Functional Interactions in Cytochrome P450BM3: Flavin Semiquinone Intermediates, Role of NADP(H), and Mechanism of Electron Transfer by the Flavoprotein Domain†

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ABSTRACT: Cytochrome P450BM3 is a self-sufficient soluble fatty acid hydroxylase from *Bacillus megaterium* utilizing tightly bound FAD and FMN cofactors to transfer reducing equivalents from NADPH to the heme active site. Active−inactive transitions of cytochrome P450BM3 were exploited to identify catalytic intermediates of the enzyme. Shortly upon reduction by NADPH, a two-electron reduced active P450BM3 is formed with two flavin semiquinones, anionic and neutral, present simultaneously. P450BM3 inactivated by NADPH has a three-electron reduced flavoprotein domain. NADPH is unable to reduce P450BM3 rapidly unless the flavoprotein domain is fully oxidized. During steady-state hydroxylation of a poor substrate, tetradecanol, the flavoprotein reduction state does not exceed two, with two flavin semiquinones, anionic and neutral, present. Absorbance and EPR spectroscopic characterization of both anionic and neutral flavin semiquinone is presented. NADPH and NADH were compared as electron donors for P450BM3-catalyzed fatty acid hydroxylation and cytochrome *c* and heme iron reduction. The K_m for NADH of 3−5 mM is about 3000 times higher than the K_m of 1−1.5 μM for NADPH. Although NADH can support cytochrome *c* reduction and fatty acid hydroxylation with the rates as high as 22 and 13 s^{-1}, respectively, these turnover numbers are only about 20% of those observed with NADPH. The results suggest that nucleotide binding plays an important role in catalysis by controlling electron-transfer properties of the flavin cofactors. In W574G and G570D mutant P450BM3 enzymes that are deficient in FMN, $NADP^+$ binding stabilizes fully reduced FAD. P450BM3 catalyzes single-turnover and steady-state laurate hydroxylation with near stoichiometric product formation at NADPH concentrations below that of the enzyme. A mechanism of electron transfer by the flavoprotein domain of P450BM3 is proposed with the reduction state of the flavoprotein domain cycling in a 0−2−1−0 sequence. We also propose that an interaction of bound $NADP^+$ with anionic FAD semiquinone is essential for splitting a pair of electrons that are then transferred in two one-electron transfer steps to the heme catalytic site.

The soluble cytochrome P450BM3[1] is a self-sufficient fatty acid hydroxylase from *Bacillus megaterium* (Narhi & Fulco, 1986). The enzyme consists of two distinct domains, an FAD and FMN-containing reductase and a hemoprotein (CYP102), fused in a single polypeptide (Narhi & Fulco, 1986, 1987). Both hemoprotein and flavoprotein domains of P450BM3 are homologous to eukaryotic microsomal P450 enzymes of the CYP4 family and microsomal P450 reductase, respectively (Porter, 1991; Nelson et al., 1996). P450BM3 is also structurally analogous to nitric oxide synthases that are fusion proteins between a P450-like hemoprotein and an FAD/FMN flavoprotein [for reviews, see Griffith and Stuehr (1995) and Marletta (1993)] and possibly to fungal cytochrome P450foxy, a catalytically self-sufficient fatty acid hydroxylase (Nakayama et al., 1996).

The crystal structures of the heme domain of P450BM3 with and without bound substrate revealed that fatty acid substrate binding is associated with significant conformational changes (Ravichandran et al., 1993; Li & Poulos, 1995). Furthermore, NMR studies showed that catalysis involves 6 Å movements of the substrate within the catalytic site (Modi et al., 1995, 1996). Interaction of P450BM3 with fatty acids, substrate specificity, and regiospecificity of hydroxylation have been well characterized (Muira & Fulco, 1974; Matson et al., 1977; Boddupalli et al., 1990, 1992b; Shirane et al., 1993; Black et al., 1994; Davis et al., 1996; Capdevila et al., 1996). However, the interaction of the enzyme with its electron donor NADPH has not been characterized in detail.

Engineering of P450 enzymes with high catalytic turnover and low uncoupling is an important goal in biotechnology and bioremediation research. Because of the high efficiency of electron utilization (Matson et al., 1977; Boddupalli et al., 1990) and of the high rates of catalysis by intact P450BM3 (Narhi & Fulco, 1986), a better understanding of the mechanism of electron transfer by the flavoprotein domain in P450BM3 may provide important information on

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[1] Abbreviations: P450BM3, cytochrome $P450_{BM3}$, isolated from *Bacillus megaterium*; BMR, flavoprotein domain of the P450BM3; DCPIP, 2,6-dichlorophenolindophenol.

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mechanisms of electron transfer and uncoupling in other P450 systems and in nitric oxide synthases.

Interestingly, reconstitution of the isolated flavoprotein (BMR) and hemoprotein domains of P450BM3 yields a very poor monooxygenase system (Narhi & Fulco, 1987; Li et al., 1991; Miles et al., 1992; Boddupalli et al., 1992a), suggesting that the natural fusion protein has unique properties. Indeed, in intact P450BM3, conformation and electron transfer properties of the flavoprotein domain are controlled by the conformation of the hemoprotein domain (Munro et al., 1994; Murataliev & Feyereisen, 1996). Under equilibrium conditions, redox properties of the flavin cofactors in the intact P450BM3 and in BMR are also dramatically different, as BMR forms a neutral flavin semiquinone upon anaerobic titration (Sevrioukova & Peterson, 1995; Sevrioukova et al., 1996a), while intact P450BM3 does not form a flavin semiquinone (Peterson & Boddupalli, 1992). The formation of a flavin semiquinone in intact P450BM3, recently demonstrated by EPR spectroscopy (Munro et al., 1996), is also in contrast with the results of anaerobic titration (Peterson & Boddupalli, 1992).

Uncertainty in the identification of catalytic intermediates of P450BM3 relates to the active−inactive transitions resulting from reduction of the flavoprotein domain (Narhi & Fulco, 1986; Murataliev & Feyereisen, 1996). The inhibited three-electron reduced enzyme does not transfer electrons from $FMNH_2$ to the heme iron (Murataliev & Feyereisen, 1996), probably as a result of conformational changes that alter the relative orientation of the two domains. A three-electron reduced flavoprotein ($FADH^{-}-FMN^{\bullet -}$) was proposed recently as a catalytic intermediate of P450BM3 (Sevrioukova et al., 1996a). However, the redox potentials of the flavin cofactors invoked by Sevrioukova et al. (1996a) would strongly favor formation of the inactivated enzyme and make hydride ion transfer from NADPH thermodynamically unfavorable. Thus, our current understanding of P450BM3 mechanism remains unclear.

