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Indole Hydroxylation by Bacterial Cytochrome P450 BM-3 and Modulation of Activity by Cumene Hydroperoxide

Qing-Shan LI,[1] Jun OGAWA,[1] Rolf D. SCHMID,[2] and Sakayu SHIMIZU[1,†]

[1]*Division of Applied Life Sciences, Graduate School of Agriculture, Kyoto University, Sakyo-ku, Kyoto 606-8502, Japan*
[2]*Institut für Technische Biochemie, Universität Stuttgart, Allmandring 31, D-70459 Stuttgart, Germany*

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Cytochrome P450 BM-3 from *Bacillus megaterium* catalyzed NADPH-supported indole hydroxylation under alkaline conditions with homotropic cooperativity toward indole. The activity was also found with the support of H_2O_2, *tert*-butyl hydroperoxide (tBuOOH), or cumene hydroperoxide (CuOOH). Enhanced activity and heterotropic cooperativity were observed in CuOOH-supported hydroxylation, and both the Hill coefficient and substrate concentration required for half-maximal activity in the CuOOH-supported reaction were much lower than those in the H_2O_2-, tBuOOH-, or NADPH-supported reactions. CuOOH greatly enhanced NADPH consumption and indole hydroxylation in the NADPH-supported reaction. However, when CuOOH was replaced by tBuOOH or H_2O_2, heterotropic cooperativity was not observed. Spectral studies also confirmed that CuOOH stimulated indole binding to P450 BM-3. Interestingly, a mutant enzyme with enhanced indole-hydroxylation activity, F87V (Phe87 was replaced by Val), lost homotropic cooperativity towards indole and heterotropic cooperativity towards CuOOH, indicating that the active-site structure affects the cooperativities.

Key words: cytochrome P450; P450 BM-3; cumene hydroperoxide; cooperativity; peroxide shunt pathway

Cytochrome P450 monooxygenases (P450s) have vast potential for environmental treatment, drug design, and pharmaceutical and chemical syntheses.[1)] In this regard, the active site structures and interactions with substrates of P450s have been the focus of much interest. Several mammalian P450s with a large active site exhibit homotropic cooperativity towards a number of substrates, and their activities are also influenced by effectors. P450 3A4, the most important and abundant P450 in the liver, is one of the most extensively studied. In addition to its homotropic cooperativity towards several substrates, including progesterone,[2–4)] testosterone,[5)] 17β-estradiol,[5)] aflatoxin B1,[5)] and amitriptyline,[5)] the enzyme activity is influenced by several effectors. α-Naphthoflavone (ANF) stimulates P450 3A4 activity on the oxidation of progesterone,[2–4)] testosterone,[4)] estradiol,[6)] aflatoxins,[7,8)] polycyclic aromatic hydrocarbons,[9,10)] carbamazepine,[11)] and acetaminophen.[12)] Kinetic analysis of 7,8-benzoflavone and phenanthrene oxidation by P450 3A4 indicated that they increase each other's k_{cat} values without changes in their K_m values, suggesting that the two substrates are simultaneously present in the same active site.[10)] The simultaneous binding of two substrates in one active site has also been observed in the crystal structures of P450 eryF/9-aminophenanthrene and P450 ery F/androstenedione.[13)] Based on these results, it is proposed that a P450 enzyme with an active site large enough to accommodate multiple ligands will exhibit this type of cooperativity.[13)]

A well studied bacterial P450, P450 BM-3 from *Bacillus megaterium*, catalyzes the subterminal hydroxylation of long-chain saturated fatty acids, and the corresponding amides and alcohols, or produces the epoxides of medium and long-chain unsaturated fatty acids.[14–17)] It catalyzes the oxidation of saturated or unsaturated fatty acids without cooperativity, and only one substrate has been observed in the active site in the structure of the P450 BM-3 heme domain complexed with palmitoleic acid.[18)]

Here, we report that homotropic cooperativity is involved in indole hydroxylation catalyzed by P450 BM-3 under alkaline conditions. The activity was greatly stimulated by cumene hydroperoxide (CuOOH) through heterotropic cooperativity. In addition, we created a mutant enzyme that shows high activity and affinity for indole.[19)] But, we found in this study that the mutant F87V (Phe87 was replaced by Val) does not show homotropic cooperativity towards indole and also loses heterotropic cooperativity towards CuOOH. These results suggest that the environment of the binding site influences cooperativity.

