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PAPER

Oxidation of 10-undecenoic acid by cytochrome $P450_{BM-3}$ and its Compound I transient†

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Oxidations of 10-undecenoic acid by cytochrome $P450_{BM-3}$ and its Compound I transient were studied. The only product formed in Compound I oxidations was 10,11-epoxyundecanoic acid, whereas the enzyme under turnover conditions gave the epoxide and 9-hydroxy-10-undecenoic acid in a 10 : 90 ratio. Kinetic studies at 0 °C of oxidations by Compounds I formed by MCPBA oxidation and by a photo-oxidation pathway gave the same results, displaying saturation kinetics that yielded equilibrium binding constants and first-order oxidation rate constants that were experimentally indistinguishable. Oxidation of 10-undecenoic acid by Compound I from CYP119 generated by MCBPA oxidation also gave 10,11-epoxyundecanoic acid as the only product. CYP119 Compound I bound the substrate less strongly but reacted with a faster oxidation rate constant than $P450_{BM-3}$ Compound I. The kinetic parameters for oxidation of the substrate by $P450_{BM-3}$ under turnover conditions were similar to those of the Compound I transient even though the products differed.

Introduction

The heme-containing cytochrome P450 (CYP or P450) enzymes are nature's primary oxidants.[1] The oxidizing transients formed in these enzymes have not been detected under turnover conditions, but they have long been assumed to be oxoiron(IV) porphyrin radical cations, known as Compounds I, similar to the oxidized transients formed in other heme-containing enzymes, peroxidase and catalase enzymes.[2] Nonetheless, the wide diversity of P450-catalyzed reactions[3] and experimental results accumulated in the past two decades suggest that multiple oxidant types are produced in the P450s.[4] Noteworthy were studies of P450s and mutants lacking a highly conserved threonine in the active site where dramatic variations in product distributions were found for the wild type and mutant enzymes.[5–10] The typical explanation of the product variations is that the mutations altered the partition between Compound I and an alternate oxidant, a hydroperoxy-iron species or its kinetic equivalent, iron-complexed hydrogen peroxide.[4]

Given the inability to detect the P450 oxidants under turnover conditions and the possibility that multiple oxidizing species might be formed in P450s, systematic studies of reactions by a single P450 oxidant that can be compared to results of oxidations under turnover conditions are desired. The P450 Compound I transient is commonly thought to be produced by oxidation of resting enzyme with peroxy acids,[11] and evidence for formation of Compound I by *m*-chloroperoxybenzoic acid (MCPBA) oxidations of P450s has been reported. Egawa and co-workers reported in 1994 that MCPBA oxidation of $P450_{cam}$ (CYP101) gave a species identified by UV-visible spectroscopy as Compound I.[12] Similarly, Sligar and co-workers reported in 2002 that CYP119, a P450 from a thermophile, was oxidized to its Compound I transient,[13] and more recently Rittle and Green studied the EPR and Mössbauer spectra of this intermediate.[14] Dawson and co-workers reported that Compound I transients could be detected in a mutant of $P450_{BM-3}$ (CYP102A1).[15] An alternative entry to P450 Compound I transients involves a photooxidation method where a Compound II species, an oxoiron(IV) neutral porphyrin species, is photooxidized to give Compound I,[16] although this approach has been criticized.[17]

In the present work, we report oxidations of 10-undecenoic acid by a P450 Compound I transient formed by both MCPBA oxidation of the resting enzymes and photooxidation of the Compound II intermediate, and we compare the results to those for oxidations by the P450 under turnover conditions. The Compound I transient formed by the two methods gave the same results, oxidizing 10-undecenoic acid to the corresponding epoxide with the same rate constants, but oxidation of the substrate under natural conditions gave predominantly the allylic alcohol product 9-hydroxy-10-undecenoic acid. This study provides benchmark kinetic results for analysis of a P450 Compound I oxidation and raises new questions about P450 mechanisms that should be addressed in future studies. In addition, the change in product ratio for Compounds I and the enzyme under turnover conditions

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† Electronic Supplementary Information (ESI) available: Representative kinetic results and NMR spectra of authentic samples of oxidation products **2** and **3**.

suggests that multiple oxidizing agents can be formed in the P450 under natural conditions, which is consistent with the results of previous studies.[4]

Experimental

Enzymes

Cytochrome P450$_{BM-3}$ (CYP102A1) was expressed and purified as previously described.[18] The enzyme had $Rz = 0.8$.

The heme domain from cytochrome P450$_{BM-3}$ (termed CYP102$_{HD}$) was expressed in *E. coli* and purified as previously described.[18] The enzyme had $Rz = 1.5$.

The cytochrome P450 enzyme CYP119 was expressed in *E. coli* and purified as previously described.[19] The purified enzyme had $Rz = 1.7$.