The recognition of the reductive inactivation of P450BM3 and the definition of conditions for rapid recovery of hydroxylase activity (Murataliev & Feyereisen, 1996) allowed us to produce forms of the enzyme with high or inhibited hydroxylase activity and to identify and characterize active P450BM3 species that can be considered as catalytic intermediates. Optical and EPR spectroscopic studies under aerobic conditions demonstrated formation of two types of flavin semiquinones, neutral and anionic, in the active two-electron reduced P450BM3. Inactivation of P450BM3 by NADPH produces a three-electron reduced enzyme with all the electrons located in the flavoprotein domain. During steady-state hydroxylation of tetradecanol, the state of flavoprotein domain reduction does not exceed two. Our results also indicate that nucleotide binding may control electron-transfer properties of flavin cofactors. We propose a mechanism for electron transfer by the flavoprotein domain of P450BM3.

MATERIALS AND METHODS

Enzymes. The wild-type P450BM3 as well as W574G and G570D mutant enzymes (Klein & Fulco, 1993) were purified as described earlier (Murataliev & Feyereisen, 1996). Rabbit muscle lactate dehydrogenase (L-2518) and immobilized calf intestinal alkaline phosphatase (P-0762) were purchased from Sigma. Yeast glucose-6-phosphate dehydrogenase (G-7877, Sigma) was precipitated from ammonium sulfate suspension by centrifugation, dissolved in 0.1 M Tris-HCl pH 7.7 buffer, and dialyzed against three changes of a 1000-fold volume of the same buffer. Dialyzed glucose-6-phosphate dehydrogenase retains about 3−5 nmol of NADPH/mg of the enzyme as measured by fluorescence of NADPH ($\lambda_{ex} = 340$ nm, $\lambda_{em} = 455$ nm) in the presence of 2 mM glucose-6-phosphate. To remove NADPH, glucose-6-phosphate dehydrogenase was incubated overnight at 4 °C in 0.1 M Tris-HCl buffer, pH 7.7, at a concentration of 0.5−1.0 mg/mL with 10 U/mL immobilized alkaline phosphatase, and no NADPH was detected after the treatment. Immobilized alkaline phosphatase was removed by filtration of the suspension through a 0.45-μm filter, and the filtrate yielded an enzyme preparation we will refer to as NADPH-depleted glucose-6-phosphate dehydrogenase.

Spectral Measurements. All spectral assays were carried out under aerobic conditions. Unless stated otherwise, NADPH was added in the presence of NADPH-regenerating system containing 2 mM glucose-6-phosphate and 2.0 U/mL glucose-6-phosphate dehydrogenase. The reaction mixture (750 μL final volume) contained P450BM3 in 0.1 M Tris-HCl pH 7.7 buffer, and NADPH was added as a 10 mM solution to a final concentration as indicated in the legend to the figures. The volume change after all additions was less than 5%. The spectra were recorded at 960 nm/min in 210 nm intervals on a Lambda 19 Perkin Elmer spectrophotometer, and data were collected into a computer. Spectra recorded at different wavelength ranges were overlapped on a single graph to give the final range of 320−650 nm. Absorbance difference spectra were obtained by subtracting absolute absorbance spectra. The reference cell contained 0.05 μM P450BM3 to reduce DCPIP, and all the additions that were made in the sample cell.

Activity Assays. We found that 0.1 K-phosphate buffer caused inhibition of P450BM3 and glucose-6-phosphate dehydrogenase activities as compared to the activities in 0.1 M Tris-HCl buffer, pH 7.7. We used 0.1 M Tris-HCl pH 7.7 buffer for all incubations and activity assays. Otherwise, P450BM3 catalytic activities were measured as detailed earlier (Murataliev & Feyereisen, 1996) using [^{14}C]laurate of specific activity of 85 dpm/nmol. Alternatively, laurate and palmitate hydroxylation was measured by GC-FID of fatty acid methyl esters prepared by methylation with diazomethane. Reaction rate was calculated based on the integrated peaks areas of the product formed and substrate remaining. With laurate as a substrate, the latter procedure differed by less than 10% from the assay with [^{14}C]laurate.

With tetradecanol as a substrate (Miura & Fulco, 1975), hydroxylase activity was measured by NADPH oxidation at 340 nm. Tetradecanol was hydroxylated by P450BM3 with a turnover number of about 731 ± 57 (4) min^{-1}, and the activity was nearly saturated at 20 μM. In our spectral studies, tetradecanol was used at 330 μM to ensure that all the substrate is not consumed during experiment. Due to the low solubility of tetradecanol, 330 μM represents a nominal concentration.

Stopped-Flow Experiments. Stopped-flow experiments were carried out as described earlier (Guzov et al., 1996). For single-turnover cytochrome *c* reduction assays, one syringe contained 10−15 μM P450BM3, and the second syringe contained 50 μM cytochrome *c* and 5−10 μM

NADPH. Equal volumes of the solutions were mixed, and the absorbance change was recorded at 550 nm. Heme reduction was measured by formation of the carbonyl−ferrous adduct at 448 nm in CO-saturated buffer. One syringe contained 5−10 μM P450BM3 and 1 mM laurate, and the second syringe contained 1 mM laurate, NADPH, or NADH. The reactions followed first-order kinetics.

Determination of the Reduction State of P450BM3. NADH was used as an electron donor in these experiments because unreacted NADH can be rapidly removed by lactate dehydrogenase, and the traces of remaining NADH caused no detectable cytochrome *c* reduction. Similar conditions could not be achieved with NADPH because even traces of NADP(H) produced significant cytochrome *c* reduction that interfered with the assay. P450BM3 (16 μM) was incubated with 500 μM NADH for 10 s or 6 min. Fifty microliters of the solution was then mixed with 750 μL of the reaction mixture containing 15−25 μM cytochrome *c*, 25 mM pyruvate, and 20 U/mL lactate dehydrogenase, and the reaction was measured at 550 nm ($\epsilon = 21$ mM^{-1} cm^{-1}). No significant cytochrome *c* reduction was observed when a solution of P450BM3 and lactate dehydrogenase was mixed with a solution containing NADH, pyruvate, and cytochrome *c*. The reference cell contained 1.0 μM oxidized P450BM3 in the same reaction mixture. Flavin semiquinone absorbance did not interfere with the assay because of its low extinction coefficient relative to that of the reduced minus oxidized cytochrome *c*. Also, under the conditions of these experiments all the electrons were transferred to cytochrome *c* from P450BM3 thus regenerating oxidized enzyme.