† To whom correspondence should be addressed. Tel: +81-75-753-6115; Fax: +81-75-753-6128; E-mail: sim@kais.kyoto-u.ac.jp

Abbreviations: P450, cytochrome P450; WT, wild type of P450 BM-3; F87V, Phe87Val mutant of P450 BM-3; CuOOH, cumene hydroperoxide; tBuOOH, *tert*-butyl hydroperoxide

Materials and Methods

Preparation of WT and F87V enzymes. WT and F87V enzymes were obtained as described previously.[19] The expression and purification of P450, and determination of the concentration of P450 were carried out as described previously.[20]

Enzyme activity assay. Indole hydroxylation activity was evaluated by monitoring blue color development due to indigo formation through P450-catalyzing hydroxylation and successive chemical dimerization.[19] Kinetic analysis was performed as described previously.[21] Nonlinear regression was used to determine V_{max} and S_{50}, using the equation $v = (V_{max}S^n)/(S_{50}{}^n + S^n)$. The Hill coefficient (n) was determined by plotting $\log[v/(V_{max} - v)]$ *vs.* $\log S$.

Hydroperoxide-supported reaction: To measure indole hydroxylation supported by H_2O_2, *tert*-butyl hydroperoxide (tBuOOH; Katayama Kougyo, Osaka, Japan), or CuOOH (Aldrich, Milwaukee, WI, U.S.A.), 60 µl of a 45 µM P450 sample, was mixed with 480 µl of 0.1 M Tris/HCl buffer (pH 8.6) containing 0.03 to 30 mM indole. After 2 min, 60 µl of a hydroperoxide solution was added to start the reaction. The concentrations of hydroperoxides in the reaction mixtures are indicated in Fig. 2, Table 2, and Table 3. The reaction was carried out at 25 °C and indigo formation was monitored at 670 nm for 10 min. The amount of indigo was calculated using $\varepsilon_M = 13400\,M^{-1}\,cm^{-1}$, which was obtained with pure indigo. Three types of controls were carried out under the same conditions except for the absence of one of these compositions, P450, indole, or hydroperoxides.

NADPH-supported reaction: To measure the NADPH consumption rate in indole hydroxylation, 60 µl of a 10 µM P450 sample was mixed with 480 µl of 0.1 M Tris/HCl buffer (pH 8.6) containing 0.03 to 30 mM indole, and after 2 min standing, 60 µl of 3.5 mM NADPH was added to start the reaction. NADPH consumption was monitored at 340 nm for 30 s at 25 °C and the NADPH concentration was calculated using $\varepsilon_M = 6200\,M^{-1}\,cm^{-1}$.[22] To measure the indole hydroxylation supported by NADPH, 60 µl of a 45 µM P450 sample was mixed with 480 µl of 0.1 M Tris/HCl buffer (pH 8.6) containing 0.3 to 30 mM indole, and after 2 min standing, 60 µl of 10.5 mM NADPH was added to start the reaction, followed by standing for 1 h at 25 °C. The total amount of indigo produced was determined at 670 nm. To monitor the effects of CuOOH on NADPH consumption or on NADPH-supported indole hydroxylation, the procedures were the same as those without the addition of CuOOH, except that 1 mM CuOOH was added 1 min before the addition of NADPH and the measurement of indigo was carried out after the reaction mixture had stood for 15 min at 25 °C. Four kinds of control experiments were carried out under the same conditions, except for the absence of one of these compositions: P450, indole, CuOOH, or NADPH. The NADPH-supported indole hydroxylation with 1 mM CuOOH was calculated by subtracting the values of the control without NADPH from the measured data.