General

10-Undecenoic acid (1) and *m*-chloroperoxybenzoic acid (MCPBA) were commercial samples. Sodium peroxynitrite solutions were prepared by the method of Uppu and Pryor.[20] NMR spectra were recorded at 500 MHz (^{1}H) or 125 MHz (^{13}C) on $CDCl_3$ solutions containing tetramethylsilane (TMS) as a standard.

Preparation of authentic samples of oxidation products

10,11-Epoxyundecanoic acid (2) was prepared in 63% yield by MCPBA oxidation of acid **1** by a reported method.[21] The isolated acid **2** was characterized by NMR spectroscopy and shown to be spectroscopically identical to the compound previously reported.[21] GC and NMR analysis indicated that the authentic sample was >98% pure.

9-Hydroxy-10-undecenoic acid (3) was prepared by selenium dioxide oxidation of acid **1** using the general method of Sharpless and Lauer.[22] The product was isolated in 48% yield and was characterized by its ^{1}H and ^{13}C NMR spectra, which matched those previously reported.[23] No detectable amount of the isomeric 11-hydroxy-9-undecenoic acid was observed.

Product studies

Samples of Compounds I were prepared by mixing the P450 enzyme (10 nmol) with two equivalents of MCPBA in 100 mM phosphate buffer (pH 7) at *ca.* 4 °C in the stopped-flow apparatus used for kinetic studies (see below). The enzyme concentration in the preparation stage was 10 μM. The samples were monitored at 416 nm (CYP119) or 419 nm (CYP102$_{HD}$) until signal decay ceased (*ca.* 100 ms for CYP119 and 700 ms for CYP102$_{HD}$). The samples were then mixed with equal volume buffer solutions containing *ca.* 1 mM acid **1** and allowed to react for several minutes. The product mixtures were extracted with CH_2Cl_2, and palmitic acid standard was added to the mixture. The product mixture was treated with excess diazomethane and anlyzed by GC on a bonded phase Carbowax column. Typical results are shown in Fig. 1, and the yields are reported in Table 1.

A similar set of reactions was performed where the substrate was present in the mixture of enzyme before addition of 10 equivalents of MCPBA. Product work-up and analysis was the same as above.

Table 1 Yields of products from oxidations of 10-undecenoic acid[a]

Enzyme	Method	Yield (%)
CYP102$_{HD}$	MCPBA A[b]	48
	MCPBA B[c]	80
	photo[d]	50
CYP102A1	turnover[e]	50
CYP119	MCPBA A[b]	40
	MCPBA B[c]	78
	photo[d]	50

[a] Yield of acid **2** unless noted. [b] Single turnover for oxidations of MCPBA-generated Compound I; yield based on Compound I. [c] Multiple turnover for oxidations with excess MCPBA; yields based on MCPBA. [d] Single turnover for photo-generated Compound I; yields based on Compound I. [e] Multiple turnover for oxidations under turnover conditions; the products were **2** and **3** in a 10 : 90 ratio; yield based on NADPH.

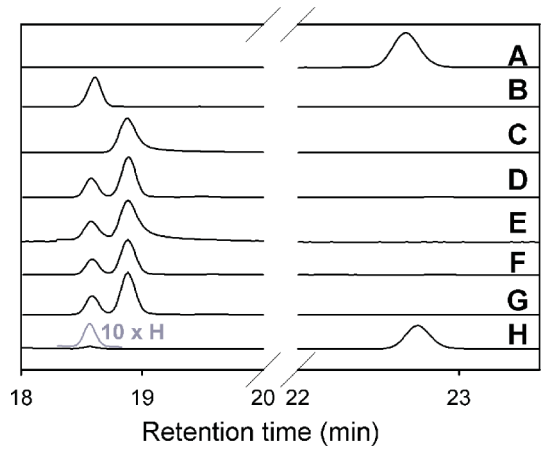


Fig. 1 GC results from analyses of oxidations of 10-undecenoic acid (Carbowax column). (**A**) Authentic methyl 9-hydroxy-10-undecenoate. (**B**) Authentic methyl 10,11-epoxyundecanoate. (**C**) Methyl palmitate (standard) which is present in D–G. (**D**) Methyl esters of products from reaction of MCPBA-generated CYP102$_{HD}$ Compound I. (**E**) Methyl esters of products from reaction of photooxidation-generated CYP102$_{HD}$ Compound I. (**F**) Methyl esters of products from reaction of MCPBA–generated CYP119 Compound I. (**G**) Methyl esters of products from photooxidation-generated CYP119 Compound I. (**H**) Methyl esters of products from reaction of CYP102A1 under turnover conditions; note the 10x expansion in grey for the region for elution of the minor product.