Kinetics of Semiquinone Formation and Oxidation by G570D and W574G Mutant P450BM3. The reaction was carried out in a final volume of 800 μL in a spectrophotometer cell in the presence of 25 mM pyruvate. Reduction of the mutant enzymes was initiated by the addition of NADH or NADPH with no regenerating system present, and the reaction was monitored at 590 nm at a data sampling rate set to 10/s. As the absorbance reached a plateau, lactate dehydrogenase was added to oxidize the excess NAD(P)H. In a separate experiment, it was confirmed that lactate dehydrogenase in the presence of pyruvate can oxidize NADPH, although with rates considerably slower than NADH. The high lactate dehydrogenase concentration (105 NADH units/mL) used in these experiments was sufficient to oxidize 100 μM NADPH in 15 s. The reactions followed first-order kinetics.

EPR Measurements. P450BM3 was incubated under the conditions described in the legends to figures and in the text. The enzyme samples were placed into a 4 mm quartz tube and frozen in liquid nitrogen. EPR spectra were measured at 77 K with a Bruker Model ESP 300E spectrometer. The magnetic field was scanned between 3260 and 3460 G with a modulation amplitude of 4.027 G at a microwave frequency of 100 kHz with a modulation frequency of 9.45 Hz. The spin concentration of free radical was determined by comparison of a double integral of the first derivative of the sample spectrum with that of standards using standard Bruker software. Five and 50 μM solutions of 2,2,6,6-tetramethyl-1-piperidine-*N*-oxyl (TEMPO) in 98% methanol/water at 77 K were used as the standards. Line width was measured between the extrema of first derivatives of the recorded spectra.

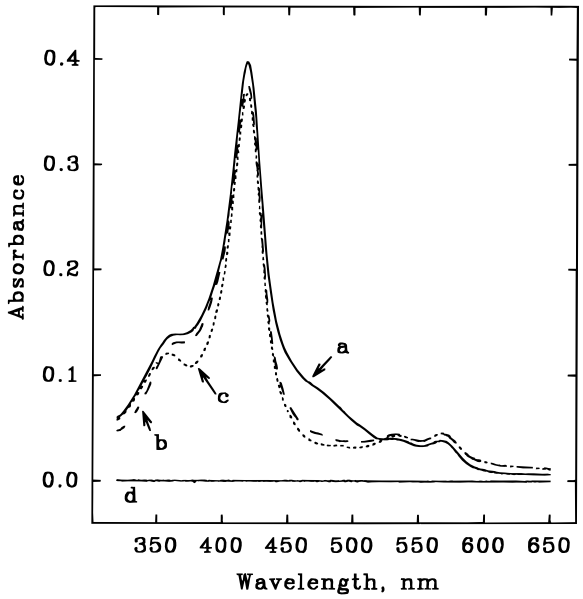

FIGURE 1: Absolute absorbance spectra of oxidized P450BM3 and of the enzyme incubated with NADPH for 10 s or 5 min. The enzyme was incubated in a spectrophotometer cell at a concentration of 3.2 μM, and spectra were recorded before (spectrum a), 10 s after (spectrum b), and 5 min after (spectrum c) addition of NADPH. Spectrum d, background. NADPH was added in both sample and reference cells simultaneously with glucose-6-phosphate and glucose-6-phosphate dehydrogenase to give final concentrations of 40 μM, 2 mM, and 2.0 U/mL, respectively.

Other Procedures. First-order rate constants were obtained by a best fit to the exponential function. The values presented are mean ± SD of (*n*) measurements. Protein concentration was determined by the Lowry et al. (1951) procedure using bovine serum albumin as standard. The molar concentration of P450BM3 was calculated based on the protein content using a molecular mass of 119 000 Da (Narhi & Fulco, 1986).

RESULTS

P450BM3 Flavin Reduction by NADPH. Inactivation of P450BM3 by NADPH in the absence of a fatty acid substrate is a slow process (Murataliev & Feyereisen, 1996); therefore, we compared the absorbance spectrum of the oxidized enzyme (Figure 1, spectrum a) to the spectrum of the active enzyme obtained after 10 s exposure to NADPH (spectrum b) and to that of the inactive P450BM3 obtained after 5 min incubation with NADPH (spectrum c). A 10 s exposure to NADPH results in a decrease of the absorbance below 500 nm and an increase above 500 nm. After 5 min incubation, absorbance in the 350−500 nm range decreased further, with no significant changes above 500 nm. No further spectral changes were observed after 5 min of incubation, when inactivation is completed. There were no changes in the position of the Soret band, suggesting that the spectral changes associated with P450BM3 inactivation do not involve the heme.

Absorbance difference spectra allow a more detailed analysis of the active−inactive transition (Figure 2). A short-time exposure to NADPH causes an increase of absorbance in the 500−650 nm region, typical for a neutral flavin semiquinone, and a decrease of absorbance between 400 and 500 nm, reflecting total flavin reduction (spectrum a). Absorbance in the 350−400 nm region decreases only