Spectral binding studies. Binding spectra were recorded with a Shimadzu MultiSpec-1500 spectrophotometer. For analysis of indole binding to WT or F87V, various concentrations of indole in 0.1 M Tris/HCl (pH 8.6) were prepared (see the legend to Fig. 3), and 120 µl of a 20 µM P450 sample was added to 480 µl of the indole solutions. The difference spectra were recorded from 340 to 500 nm, using the same solution without indole as the reference. For CuOOH stimulation of indole binding to WT, 400 µl of 0.45 mM indole in 0.1 M Tris/HCl (pH 8.6) was mixed with 100 µl of a 24 µM P450 sample and 100 µl of 6 mM CuOOH in 0.1 M Tris/HCl. The spectra were recorded 30 s after all the components had been mixed together, using the same solution without indole and CuOOH as a reference at 25 °C. The change in absorbance (A) was determined by subtracting the absorbance at 425 nm (the trough) from that at 390 nm (the peak). The concentration of indole added for binding was used as the concentration of free indole for data analysis, *i.e.*, $\text{ligand}_{free} = \text{ligand}_{total}$. Calculation was carried out according to the method described previously.[21,22] ΔA_{max} was determined by nonlinear regression of the plots of ΔA *vs.* S using the equation $\Delta A = \Delta A_{max}S^n/K_s{}^n + S^n$.

HPLC analysis. HPLC analysis was performed to determine the amounts of degradation products of CuOOH. The reaction samples were extracted twice with chloroform, 6 ml in total, evaporated to dryness, and then dissolved in 5% dimethyl sulfoxide for HPLC analysis. HPLC analysis was carried out on a C18 reverse phase column (Cosmosil $5C_{18}$ AR-II, 4.6 mm I.D. × 250 mm; Nacalai Tesque, Kyoto, Japan), with elution at 1.0 ml/min with a methanol/water gradient containing 1% acetic acid. The gradient of methanol was as follows: 0–5 min, 0% (v/v); 5–50 min, 0–80%; and 50–60 min, 80%. The eluent was monitored at 254 nm.

Results

Detection of indole hydroxylation activity of WT P450 BM-3

Several mutants of P450BM-3, such as F87V, have been reported to be able to hydroxylate indole, while WT could not.[19] After extensive tests under various conditions, we found that WT also catalyzes NADPH-supported indole hydroxylation under high pH conditions. However, it is not practical to determine the indole hydroxylation activity of WT by measuring indigo formation, because the amount of indigo formed with NADPH is too small. Therefore the activity was measured by monitoring NADPH consumption. The kinetic data are summarized in Table 1. With the support of NADPH, WT exhibited a typical "S" curve

Table 1. Kinetic Data for Indole Hydroxylation Catalyzed by WT and F87V

	WT			F87V		
	k_{cat}[a]	S_{50}[b] (mM)	n	k_{cat}[a]	K_m[b] (mM)	n
NADPH	323 ± 31	13.2 ± 2.1	1.78 ± 0.24	383 ± 42	2.1 ± 0.3	1.01 ± 0.02

[a]The k_{cat} unit for NADPH is nmol NADPH/min/nmol P450.
[b]S_{50} and K_m are all for indole.

Table 2. Kinetic Analysis of Indole Hydroxylation Catalyzed by WT and F87V

	WT		F87V	
	k_{cat} of indole oxidation (nmol indigo/min/nmol P450)	K_m for hydroperoxide (mM)	k_{cat} of indole oxidation (nmol indigo/min/nmol P450)	K_m for hydroperoxide (mM)
H_2O_2	0.32 ± 0.05	20.4 ± 2.7	29.4 ± 2.2	25.6 ± 4.3
tBuOOH	0.24 ± 0.03	26.3 ± 3.5	10.9 ± 1.4	23.9 ± 3.9
CuOOH	30.7 ± 3.2	14.4 ± 1.1	45.5 ± 5.1	22.3 ± 3.1

The hydroperoxide concentrations in the reaction mixtures were in the range of 1 to 30 mM. The indole concentrations were fixed at 11.2, 11.2, 6.4, 0.9, 6.4, and 6.4 mM in the reactions with WT and H_2O_2, WT and tBuOOH, WT and CuOOH, F87V and H_2O_2, F87V and tBuOOH, and F87V and CuOOH respectively.