A similar set of reactions was performed where the Compound I transients were prepared by the photooxidation method.[24] In short, the P450 enzyme (10 nmol) in 100 mM phosphate buffer (pH 7) at 4 °C containing 10 equivalents of acid **1** was treated with 15 equiv. of sodium peroxynitrite (PN) solution. The final enzyme concentration was 5 μM. When decay of PN was complete as determined by following the signal at 300 nm, the mixture was irradiated with *ca.* 4 J of 365 nm light. Product work-up and analysis was the same as above.

For CYP102A1 product studies, 10 nmol of enzyme and excess acid **1** (*ca.* 2 μmol) in 100 mM phosphate buffer (pH 7) at 0 °C was mixed with a buffer solution containing 320 nmol of NADPH. The final volume after mixing was 2 mL. After decay of NADPH was complete (as determined by monitoring at 340 nm), the mixture was worked up and analyzed as described above.

Table 2 Equilibrium binding constants and rate constants for reactions of 10-undecenoic acid

Enzyme	Method	T/°C[a]	K_{bind}/M^{-1}[b]	k_{ox}/s^{-1}[b]
CYP102$_{HD}$	MCPBA[b]	0	730 ± 50	1.08 ± 0.04
CYP102$_{HD}$	photo[d]	0	750 ± 300	0.96 ± 0.20
CYP102A1	turnover[e]	0	2000 ± 400	1.9 ± 0.4
CYP119	MCPBA[c]	4	330 ± 80	26 ± 5

[a] ± 1 °C. [b] Errors are 1σ. [c] Production of Compound I by MCPBA oxidation of resting enzyme. [d] Production of Compound I by photooxidation of Compound II. [e] Method of initial rates for decay of NADPH analyzed by Michaelis–Menten kinetics.

Kinetic studies

Kinetic studies with MCPBA-generated Compound I were performed in a commercial four-syringe stopped-flow mixing kinetic spectrometer (Applied Photophysics, model SX-18) equipped with either diode array detection or photo-multiplier detection. In a typical study, the enzyme (10 nmol) in 100 mM phosphate buffer (pH 7) at 0 or 4 °C was mixed with 2 equivalents of MCPBA in the first push of the syringes and aged for 100 ms (CYP119) or 700 ms (CYP102$_{HD}$). The aged Compound I solution was then mixed with the solution of substrate in the reactor cell, and the reaction was monitored as above. The final enzyme concentration was 5 μM. The first-order rate constant for decay of Compound I in the absence of substrate was subtracted from the first-order rate constant for decay in the presence of substrate to give the results shown in Fig. 2–3. This data was solved according to eqn (1) in the text to give the values of K_{bind} and k_{ox} listed in Table 2.

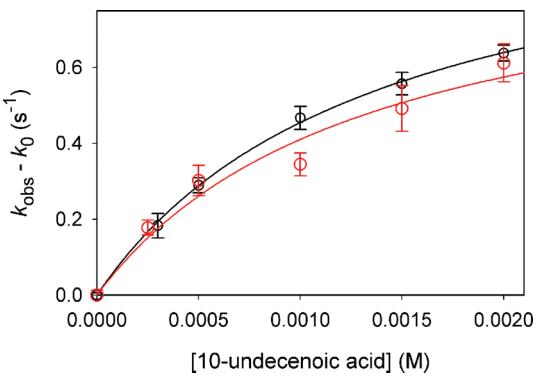


Fig. 2 Rate constants for reactions of CYP102$_{HD}$ Compound I with 10-undecenoic acid at 0 °C. Black symbols and line: MCPBA-generated Compound I. Red symbols and line: photooxidation-generated Compound I. The lines are from the equilibrium binding constants and rate constants listed in Table 2.

Kinetic studies with Compound I from CYP102$_{HD}$ generated by photooxidation were conducted as previously described.[24,25] The reactions were studied at 0 °C. The enzyme concentrations were 5 μM in 100 mM phosphate buffer (pH 7). The data was analyzed as above using eqn (1), and the values for K_{bind} and k_{ox} are in Table 2.

Kinetic studies with CYP102A1 were conducted in a temperature-regulated reactor mounted in an Agilent 8453 spectrometer. The enzyme (1 nmol) and substrate were equilibrated in 100 mM phosphate buffer (pH 7), and NADPH (320 nmol) was added. The final volume was 2.0 mL. Decay of NADPH was monitored at 340 nm, and rate constants for decay of NADPH were determined from the initial slopes of the kinetic traces and analyzed by eqn (1) to give the values in Table 2.