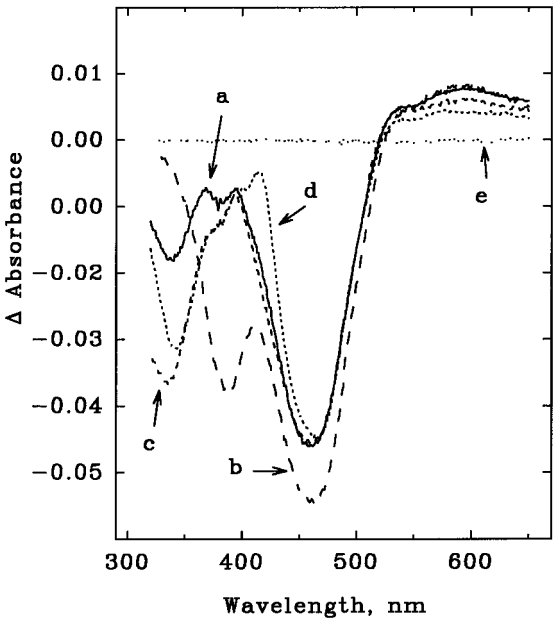

FIGURE 2: Absorbance difference spectra of reduced active (a), reduced inactive P450BM3 (b), enzyme reactivated by DCPIP (c), and during steady-state state catalysis (d) each minus oxidized P450BM3. The conditions were as described in Figure 1. Reference cell contained 0.05 μM P450BM3 to reduce added DCPIP. The spectra were recorded 10 s (spectrum a) and 5 min (spectrum b) after addition of NADPH. 200 μM DCPIP was then added in the reference and sample cells, and spectrum c was recorded about 10 s later. All DCPIP added was reduced within the mixing time. For steady-state catalysis conditions, P450BM3 was incubated at 4 °C with 330 μM tetradecanol; 40 μM NADPH and regenerating system were added to start the reaction, and spectrum was recorded 30 s later (spectrum d). The difference spectra were obtained by subtracting the spectrum of the oxidized P450BM3. Spectrum e, background.

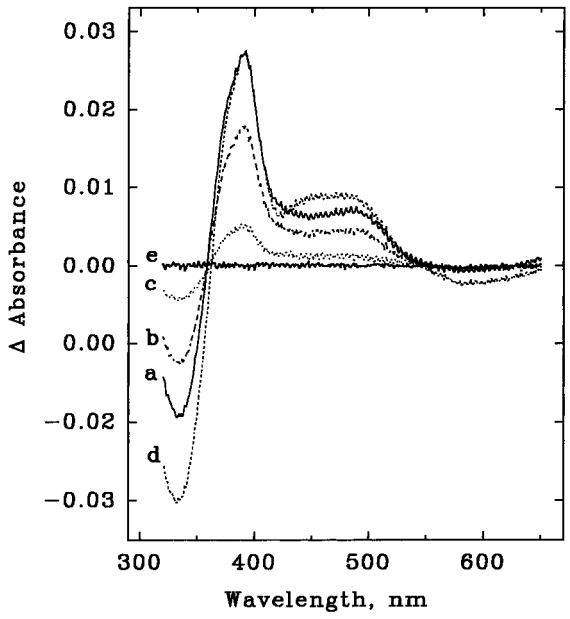

FIGURE 3: Spectral changes associated with P450BM3 inactivation by NADPH and reactivation by DCPIP. Experimental details are described in the legend to Figures 1 and 2. The spectrum obtained after 5 min incubation was subtracted from the spectra obtained 10 (spectrum a), 30 (spectrum b), and 120 (spectrum c) s after addition of NADPH and regenerating system and 10 s after DCPIP addition (spectrum d).

slightly within the first few seconds, indicating that an anionic flavin semiquinone is formed. After a 5 min incubation (spectrum b), when conversion to the inactive state is completed, the absorbance in the 500−700 nm region is still increased, indicating that a flavin semiquinone is still present in the inactive P450BM3. Total flavin reduction is increased, as evidenced by further absorbance decrease at 470 nm, while the absorbance between 350 and 400 nm is decreased significantly. An increased absorbance in the 500−600 nm range, characteristic for a neutral flavin semiquinone, is clearly observed in both active and inactive states of the enzyme.

Inactive P450BM3 can be rapidly reactivated by artificial electron acceptors, such as DCPIP (Murataliev & Feyereisen, 1996). DCPIP was added to P450BM3 inactivated by a 5 min incubation with NADPH, and a spectrum of the reactivated enzyme revealed spectral properties similar to that of the active, reduced enzyme (Figure 2, spectrum c). Thus, reactivation of P450BM3 is accompanied by a restoration of the spectral properties of the active enzyme, including the increased absorbance around 380 nm.

We compared the difference spectra of the active and inactive enzyme forms with that of P450BM3 during catalytic turnover. Due to the high catalytic activity and high concentration of P450BM3 in the reaction mixture, a poor substrate was desirable for such an experiment. Tetradecanol, a poor substrate for P450BM3 (Miura & Fulco, 1975), was hydroxylated with a turnover number of 30 min^{-1} at 4 °C, as measured by NADPH oxidation. This substrate allowed us to obtain an absorbance difference spectrum of P450BM3 under steady-state catalysis conditions. P450BM3 was incubated at 4 °C with a nominal concentration of 330 μM tetradecanol, and the difference spectrum (Figure 2, spectrum d) was recorded 30 s after addition of 40 μM NADPH. The spectrum of the enzyme during steady-state hydroxylation is clearly similar to the spectra of the active P450BM3 (spectra a and c), but different from that of the inactive enzyme (spectrum b). Presence of a neutral flavin semiquinone (absorbance at 510−650 nm) and increased absorbance at 380 nm as compared to the inactive enzyme are evident under conditions of substrate hydroxylation. However, during steady-state hydroxylation, the amount of neutral flavin semiquinone is decreased as compared to that of P450BM3 reduced in the absence of substrate. This observation is consistent with electron utilization in hydroxylation reaction.

The active and inactive forms were then compared by subtracting the spectrum of the inactive enzyme from the spectra obtained at 10, 30, and 120 s after NADPH addition and from the spectra of the enzyme reactivated by DCPIP (Figure 3). Inactivation is accompanied by the disappearance of an enzyme species with characteristic spectral properties, and reactivation by DCPIP restores the specific absorbance spectrum of this enzyme species. This enzyme species is characterized by a high absorbance at about 380 nm and a low absorbance at 500−700 nm, spectral properties typical of an anionic semiquinone of the enzyme-bound FAD (Massey & Palmer, 1966; Iyanagi, 1977) or free lumiflavin (Ehrenberg et al., 1967).