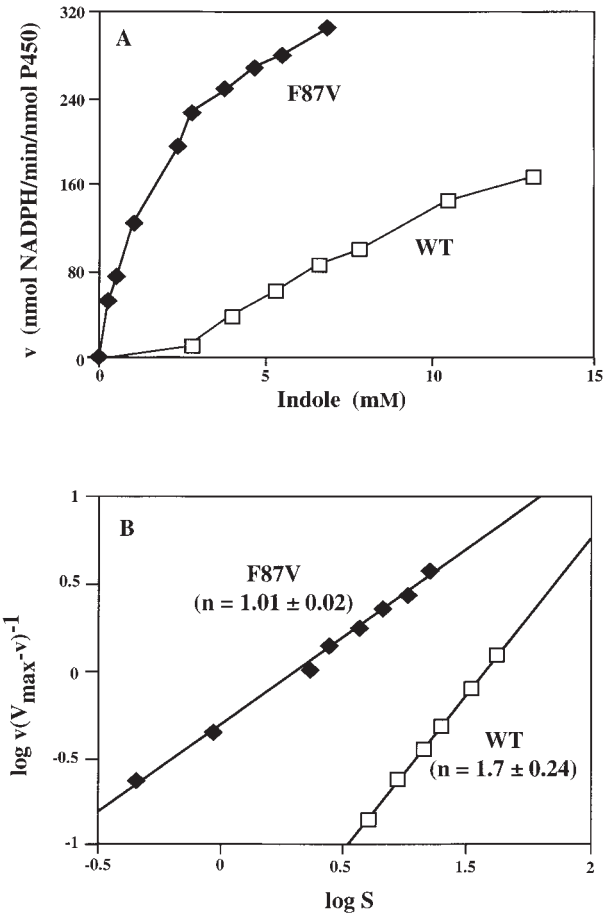

Fig. 1. NADPH Consumption by WT and F87V in Indole Hydroxylation with a Series of Concentrations of Indole (A), and Linear Regression of Plots of $\log[v/(V_{max} - v)]$ *vs.* $\log S$ (B).

Each point presents the average value for three independent measurements.

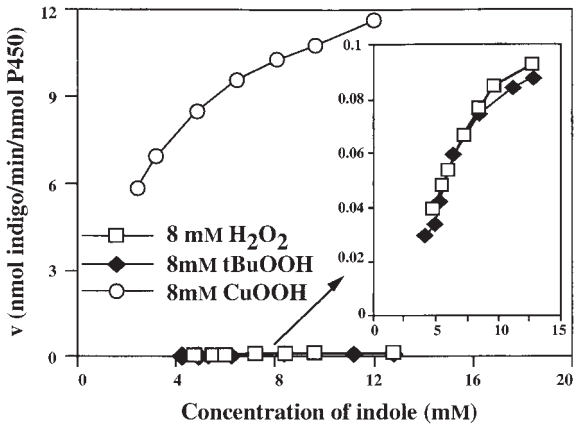

Fig. 2. Turnover Numbers of WT in Indole Hydroxylation Supported by 8 mM H_2O_2, 8 mM tBuOOH and 8 mM CuOOH with a Series of Concentrations of Indole.

Each point represents the average value for three independent measurements.

for NADPH consumption with different concentrations of indole, but F87V did not (Fig. 1A). The K_m value of F87V was less than 16% of the S_{50} for indole of WT (Table 1). The Hill coefficient was n = 1.78 ± 0.24 for WT and n = 1.01 ± 0.02 for F87V (Fig. 1B). In sum, WT exhibited homotropic cooperativity toward indole, but F87V did not.

