 2088–2089