These spectral studies thus revealed that the active, reduced P450BM3 contains an anionic flavin semiquinone and at the same time it contains a neutral flavin semiquinone, as judged from the increased absorbance at 500−650 nm (Figures 1 and 2). The active, reduced enzyme therefore has a two-electron reduced flavoprotein domain containing both anionic

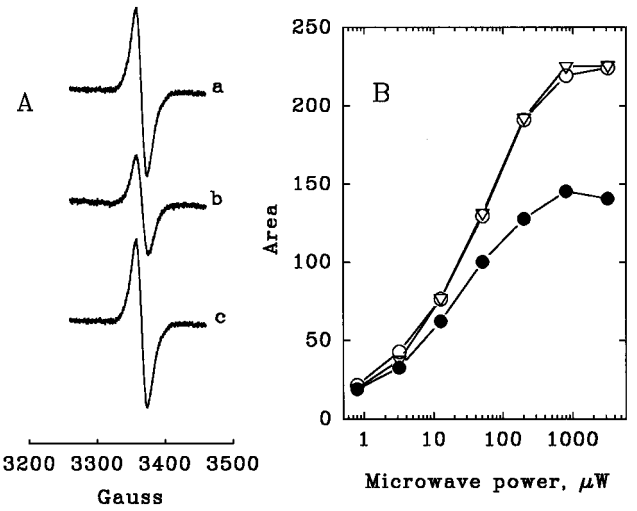

FIGURE 4: Characterization of P450BM3 flavin semiquinones by EPR spectroscopy. (A) EPR spectra of different P450BM3 preparations. P450BM3 was incubated at 4 °C at a concentration of 16 μM in the presence of 100 μM NADPH, 10 mM glucose-6-phosphate, and 5.0 U/mL glucose-6-phosphate dehydrogenase. Samples were frozen in liquid nitrogen after 15 s (a) and 5 min (b) incubation. 330 μM tetradecanol was included in the above reaction mixture (c),and sample was frozen after 15 s incubation. The spectra shown were recorded at saturating microwave power of 3170 μW. (B) Saturation of the EPR signal intensity as a function of microwave power. The spectra of the samples were measured at different microwave power, and the area of the spectra was measured. Samples were frozen in liquid nitrogen after 15 s (○) and 5 min (●) incubation with NADPH and after 15 s incubation with tetradecanol (▽).

Table 1: EPR Characterization of Flavin Semiquinone Species of P450BM3

P450BM3 preparation	SQ[a] (mol/mol)	line width, (G)	*g*-factor
1 WT, active, reduced (NADPH)	1.22	15.7	2.005
2 WT, inactive, reduced (NADPH)	0.93	19.2	2.006
3 WT, active, steady-state (NADPH)	1.06	16.8	2.006
4 WT, active, reduced (NADH)	1.08	16.6	2.005
5 WT, inactive, reduced (NADH)	0.93	18.3	2.006
6 W574G, NADPH	0.90	18.9	2.007
7 G570D, NADPH	0.89	19.1	2.006

[a] Flavin semiquinone radical. See legend to Figure 4 for the experimental details. Spin concentration and line width of the spectra were determined at nonsaturating microwave power of 0.7 μW.

and neutral flavin semiquinones. The inactive enzyme still contains a neutral flavin semiquinone and is more reduced than the active P450BM3. Thus, the inactive P450BM3 must be a three-electron reduced enzyme. These conclusions are confirmed by the results presented below.

Characterization of Flavin Semiquinones by EPR Spectroscopy. We used EPR to observe and characterize flavin semiquinones in (i) active P450BM3 obtained after a 15 s exposure to NADPH; (ii) inactive enzyme obtained by 5 min incubation with NADPH; and (iii) active P450BM3 under steady-state turnover conditions, as specified in the legend to Figure 2. Figure 4A shows EPR spectra obtained for the three enzyme preparations, and Table 1 summarizes semiquinone content in the samples. The stoichiometries of flavin semiquinone in the active enzyme species (Table 1) are likely underestimated because of some uncertainty with the time required for sample mixing, incubation, transfer into an EPR tube, and freezing. A fraction of the enzyme has already been inactivated, a fraction of the two-electron reduced enzyme has one of the flavin cofactors fully reduced as a result of the redox equilibrium, and a fraction of the enzyme is oxidized by oxygen (see below). Thus, the stoichiometries presented in Table 1 serve to demonstrate that a major fraction of the enzyme contains flavin semiquinones.

In EPR spectroscopy, anionic and neutral flavin semiquinones have the same *g*-factor (Eriksson & Ehrenberg, 1964) but can be distinguished by different line widths of the spectra (Palmer et al., 1969). The line width is defined as the distance, in Gauss, between the positive and negative extrema in the first derivative presentation and reflects different hyperfine interaction constants in these two flavin species (Ehrenberg et al., 1967; Muller et al., 1969, 1970). A 19 G line width is characteristic for a neutral semiquinone, a 15 G line width is characteristic for an anionic semiquinone, and intermediate values are indicative of a mixture of the two types of semiquinone. The EPR spectra line width of the inactive P450BM3 was 19.2 (Table 1), characteristic for the neutral flavin semiquinone. The two active enzyme forms produced spectra with intermediate line width values of 15.7 and 16.8, suggesting the simultaneous presence of two types of flavin semiquinones. These findings are consistent with the spectral characterization of the active−inactive transitions of P450BM3 (Figures 2 and 3).

In addition to a different line width, anionic and neutral semiquinones show different saturation curves as the microwave power is increased (Palmer et al., 1969), and these are shown for the active and inactive P450BM3 preparations in Figure 4B. The double integrals at unsaturating microwave power were almost the same for all the samples. When microwave power was increased, the double integrals of the samples containing active enzyme increased in the same way, but the two differed considerably from that of the sample containing inactivated P450BM3. At saturating power, the area of the spectra of the active enzyme samples were twice as large as those of the inactivated P450BM3. This indicates that flavin radicals in the active enzyme preparations are different from those in the inactive enzyme.

The results of absorbance and EPR spectroscopic studies of the flavin semiquinone forms of active and inactive P450BM3 show that (i) P450BM3 readily forms flavin semiquinone species upon reduction with NADPH under aerobic conditions; (ii) significant levels of flavin semiquinone are present in all the enzyme preparations tested; (iii) two different types of flavin semiquinone, anionic and neutral, were detected and characterized; (iv) active, reduced P450BM3 forms the same flavin semiquinone intermediates in the presence or absence of tetradecanol.