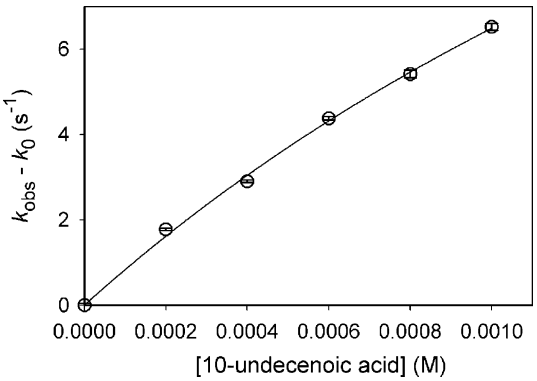


Fig. 3 Rate constants for reactions at 4.2 °C of CYP119 Compound I with 10-undecenoic acid. The line was generated from the equilibrium binding constant and rate constant listed in Table 2.

Results

Enzymes

The enzymes used in these studies were cytochrome P450$_{BM-3}$ (CYP102A1), the heme domain of this P450, and CYP119. CYP102A1 from *Bacillus megaterium* and its mutants have been widely studied in part due to its potential for commercial applications in bioremediation or synthesis of fine chemical intermediates.[26] It is one of the few known P450 fusion proteins containing both a heme domain (or oxidase domain) analogous to a conventional P450 and a reductase domain analogous to P450 reductase. Thus, CYP102A1 is active in the presence of NADPH with no additional enzymes. The heme domain of CYP102A1 has been termed CYPBMP, but we label it CYP102$_{HD}$ here for clarity. This enzyme is similar to a conventional P450 enzyme.

CYP119 is a P450 from the thermophile *Sulfolobus acidocaldarius*.[27] A unique aspect of this P450 is that the Compound I transient is known to be formed by oxidation of the resting enzyme with *m*-chloroperoxybenzoic acid (MCPBA), and the UV-visible spectrum of the CYP119 Compound I was reported by Sligar and co-workers.[13] More recently, Rittle and Green reported EPR and Mössbauer spectroscopic studies of the Compound I species.[14]

Compound I production in MCPBA oxidations

The formation of Compound I by MCPBA oxidation of CYP119 was reported by Kellner *et al.* in 2002.[13] The spectroscopic signature for P450 Compound I has long been assumed to be a long-wavelength Q-band absorbance centered at about 690 nm,[11] but that absorbance is weak, and kinetic studies are more conveniently performed at *ca.* 416 nm where a growth in signal is observed when Compound I reacts to give resting enzyme.[13,14,24]

In studies of CYP119 Compound I, we used the same methods as described by Rittle and Green.[14] The only difference was that they used diode array detection with a minimum integration time of *ca.* 1 ms,[14] whereas we used photomultiplier tubes with 2 ns rise times, which increased our dynamic kinetic range by a factor of greater than 10^6 and permitted studies with higher

substrate concentrations than they employed. Nonetheless, we measured kinetics with very low substrate concentrations as in the Rittle and Green's studies[14] so that we could determine apparent second-order rate constants under their conditions. For lauric acid oxidations at concentrations they used, we obtained an apparent second-order rate constant of $k_{app} = (1.2 \pm 0.3) \times 10^7$ M^{-1} s^{-1}, which compares favourably with the value of 1.1×10^7 M^{-1} s^{-1} reported by Rittle and Green.[14]

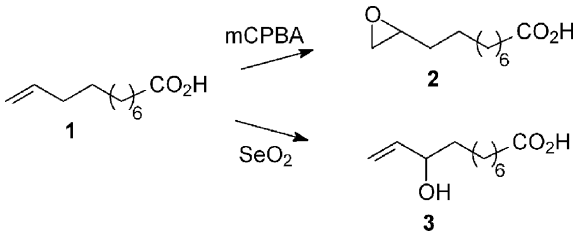
The Compound I transient from $CYP102_{HD}$ was studied in the same manner as that from CYP119. MCPBA oxidations of this enzyme were reported not to give a Compound I transient as determined in freeze-quench studies with EPR and Mössbauer spectroscopy analyses with very short mixing times,[28,29] but UV-visible spectroscopy studies of MCPBA oxidation of the CYP102A1 F87G mutant revealed a Compound I transient with a Soret band absorbance at 406 nm[15] that resembled the Soret band observed when CYP119 was oxidized with MCPBA.[13] In our studies at 0 °C, reaction of $CYP102_{HD}$ with MCPBA gave a spectroscopic signature essentially the same as that reported for CYP119 Compound I by Raner *et al.*[15] A Soret band absorbance at 406 nm was generated, and this feature decayed to give an absorbance at 420 nm from the resting enzyme. An important point was that we used highly purified enzyme and only 2 equivalents of MCPBA. These conditions match those reported by Rittle and Green,[14] and, as in their case with CYP119, gave strong signals in the Soret band region in kinetic studies. When large excesses of MCPBA were used, extensive decomposition of the enzyme was observed after complete decay of the peroxy acid.