Hydroperoxide-supported indole hydroxylation by WT and F87V

Indole hydroxylation by WT was further investigated with the support of different hydroperoxides. We observed that CuOOH strongly supported the reaction with a k_{cat} value nearly 100-fold of that obtained in the tBuOOH or H_2O_2-supported reaction (Table 2 and Fig. 2). In the absence of P450 BM-3 enzymes, no indigo was formed with any of these hydroperoxides (data not shown), suggesting that the hydroxylation was actually an enzyme-catalyzed reaction. Kinetic analysis indicated that the K_m values of CuOOH, tBuOOH, and H_2O_2 for WT were close to each other (Table 2). The small differences in affinities of these hydroperoxides to WT appear not to be responsible for the large differences

Table 3. Kinetic Analysis of Hydroperoxide-Supported Indole Hydroxylation by WT

	H_2O_2 (mM)	tBuOOH (mM)	CuOOH (mM)		
	16	16	1	2	4
k_{cat} of indole oxidation (nmol indigo/min/nmol P450)	0.26 ± 0.04	0.27 ± 0.04	2.20 ± 0.20	3.56 ± 0.24	7.70 ± 0.52
S_{50} for indole (mM)	7.71 ± 0.54	7.54 ± 0.67	1.76 ± 0.19	1.75 ± 0.22	3.34 ± 0.27
Hill coefficient (n)	1.96 ± 0.34	1.98 ± 0.29	1.37 ± 0.10	1.16 ± 0.07	1.02 ± 0.03

in indole hydroxylation activities. These results suggest that, rather than as oxygen donor, CuOOH may play another important role(s) in WT-catalyzing indole hydroxylation. As described below, CuOOH enhanced the activity through increasing the binding affinity of indole to WT.

On the other hand, as shown in Table 2, all three hydroperoxides effectively supported indole hydroxylation catalyzed by a mutant enzyme F87V, which has a substitution of Phe87 by Val and shows increased indole hydroxylation activity supported by NADPH.[19)]

CuOOH supports WT-catalyzing indole hydroxylation through a P450-type reaction

Since the CuOOH-supported hydroxylation activity of WT to its native substrates is very low,[22)] it should be clarified whether CuOOH supports indole hydroxylation through a P450-type reaction. The degradation products of CuOOH produced through indole hydroxylation by WT were analyzed by HPLC. The results indicated that 13 and 87% of the CuOOH was converted to acetophenone and cumyl alcohol respectively (these compounds were not contained in the original CuOOH solution). The latter was produced through heterolytical cleavage of the o–o bond of CuOOH, *i.e.*, a P450-type reaction.[23)] The molar amount of cumyl alcohol was 2.85-fold that of indigo, for one mol of which at least two mols of CuOOH were required. The small amount of acetophenone and a little more of cumyl alcohol might be products derived from the CuOOH decomposed by WT without any contribution to the indole hydroxylation, because P450 can catalyze the transformation of CuOOH into the two compounds without the participation of any other substrate.[23)] These results suggest that WT-catalyzing indole hydroxylation involves heterolytical cleavage of the o–o bond of CuOOH, *i.e.*, through a P450-type reaction, not a peroxidase-type reaction.

Kinetic analysis of CuOOH-supported indole hydroxylation by WT

Kinetic analysis of WT-catalyzing indole hydroxylation was performed with the support of 16 mM H_2O_2, 16 mM tBuOOH, or 1, 2 or 4 mM CuOOH. With 16 mM tBuOOH or 16 mM H_2O_2, WT exhibited a typical "S" curve for indigo formation with different concentrations of indole. With 1 mM of CuOOH, an "S" curve for indigo formation was also observed. The "S" curve gradually changed to a Michaelis–Menten type curve when higher concentrations (2 or 4 mM) of CuOOH were used (data not shown). The Hill coefficients (n), indicators of the degree of cooperativity, were 1.96 ± 0.34 and 1.98 ± 0.29 for the H_2O_2- and tBuOOH-supported reactions (Table 3) respectively, but decreased greatly to 1.37 ± 0.10 and further to 1.02 ± 0.03 when the reactions were supported by 1 and 4 mM CuOOH respectively. The S_{50} values of indole for WT were 7.71 ± 0.54 and 7.54 ± 0.67 mM for the H_2O_2- and tBuOOH-supported reactions respectively, but also decreased greatly to 1.76 ± 0.19 and 1.75 ± 0.22 mM for the reactions supported by 1 and 2 mM CuOOH (Table 3) respectively. These results indicate that indole binds to WT with homotropic cooperativity and that CuOOH can significantly stimulate indole binding to WT and reduce the homotropic cooperativity of indole.