P450BM3 Reduction and Inactivation by NADH. Preliminary experiments showed that P450BM3 can also be inactivated by NADH. Incubation of P450BM3 with 0.4−2 mM NADH in the absence of a fatty acid substrate inhibited subsequent hydroxylase activity measured in the presence of NADPH. The rate constant of inactivation by NADH ($k \approx 0.7$ min^{-1}) was very close to that found with NADPH ($k \approx 1$ min^{-1}; Murataliev & Feyereisen, 1996), and about 85% of the enzyme activity was lost after a 5 min incubation with either nucleotide. Absorbance spectroscopy confirmed that incubation of P450BM3 with NADH produced spectral changes similar to those found with NADPH (data not shown). Formation of the flavin semiquinones with NADH as an electron donor was also confirmed by EPR spectros-

Table 2: Reduction of Cytochrome *c* by P450BM3[a]

	conditions of P450BM3 incubation prior to assay	cytochrome *c* reduced (mol/mol of P450BM3)[b]
1	none, P450BM3 and lactate dehydrogenase added simultaneously	0.03 ± 0.02 (2)
2	500 μM NADH, 10 s	1.83 ± 0.22 (8)
3	500 μM NADH, 6 min	2.44 ± 0.24 (8)
4	500 μM NADH, 10 s, followed by a 10 s incubation with lactate dehydrogenase	0.89 ± 0.10 (6)

[a] Final concentration of P450BM3 in all experiments was 1 μM. [b] Values are mean ± SD of (*n*) experiments.

copy, and characteristics of the semiquinone species are presented in Table 1, experiments 4 and 5. A short-time exposure of P450BM3 to NADH produced an EPR spectrum with a line width of 16.6 G, indicating the presence of two types of flavin semiquinone, anionic and neutral. As in the experiments with NADPH, the signal line width was broadened to 18.3 G after 5 min incubation.

Determination of the State of Flavoprotein Reduction in Active and Inactive P450BM3 Forms. Because NADH reduction of P450BM3 inhibits hydroxylase activity and produces active and inactive reduced enzyme species similar to those formed with NADPH, and because NADH can be rapidly removed from the reaction mixture by lactate dehydrogenase, we were able to determine the state of flavoprotein reduction in the active and inactivated P450BM3 directly. P450BM3 incubated for either 10 s or 6 min with 500 μM NADH was added into a reaction mixture containing cytochrome *c* as well as lactate dehydrogenase and pyruvate to study the amount of cytochrome *c* that can be reduced.

When NADH was added into a reaction mixture containing P450BM3, cytochrome *c*, lactate dehydrogenase, and pyruvate, little cytochrome *c* was reduced (Table 2, experiment 1). Thus, any significant cytochrome *c* reduction by prereduced P450BM3 will reflect the steady-state reduction of the flavoprotein. When P450BM3 was preincubated with NADH for 10 s, 1 mol of enzyme was able to reduce 1.83 ± 0.22 mol of cytochrome *c* (experiment 2), and 2.44 ± 0.24 mol of cytochrome *c* was reduced (experiment 3) after a 6 min incubation. Because 15−20% of the enzyme remains active (Nahri & Fulco, 1986; Murataliev & Feyereisen, 1996) and partial oxidation of the reduced enzyme by oxygen occurs, the data of the Table 2 clearly show that active and inactive P450BM3 forms are two- and three-electron reduced forms, respectively. The two-electron reduced form of P450BM3 loses about one reducing equivalent within 10 s after removal of excess of NADH (experiment 4), suggesting that reduced P450BM3 readily reacts with oxygen.

Comparison of P450BM3 Oxidation and Inactivation. NADPH binding and hydride ion transfer to oxidized P450BM3 is completed within milliseconds (Munro et al., 1996), while inactivation is a much slower process (Murataliev & Feyereisen, 1996). We compared the rates of P450BM3 inactivation and transition of the two- to three-electron reduced enzyme (Figure 5, curves 1 and 2). Reduction of the flavin cofactors by 100 μM NADPH as measured by the absorbance decrease at 470 nm is biphasic. The fast phase corresponding to the two-electron reduction of oxidized P450BM3 is completed within milliseconds (not shown). The slower phase of reduction occurred with a rate constant of 0.009 ± 0.002 (4) s^{-1} similar to the rate constant

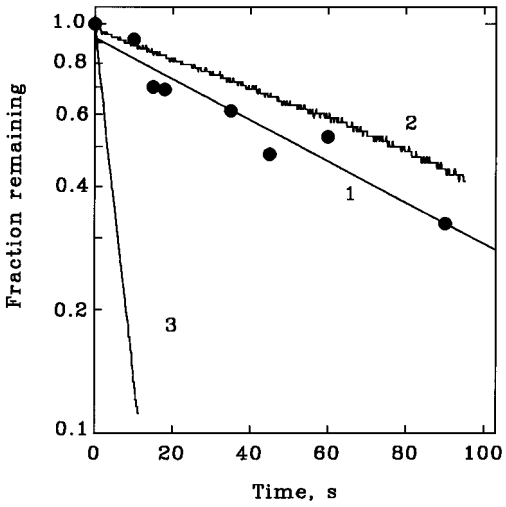

FIGURE 5: Comparison of the rates of P450BM3 inactivation, three-electron reduced enzyme formation, and two-electron reduced P450BM3 oxidation. Curve 1, inactivation. P450BM3 was incubated in the presence of 100 μM NADPH, and aliquots were withdrawn to measure the NADPH oxidation rate in the presence of 1 mM laurate. Curve 2, transition of two-electron reduced P450BM3 to a three-electron reduced inactive enzyme. The rate of flavin reduction in the presence of 100 μM NADPH was measured by the absorbance decrease at 470 nm. The first-order rate constant for curves 1 and 2 is about 0.01 s^{-1}. Curve 3, oxidation of the two-electron reduced enzyme. NADPH was added to a final concentration of 11.5 μM into a spectrophotometer cell containing 12 μM P450BM3, and the absorbance change at 590 nm was measured. First-order rate constant is 0.19 s^{-1}.

of inactivation (Murataliev & Feyereisen, 1996). Thus, slow inactivation of P450BM3, which is independent of the enzyme concentration (data not shown), correlates well with an increased reduction state of the flavin cofactors. Since NADPH is a two-electron donor, the formation of a three-electron reduced form must include either enzyme oxidation by oxygen or intermolecular electron transfer. Both processes are much slower than catalytic turnover and, therefore, are not part of a normal catalytic cycle.