Compound I formation by photooxidation

Compound I production *via* the photooxidation method was described for both CYP119 and $CYP102_{HD}$,[24,25] and the same method was used in this work. In this method, the resting enzyme is oxidized to its Compound II derivative, an oxoiron(IV) neutral porphyrin, by reaction with peroxynitrite (PN),[30] and the Compound II species is photooxidized to give Compound I.[16] At high concentrations, PN will degrade the P450 enzyme,[30] but no loss in enzyme activity was found when $CYP102_{HD}$ was treated with PN at the concentrations used in the present study.[24] The photooxidation method was criticized by Green and co-workers[17] based on an earlier report by the same group that claimed that $CYP102_{HD}$ reacted with PN to give a nitrosyl derivative and not Compound II.[31] However, it is apparent that an authentic sample of the nitrosyl derivative was not used in the Green and co-workers study[31] because both the UV-visible spectrum and life-time of the supposed nitrosyl complex did not match those previously published for a *bona fide* sample.[32] In addition, the product of $CYP102_{HD}$ from PN treatment was specifically demonstrated not to be the nitrosyl complex by both thermal and photochemical reactions,[25] and XAS studies of the Compound II species from reaction of CYP119 with PN demonstrated that the nitrosyl complex was not formed in the PN reaction.[33] Finally, the demonstrations in this work that the Compound I transients produced from both the MCPBA and photooxidation pathways give exactly the same products and kinetics in reactions with substrate **1**, as described in the sections below, further confirm that Compound I was formed in the photooxidation reaction.

Oxidation products

Most fatty acid hydroxylases oxidize fatty acids at the hydrocarbon end, and lauric acid oxidation occurs mainly at the ω-1, ω-2 and ω-3 positions with both CYP102A1[18] and CYP119.[19,34] We expected that oxidations of 10-undecenoic acid (**1**) would occur at and/or adjacent to the terminal double bond, and authentic samples of 10,11-epoxyundecanoic acid (**2**) and 9-hydroxy-10-undecenoic acid (**3**) were prepared for identification of the P450-catalyzed oxidation products (Scheme 1). For analysis of the products, authentic acids **2** and **3** were treated with excess diazomethane to give the methyl esters that were analyzed on a polar GC column.



Scheme 1

Acid (**2**) is a known compound, and we prepared a sample by the literature method.[21] Acid (**3**) is a known natural product,[23] and it was previously reported to be formed by selenium dioxide oxidation of acid **1** although details were not reported in that work.[23] We prepared **3** using the protocol reported by Sharpless and Lauer.[22] Allylic oxidations by selenium dioxide proceed by an initial ene reaction followed by a [2.3]-sigmatropic rearrangement with the result that the reactions are highly regioselective giving an allyl alcohol product with the position of the double bond unchanged from that in the initial substrate.[22] In our preparation, alcohol **3** was obtained in *ca.* 45% yield after purification by silica gel chromatography. It was characterized by its ^1H and ^{13}C NMR spectra, which matched those previously reported.[23] As expected from the mechanism of the oxidation,[22] no detectable amounts of isomeric 11-hydroxy-9-undecenoic acids were indicated.

Products from oxidations of 10-undecenoic acid under various conditions were studied by GC analysis of the corresponding methyl esters. Following the oxidation reactions, the product mixtures were treated with excess diazomethane, and the methyl ester derivatives were analyzed by GC on a polar column. Fig. 1 shows typical results. Oxidations by Compounds I formed by either the MCPBA or photooxidation method gave epoxy-acid **2** as the only detectable oxidation product. Surprisingly, oxidation of **1** by CYP102A1 under turnover conditions gave mainly the allyl alcohol product **3** with a minor amount of epoxide **2** (*ca.* 90 : 10 ratio of **3**:**2**). The sharply different product mixtures obtained from reactions of Compound I and the enzyme under turnover conditions is reminiscent of grossly different product mixtures obtained from wild-type and mutant P450s that led to conclusions that multiple oxidizing species were formed in the enzyme under natural turnover.[4] In any event, these results demonstrate the importance of isolating a single oxidizing species for characterization.

Yields from oxidations by Compounds I were determined under various conditions, and the results are listed in Table 1. We used two sets of conditions for Compounds I generated by MCPBA

oxidations. In one set, Compound I was produced before addition of substrate, and the yields are based on enzyme. In the other, excess substrate was present before addition of MCPBA, and the yields are based on MCPBA. When Compound I was generated by photooxidation, the reactions were single turnover processes, and the yields are based on enzyme. For CYP102A1 under turnover conditions, the yields are based on NADPH.

The noteworthy aspects of the product yields are that the MCPBA oxidation and photooxidation methods gave essentially the same results for single turnover reactions with yields of about 50%. In both cases, Compound I was decaying in non-productive reactions that competed with substrate oxidation.