Spectral observation of indole binding to WT and F87V in the absence of CuOOH

The homotropic cooperativity of indole binding to WT was further confirmed by spectral binding assays in the absence of CuOOH. The results indicate that indole bound to WT with half-maximal binding at 7.4 ± 0.7 mM and a Hill coefficient of 1.73 ± 0.15 (Fig. 3A and C). On the other hand, F87V did not show any cooperativity for indole binding. F87V showed high affinity to indole with half-maximal binding at 0.75 ± 0.06 mM and a Hill coefficent of 1.00 ± 0.12, *i.e.*, no cooperativity (Fig. 3B and C).

Spectral observation of indole binding to WT in the presence of CuOOH

It is difficult to carry out kinetic analysis of indole binding to WT with CuOOH addition, because CuOOH oxidizes WT and also mediates WT-catalyzed indole hydroxylation, and a steady substrate-binding spectrum is hard to obtain. However, it is still possible to observe the stimulation of indole binding to WT caused by CuOOH addition. Both indole and CuOOH can induce a type I binding spectrum with a peak at 390 nm and a trough at 425 nm. For comparison, spectra were recorded under the same conditions except that the enzyme was mixed with the substrate solution containing only indole, or CuOOH, or indole and CuOOH together. The concentrations of the substrate-binding complex were 2.4 ± 0.8, 15 ± 5 and $30 \pm 9\%$ of the total enzyme

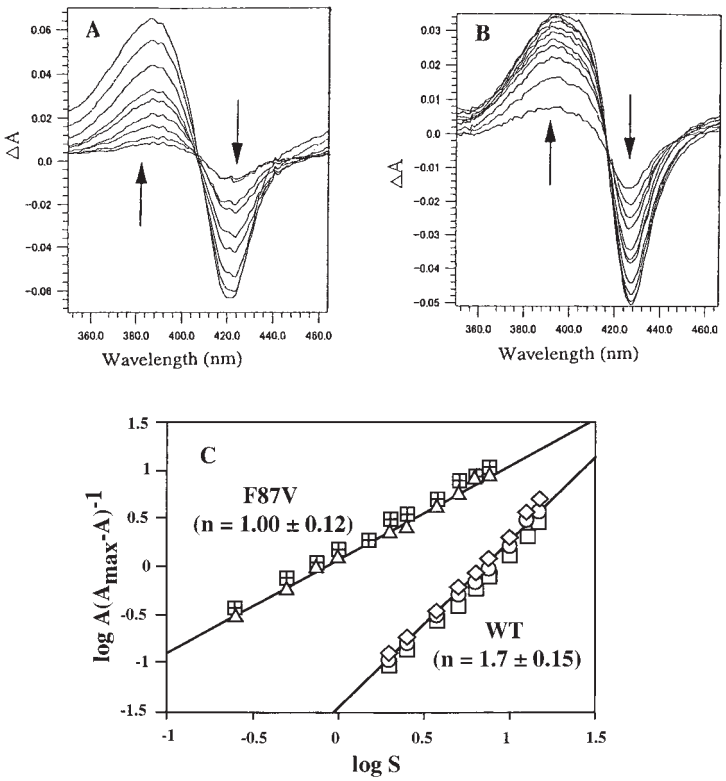

Fig. 3. Indole-Induced Spectral Shifts of P450 BM-3.

(A) Difference spectra from a single assay of 4 μM of WT to a series of concentrations of indole: 1.92, 2.4, 3.6, 4.8, 6.0, 7.2, 9.6, 12, and 14.4 mM. (B) Difference spectra from a single assay of 4 μM of F87V to a series of concentrations of indole: 0.24, 0.48, 0.72, 0.96, 1.44, 1.92, 2.4, 3.6, 4.8, 6.0, and 7.2 mM. The arrows in (A) and (B) indicate the direction of spectral changes following low to high concentrations of indole. (C) Kinetic analysis of indole binding. Hill coefficients (n) are the averages of the individual values determined from the slopes of the linear regression lines of three independent titrations for WT and two titrations for F87V.

concentration with the addition of 0.3 mM indole, 1 mM CuOOH, and both, respectively. Therefore, CuOOH stimulated indole binding to WT.