We also measured the rate of oxidation of P450BM3 that follows reduction by a substoichiometric amount of NADPH. Addition of NADPH resulted in rapid absorbance changes caused by the formation of a two-electron reduced P450BM3 followed by enzyme oxidation. The oxidation kinetics obtained at 470 nm (total flavin reduction) and at 590 nm (neutral flavin semiquinone) had the same rate constant of 0.20 ± 0.03 (8) s^{-1}. Figure 5 shows the semilogarithmic plot of flavin semiquinone oxidation (curve 3). Clearly, the inactivation rate is 20 times slower than the rate of oxidation of the two-electron reduced P450BM3. The two-electron reduced P450BM3 is rapidly oxidized by oxygen, losing about one reducing equivalent within 10 s (Figure 5; Table 2). The fact that formation of three-electron reduced P450BM3 and inactivation are considerably slower processes strongly suggests that NADPH cannot reduce partially reduced P450BM3 as fast as it reduces the fully oxidized enzyme. Indeed, under the conditions of steady-state hydroxylation of the poor substrate tetradecanol, the reduction state of the flavoprotein domain does not exceed that of the active, two-electron reduced P450BM3 (Figure 2).

Steady-State Catalysis by P450BM3 with NADPH Excess. Cytochrome *c* reductase, laurate, and palmitate hydroxylase activities were studied as a function of NADPH concentration. NADPH-depleted glucose-6-phosphate dehydrogenase

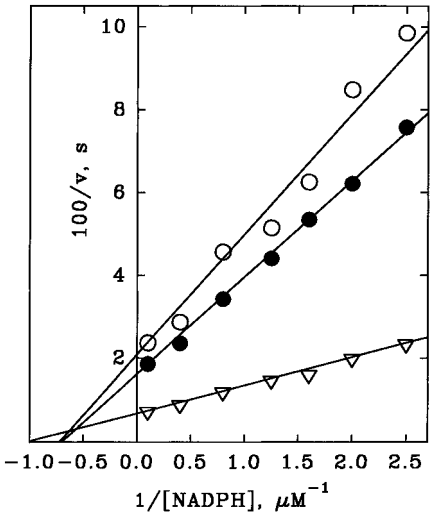

FIGURE 6: Double-reciprocal plots of P450BM3 activities as a function of NADPH concentration. Cytochrome *c* reductase (▽), laurate (○), and palmitate (●) hydroxylase activities were measured in the presence of an NADPH-regenerating system containing NADPH-depleted glucose-6-phosphate dehydrogenase as described in Materials and Methods section. Substrate concentrations were 2 mM, 250 μM, and 90 μM for laurate, palmitate, and cytochrome *c*, respectively, and the NADPH concentration was varied from 0.4 to 10 μM. The lines represent best fit to the Michaelis−Menten kinetics.

Table 3: Comparison of NADH and NADPH as Cofactors for P450BM3 Catalysis[a]

parameter	NADPH	NADH
	Cytochrome *c* Reduction[b]	
$K_{m,NAD(P)H}$, μM	1.2 ± 0.1 (3)	2691 ± 907 (6)
V_{max}, s^{-1}	102 ± 17 (3)	22 ± 5 (4)
	Laurate Hydroxylation[c]	
$K_{m,NAD(P)H}$, μM	1.5 ± 0.3 (4)	5278 ± 1431 (4)
V_{max}, s^{-1}	57 ± 13 (4)	13 ± 4 (4)
	Palmitate Hydroxylation[c]	
$K_{m,NAD(P)H}$, μM	1.4 ± 0.1 (3)	3960 ± 457 (3)
V_{max}, s^{-1}	68 ± 5 (3)	13 ± 1 (3)
heme reduction, s^{-1}	39 ± 3 (6)[d]	9 ± 0.3 (6)[d]

[a] Values are mean ± SD of (*n*) experiments. [b] Reduction in the presence of 50 μM cytochrome *c*. [c] Hydroxylation in the presence of 2 mM laurate or 250 μM palmitate. [d] In the presence of 50 μM NADPH or 19 mM NADH.

was used for an accurate measurement of the K_m for NADPH. The reactions followed Michaelis−Menten kinetics, and double-reciprocal plots of the three P450BM3 activities with NADPH as a varied substrate are shown in Figure 6. The K_m for NADPH was 1.4, 1.4, and 1.0 μM in the presence of 2 mM laurate, 250 μM palmitate, or 90 μM cytochrome *c* as substrates, with V_{max} values of 49, 62, and 153 s^{-1}, respectively. Table 3 summarizes the results of these measurements. These K_m values are in a good agreement with the high affinity of P450BM3 for NADP-type nucleotides (Black et al., 1994). High rates of fatty acid hydroxylation were confirmed by measuring substrate-dependent NADPH oxidation (data not shown). Bimolecular rate constants for NADPH binding calculated as a ratio $V_{max}/K_{m,NADPH}$ are 3.5×10^7, 4.4×10^7, and 15.3×10^7 M^{-1} s^{-1} for laurate, palmitate hydroxylation, and cytochrome *c* reduction, respectively. Laurate and palmitate hydroxylation rates by P450BM3 were independent of the protein concentration in the range of 10−250 nM.

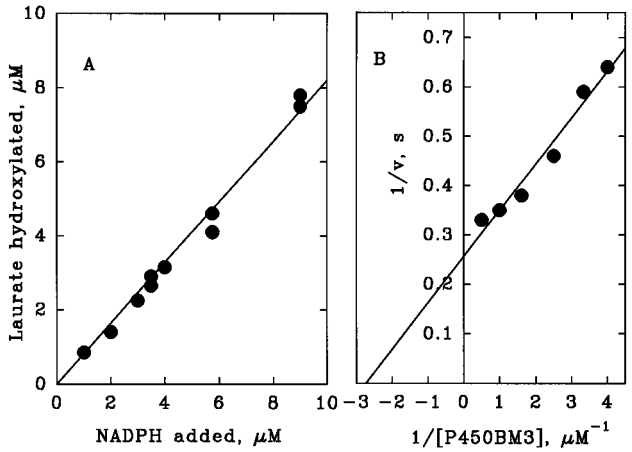

FIGURE 7: Laurate hydroxylation at [P450BM3] > [NADPH]. (A) Single turnover hydroxylation. 200 μL of a 20 μM solution of P450BM3 was mixed with 200 μL of a solution containing 110 μM [^{14}C]laurate and 2−18 μM NADPH, and the reaction was stopped with 125 μL of 1 N HCl 3−4 s later. Final concentrations of P450BM3, laurate, and NADPH were 10, 55, and 1−9 μM, respectively. (B) Steady-state hydroxylation. The reaction was carried out in the presence of 1.1 mM [^{14}C]laurate, 0.15 μM NADPH, 2.5 mM glucose-6-phosphate, and 96 U/mL of NADPH-depleted glucose-6-phosphate dehydrogenase. P450BM3 concentration was varied from 0.25 to 2.0 μM, and samples were incubated for 1 min. The reaction rates were calculated as a turnover number of added NADPH. The line is a linear regression through the data points ($r^2 = 0.983$).