Kinetics of oxidations

Compound I oxidations were studied in a stopped-flow reactor at 0 or 4 °C. The enzyme in a buffer solution was mixed with a solution containing MCPBA, and the mixture was aged until excess MCPBA decayed (*ca.* 100 ms for CYP119 and 700 ms for $CYP102_{HD}$). The aged solution containing Compound I was then mixed with a solution containing substrate **1**, and kinetics were monitored at 416 or 419 nm. At these wavelengths, decay of Compound I resulted in an increase in absorbance as noted by Rittle and Green in their kinetic studies with CYP119 Compound I.[14] Those workers used a diode array spectrometer, and we monitored some reactions with a diode array spectrometer, but our kinetic studies were performed with a photomultiplier tube due to the fast response time (2 ns rise time).

Representative kinetic traces of reactions of acid **1** at varying concentrations with Compounds I from both enzymes are shown in the Supporting Information. The observed pseudo-first order rate constants (k_{obs}) were corrected for the background first-order decay of Compound I in the absence of substrate (k_0), and the results for $CYP102_{HD}$ Compound I are shown in Fig. 2. We found saturation kinetics as is typical for enzyme reactions. This kinetic behaviour indicates equilibration of the substrate in the active site of the Compound I transient (Scheme 2) because we were studying single turnover reactions where dissociation of the enzyme from the active site was not important. The data was analyzed according to eqn (1) where K_{bind} is the equilibrium constant in units of M^{-1}, k_{ox} is a first-order rate constant in units of s^{-1}, and [Subs] is the molar concentration of substrate. The derived values for K_{bind} and k_{ox} are listed in Table 2, and these values were used to generate the lines in Fig. 2.

$$(k_{obs} - k_0) = (K_{bind}k_{ox}[Subs])/(K_{bind}[Subs] + 1) \quad (1)$$

$$\text{Cmpd I} + \text{Subs} \underset{}{\overset{K_{bind}}{\rightleftharpoons}} \text{ES}$$

$$\text{ES} \xrightarrow{k_{ox}} \text{EP}$$

$$\text{EP} \longrightarrow \text{Enz} + \text{Prod}$$

Scheme 2

The reaction of 10-undecenoic acid with $CYP102_{HD}$ Compound I was slow enough at 0 °C to permit studies with the photooxidation method for generation of Compound I. Specifically, irradiation times in the range 0.5 to 1.0 s are required to complete the photooxidation step, and the half-life for decay of $CYP102_{HD}$ Compound I in the presence of relatively high concentrations of acid **1** at 0 °C was greater than 1 s. A series of kinetic studies using the photooxidation method was performed at 0 °C, and the results are shown in Fig. 2 as the red symbols. Again, the kinetic parameters were determined from eqn (1) and are listed in Table 2. The results for the photooxidation method were less precise than those for the MCPBA oxidation method, but the results for the two methods of forming Compound I are essentially the same.

Kinetics for reaction of CYP119 Compound I with 10-undecenoic acid also were studied, and the results are shown in Fig. 3 and listed in Table 2. CYP119 Compound I binds the substrate less strongly than $CYP102_{HD}$ Compound I, resulting in larger percentage errors. Nonetheless, it is apparent that the rate constant for the CYP119 reaction is greater than that for CYP102. Due to the fast reaction, it was not possible to employ the photooxidaiton entry to CYP119 Compound I at 4.2 °C. In principle, low temperature studies could be accomplished if a cryo-solvent containing glycerol was employed.

The kinetics of reactions of CYP102A1 at 0 °C under turnover conditions were determined by following the decay of NADPH. Initial rates of decay were determined and analyzed by conventional Michaelis–Menten kinetics. The results are shown graphically in Fig. 4. The Michaelis constant was $K_M = (0.52 \pm 0.1) \times 10^{-3}$ M, which corresponds to the binding constant K_{bind} in Table 2. The V_{max} value was $(1.9 \pm 0.2) \times 10^{-6}$ mmol NADPH per nmol enzyme s^{-1}, which converts to a rate constant of 1.9 ± 0.4 s^{-1}. Because the yield of oxidized products for the enzyme under turnover was 50% based on NADPH consumption, the rate constant for oxidation is 1.0 ± 0.2 s^{-1}. The values listed in Table 2 are close to the values for the equilibrium binding constant and rate constant found in the reactions of Compound I, suggesting that the model for oxidation in Scheme 2 applies for the enzyme under turnover where the release of product is fast. One notes, of course, that the products formed under turnover consist mainly of the allyl alcohol product **3**, whereas epoxide **2** was the only product formed by Compound I oxidations.