CuOOH enhances NADPH consumption by WT with low concentrations of indole

The NADPH consumption rate of WT with the addition of 1 mM CuOOH and a series of concentrations of indole was investigated (Fig. 4). The kinetic data are presented in Table 4. The addition of CuOOH greatly enhanced the consumption of NADPH by WT in the presence of low concentrations of indole. There was no NADPH consumption observed without P450 BM-3 enzymes, suggesting that NADPH consumption was actually a P450-catalyzing reaction. The S_{50} value for indole decreased more than 80-fold, from 13.2 to 0.16 mM, without any significant difference in the k_{cat} value with the addition of 1 mM CuOOH. The Hill coefficient also largely decreased with the addition of 1 mM CuOOH (Table 4). However, the S_{50} value for indole did not change obviously with the addition of H_2O_2, tBuOOH, acetophenone, or cumyl alcohol instead of CuOOH (data not shown). These results indicate that the molecular structure of the whole CuOOH molecule, rather than the properties of a hydroperoxide or its degradation products, played a role in the heterotropic stimulation of indole binding to WT. On the other hand, the addition of CuOOH did not significantly affect the NADPH consumption rate of F87V.

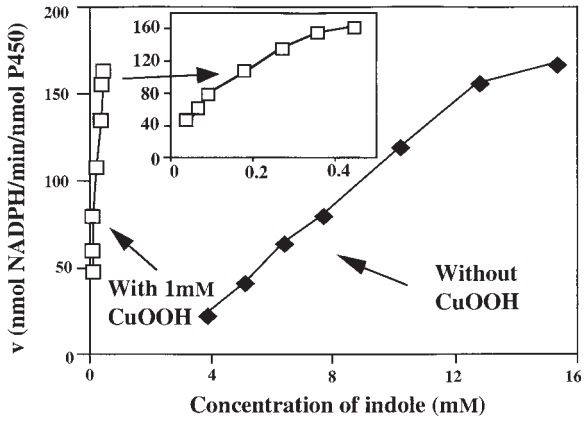

Fig. 4. Effect of 1 mM CuOOH on NADPH Consumption by WT in the Presence of Indole.

Each point represents the average value for three independent measurements.

Table 4. Effect of 1 mM CuOOH on Kinetics of NADPH Consumption by WT in the Presence of Indole

	k_{cat} of NADPH consumption (nmol NADPH/min/nmol P450)	S_{50} for indole (mM)	Hill coefficient (n)
Without CuOOH	323 ± 31	13.2 ± 2.1	1.78 ± 0.24
With 1 mM CuOOH	256 ± 29	0.16 ± 0.02	1.03 ± 0.04

The concentrations of indole used were the same as indicated in Fig. 3.

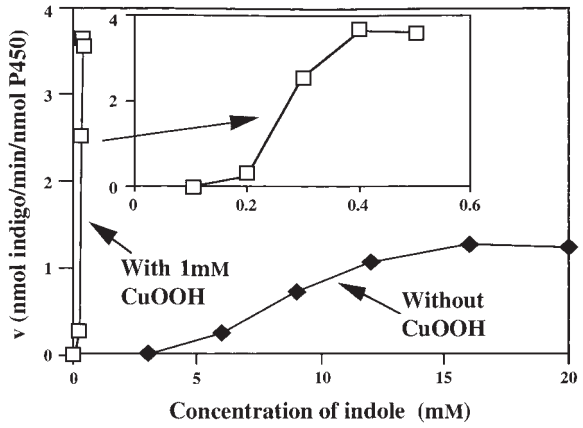

Fig. 5. Effect of 1 mM CuOOH on NADPH-Supported Indole Hydroxylation by WT.