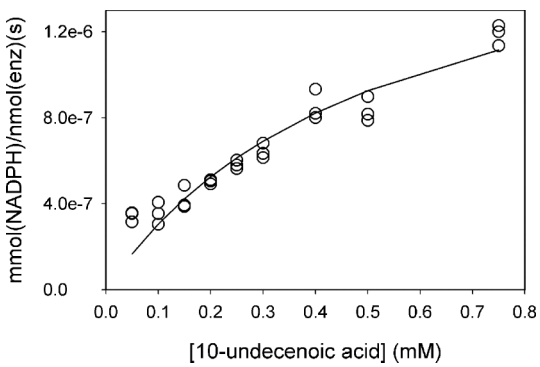


Fig. 4 Rate of NADPH consumption as a function of substrate concentration.

Discussion

The rates of oxidations of 10-undecenoic acid were optimal for two major objectives of our study. One of our primary aims was to study a substrate that reacted with P450 Compound I at a

rate appropriate for generation of Compound I by both stopped-flow mixing (MCPBA oxidation) and photooxidation methods. Whereas stopped-flow methods are appropriate for mixing and aging times of less than 1 s, the photooxidation method using lamp photolysis requires about one second irradiation time for complete conversion of Compound II.[24,25] Thus, the reactions consuming Compound I should have a half-life of at least 0.5 s for the photooxidation method, but they must also be appreciably faster than background decay reactions. For $CYP102_{HD}$ Compound I at 0 °C, the background decay reaction had a half-life of about 7 s where the fastest reaction we studied (substrate concentration of 2 mM) had a half-life of about 1 s, and both the MCPBA and photooxidation methods could be studied at the same temperature with this enzyme. It is clear from the results in Table 2 that the two methods for production of Compound I gave the same kinetic parameters.

For CYP119 Compound I, the background decay reaction at 4.2 °C and reactions with substrate were too fast for the photooxidation entry to Compound I. Studies at low temperatures might be accomplished, but that would require a drastic change in solvent to a mixture of buffer and glycerol,[24,25] and the effect of the solvent change cannot be predicted.

Another reason for using 10-undecenoic acid as a substrate was that we could use reasonably high concentrations of the salt in buffer solutions such that accurate kinetic results could be obtained. The results for $CYP102_{HD}$ clearly demonstrate saturation kinetics behaviour with a prior equilibration to give a reactive complex that subsequently reacts in a rate-limiting first-order reaction to give product (Scheme 2), which is a typical model for enzyme kinetics. Because we were studying single turnover reactions of Compound I, product release steps could not be involved in the overall kinetics, and we did not use a Michaelis–Menten approach where product release steps can be important. Our kinetic analysis *via* eqn (1) yields equilibrium binding constants (K_{bind}) and a first-order rate constant (k_{ox}).

For CYP119 Compound I, saturation kinetics behaviour is less obvious than for $CYP102_{HD}$ because the binding constant for CYP119 Compound I is considerably smaller than that for $CYP102_{HD}$. Nonetheless, curvature is present in the data shown in Fig. 3. We note that the Rittle and Green did not observe curvature in their kinetic studies with CYP119 Compound I, but they studied lower concentrations for, for example, lauric acid than we studied in this work.[14]

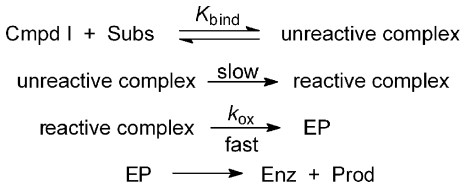
The binding constants for Compounds I from the two enzymes differ in a manner similar to those for substrate binding by the resting enzymes. CYP102A1 is a fatty acid hydroxylase that binds fatty acids relatively strongly, and the binding constant for 10-undecenoic acid by CYP102A1 at room temperature determined in this work was 1000 M^{-1}. The binding constant for $CYP102_{HD}$ Compound I at 0 °C was K_{bind} = 750 M^{-1} indicating that the substrate was held less strongly in the active site of the activated enzyme than in the active site of the resting enzyme. The function of CYP119 in nature is not known, but it has been shown to bind fatty acids less strongly than CYP102A1.[19] For CYP119 Compound I, the binding constant for **1** at 4.2 °C was K_{bind} = 330 M^{-1}, a factor of 2 smaller than that for $CYP102_{HD}$ Compound I.