Each point represents the average value for three independent measurements.

CuOOH enhances NADPH-supported indole hydroxylation by WT

Since CuOOH can stimulate NADPH consumption by WT with low concentrations of indole, whether CuOOH stimulates NADPH-supported indole hydroxylation by WT was investigated. The NADPH-supported hydroxylation of indole by WT with and without the addition of 1 mM CuOOH is shown in Fig. 5. With the addition of 1 mM CuOOH, production of indigo, the end product of indole hydroxylation, was observed even with a concentration of 0.2 mM indole. On the other hand, without CuOOH it occurred only when the concentration of indole was higher than 3 mM. Acetophenone and cumyl alcohol did not stimulate indole hydroxylation by WT (data not shown).

Discussion

In this study, we found that WT P450 BM-3 catalyzes indole hydroxylation with homotropic cooperativity toward indole under alkaline conditions. Furthermore, we found that the activity was modulated by CuOOH. CuOOH has been used extensively to support P450 activity as an oxygen donor through the peroxide shunt pathway,[23] and its effect on the inactivation of P450 activity has also been reported,[24] but no work on its role as a stimulator of P450 activity has been reported. The activity of WT in indole hydroxylation supported by CuOOH is much higher than that supported by tBuOOH, H_2O_2, or NADPH, suggesting that CuOOH might play another role(s) in the reaction in addition to that of oxygen doner.

The very close K_m values of the three hydroperoxides for WT show that the affinities of the three hydroperoxides to WT are similar. Kinetic analysis showed that CuOOH reduces the Hill coefficient and S_{50} of indole hydroxylation by WT, as a P450 3A4 activator does.[2–4,21] These results clearly support the view that CuOOH acts as a heterotropic stimulator in indole hydroxylation by WT, in addition to its role as an oxygen donor. NADPH consumption and NADPH-supported indole hydroxylation by WT under low indole concentrations can significantly increase with the addition of CuOOH, but not with the addition of the products derived from CuOOH, *viz.*, acetophenone or cumyl alcohol. These results indicate that the whole molecule acts as a stimulator, *i.e.*, the stimulation might be caused by CuOOH binding. But the coupling efficiency was still low, approximately 3%, as found with several aromatic substrates in WT-catalyzing hydroxylation.[25,26]

Cooperativity has been observed for P450 3A4 and other mammalian P450s with a big active site. The large active site is probably related to the ability to recognize a wide range of substrates, but it reduces the affinity of substrate binding. An effector is supposed to reduce the effective size of the active site and to change the binding environment, which is expected to result in an increase in the binding affinity of the first substrate on the binding of the second, thereby yielding homotropic or heterotropic cooperativity.[21] P450 BM-3 has a small active site and catalyzes the hydroxylations of a limited number of native substrates without cooperativity.[14–17] These characteristics of P450 BM-3 are in significant contrast with those of P450 3A4. We created various mutant enzymes.[19] By analysis of these mutants, we found that removing the aromatic ring of Phe87 from the heme pocket of P450 BM-3 by replacement of Phe87 with Val greatly increased the activities of this enzyme towards a variety of different compounds such as indole, phenol derivatives, and polycyclic aromatic compounds.[19,25,26] Phe87 is an important barrier that prevents these aromatic compounds from accessing the active site of P450 BM-3. The P450 BM-3 structure also indicates that the aromatic ring of Phe87 extends into the heme pocket and is located above the porphyrin.[18] CuOOH binding in the active site could move Phe87 to a new position and thus create a space for indole binding, which would result in high affinity for indole. Replacement of Phe87 by Val resulted in F87V losing homotropic cooperativity as well as heterotropic stim-

ulation by CuOOH, but greatly increased its affinity to indole with a K_m value of 0.75 mM and also the coupling efficiency of NADPH-consumption and indole hydroxylation.

Studies on substrate interactions with P450 BM-3, combined with structural analysis, will provide information to clarify the mechanisms of the substrate binding and hydroxylation specificity in a P450 reaction. Such information will be very useful for the redesign of novel substrate specificity of a P450 and the P450 related drug design.

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