The first-order rate constants for reactions of Compounds I with acid **1** were similar with a difference in free energies of activation at 0–4 °C of only 1.5 kcal/mol. First-order reactions typically have small entropy of activations resulting in pre-exponential terms in their Arrhenius functions of approximately log A = 13, which is the value when $\Delta S^{\ddagger} = 0$ e.u. Indeed, variable temperature studies of oxidations by Compounds I from CYP119 and $CYP102_{HD}$ found log $A \approx 13$ for epoxidations of styrene and benzylic C–H oxidations of benzyl alcohol.[24,25] If one assumes that the entropy of activation is small for oxidations of substrate **1** by Compounds I, then the difference in activation energies for oxidations also is 1.5 kcal mol^{-1}, and the oxidation reactions have activation energies of $E_a \approx$ 16.5 kcal mol^{-1} for $CYP102_{HD}$ Compound I and $E_a \approx$ 15.0 kcal mol^{-1} for CYP119 Compound I.

The difference in activation energies for reactions of 10-undecenoic acid with the two Compounds I species can be compared to the activation energies for epoxidation reactions of styrene with the same two P450 Compounds I. Specifically, for styrene, E_a = 15.2 kcal mol^{-1} for $CYP102_{HD}$ Compound I, and E_a = 14.6 kcal mol^{-1} for CYP119 Compound I.[25] The increased activation energies for oxidations of the unactivated double bond in acid **1** compared to the activated double bond in styrene is consistent with previously reported kinetics of alkene oxidations by Compound I models. For example, 5,10,15,20-tetramesitylporphyrin-iron(IV)-oxo radical cations with chloride and perchlorate counterions oxidized styrene 3 times faster than they oxidized cyclohexene ($\Delta\Delta G^{\ddagger} \approx 0.6$ kcal mol^{-1}).[35]

Oxidation of 10-undecenoic acid by CYP102A1 under turnover conditions gave unusual results that require comment. Both epoxide **2** and allylic alcohol **3** were formed in the reactions, and the allyl alcohol was by far the major product. Any explanation for this change in product distribution will be speculative at this time, but, given the high regioselectivity found for Compounds I in forming only epoxide **2**, it is possible that the formation of product **3** indicates production of a second oxidant in the P450 under turnover conditions in addition to Compound I. A second oxidant might be a hydroperoxy-iron species or its kinetic equivalent iron-complexed hydrogen peroxide as first proposed nearly two decades ago by Pratt.[36] It is noteworthy that changes in product selectivity were found in studies of wild-type and mutant P450s where a highly conserved threonine was removed from the active site,[6–8,10] and those observations generally were rationalized in terms of formation of multiple oxidants.[4]

Despite the dramatic change in product distribution, the kinetics of oxidations of acid **1** under turnover by CYP102A1 were similar to those for reactions of $CYP102_{HD}$ Compound I. At 0 °C, the binding constant was within a factor of 2 of that for Compound I, and the first-order rate constant at 0 °C was similar to that found for Compound I. The similarity in binding constants might not be surprising, but the similarity in rate constants is unexpected given that the reactions are different. It is possible that the reactions coincidentally have the same rate constants, but there is another possible explanation for this finding. In principle, the reactions could be more complex than shown in Scheme 2 and involve a rate-limiting first-order reaction that precedes the oxidation step as shown in Scheme 3. In this complex model, equilibrium substrate binding is followed by rate-limiting process, a conformational change for example, and then a fast oxidation step. For Scheme 3, the kinetics measured would be the equilibrium constant for binding and the first-order rate constant for conformational change.



Scheme 3

Scheme 3 might be unnecessarily complex, but there is some support for this type of P450 oxidation model. For the kinetics measured in this work, it is difficult to rationalize why Compound I from CYP119 should be 25 times more reactive than Compound I from $CYP102_{HD}$, which seems to be a rather large difference for two similar reactive species. Moreover, the experimental first-order rate constants seem to be too small to be those for the epoxidation reaction given that P450 Compounds I can oxidize unactivated C–H bonds readily. In addition, Green and co-workers found apparent second-order rate constants for CYP119 Compound I oxidations of lauric acid and its perdeuterated isotopomer that displayed no kinetic isotope effect (KIE) (*i.e.* $k_H/k_D = 1$),[14] whereas large KIEs were found for oxidation of benzyl alcohol and its deuterated analog by the CYP119 Compound I,[24] and Compound I models oxidized benzyl alcohol with a very large KIE indicating substantial tunnelling.[37] The mechanistic details for the P450 Compounds I oxidations clearly require more study.

Conclusions

In conclusion, this work adds to the nascent field of cytochrome P450 Compound I kinetics and provides benchmark binding and kinetic data for computational studies. At the same time, it shows that more mechanistic work will be required to understand the details of these reactions. One of the more important points of the work is the demonstration that the MCBPA oxidation method and the photooxidation method give the same results when the experimental conditions are the same. The MCPBA method requires stopped-flow mixing units that are not available in most laboratories and cannot be used readily for studies at temperatures below 0 °C. The photooxidation method is most readily applicable for low temperature studies, although it requires a kinetic unit assembled in house.[24] Together, the methods will permit kinetic studies over a wide range of temperatures.

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