Cytochrome c Reduction and Laurate Hydroxylation at [P450BM3] > [NADPH]. When a substoichiometric amount of NADPH was added to an excess of P450BM3, 1.94 ± 0.12 mol of cytochrome *c* was rapidly reduced per mol of NADPH. At 25 μM cytochrome *c*, the rate constant of a single-turnover cytochrome *c* reduction by P450BM3 in the presence of substoichiometric NADPH concentrations was 52.2 ± 5.2 (6) s^{-1} as determined by stopped-flow measurements (7.5 μM enzyme), compared to the steady-state rate of 55.6 ± 3.0 (3) s^{-1} (5 nM enzyme). This demonstrates that electrons derived from NADPH are equally reactive toward cytochrome *c* in the presence of either P450BM3 or NADPH excess and that cytochrome *c* reduction rate is independent of the P450BM3 concentration from 5 nM to 7.5 μM.

A number of similar single-turnover experiments were carried out with P450BM3 concentration varied from 1.0 to 7.5 μM and the P450BM3 ratio to NADPH ranging from 1:0.15 to 1:0.9. A stoichiometric reduction of cytochrome *c* was observed under all these experimental conditions. However, when P450BM3 was added into the reaction mixture 10 s after reduction by a substoichiometric amount of NADPH, only 0.75 ± 0.26 mol of cytochrome *c* was reduced per mole of NADPH. This shows that the two-electron reduced P450BM3 is readily oxidized, producing a one-electron reduced flavoprotein, which is still able to reduce cytochrome *c*. This observation is in agreement with the results of Figure 5 and Table 2, showing that the two-electron reduced P450BM3 is readily oxidized by oxygen.

P450BM3 is also able to catalyze single-turnover fatty acid hydroxylation when the enzyme is present in excess over NADPH (Figure 7A). At a P450BM3 concentration of 10 μM, the addition of 1−9 μM NADPH resulted in laurate hydroxylation with an efficiency of about 80%. The reaction was completed within 3−4 s, the minimal time required for

mixing the reagents and stopping the reaction. A low laurate concentration (55 μM) was used for an accurate measurement of the low amount of product formed in this experiment. This laurate concentration is below the K_m for laurate (180 μM; Murataliev & Feyereisen, 1996), and about 80% of the enzyme is thus present in a substrate-free form under these conditions. As shown above, the two-electron reduced P450BM3 loses about one reducing equivalent within 5−10 s (Figure 5; Table 2). This loss of reducing equivalents explains why the efficiency of laurate hydroxylation in single-turnover experiments was limited to about 80%.

We attempted to determine whether P450BM3 can also catalyze steady-state laurate hydroxylation at NADPH concentrations lower than that of the enzyme. These experiments required a high-activity NADPH-regenerating system free of endogenous nucleotide. Treatment of glucose-6-phosphate dehydrogenase with immobilized alkaline phosphatase as described in Materials and Methods yielded an enzyme with no remaining traces of NADPH, which was suitable for use in these experiments. The reactions were carried out in the presence of 0.15 μM NADPH, regenerating system, 1.1 mM laurate, and 0.25−2.0 μM P450BM3.

Figure 7B shows a double-reciprocal plot of the turnover number of NADPH (as the nucleotide limits the concentration of enzyme−NADPH complex) versus P450BM3 concentration. The results show a linear dependence with a regression coefficient of 0.983. Under these conditions, a total of 20−45 μM laurate was hydroxylated during a 1 min incubation, demonstrating that the P450BM3−NADPH complex is able to make multiple catalytic turnovers. A K_m value determined as the P450BM3 concentration producing half-maximal activity was 0.30 ± 0.06 μM, in agreement with the high affinity of P450BM3 to NADP(H) (Black et al., 1994). The maximal velocity determined as the turnover number of NADPH was 3.5 ± 0.5 s^{-1}, or about 15 times lower than that found in the presence of excess NADPH. Considering the high concentrations of glucose-6-phosphate dehydrogenase used in these experiments and its ability to bind NADP(H) tightly, it is reasonable to expect that a significant amount of the added nucleotide is bound to the dehydrogenase at steady state, thus reducing the "effective" concentration of the P450BM3−NADPH complex, leading to a significant underestimation of the turnover rate. Even though the rate of catalytic turnover cannot be accurately determined from these experiments, it is clear that P450BM3 is able to catalyze fatty acid hydroxylation with no priming reaction. Another important conclusion from the experiments with P450BM3 excess is that intermolecular electron transfer between P450BM3 molecules, if it occurs, is not catalytically significant. This follows from the fact that NADPH dependency on Figure 7A and a double-reciprocal plot of Figure 7B are both linear.

NADH and NADPH as Electron Donors in P450BM3 Catalysis. NADH was reported to be a poor electron donor for fatty acid hydroxylation catalyzed by P450BM3 (Klein & Fulco, 1994). We compared NADH and NADPH in the cytochrome *c* reductase and fatty acid hydroxylase activities of P450BM3. The reactions followed Michaelis−Menten kinetics with both nucleotides, producing linear double-reciprocal plots as a function of NAD(P)H (results summarized in Table 3). NADH is able to support high rates of catalysis, but the K_m for NADH is about 2000−3000 times higher than that for NADPH. Maximal velocities in the presence of NADH are 4−5 times lower than in the presence of NADPH. The rates of NADPH- and NADH-dependent P450BM3 heme reduction were also compared. NADH was used at 19 mM, which is about 5 times above the K_m, to measure rates with no limitation by nucleotide binding. The rate of heme iron reduction with NADH is also 4−5 times slower (Table 3). Electron transfer from NADPH through the flavin cofactors can be as fast as 400 s^{-1} and does not limit P450BM3 turnover in hydroxylation reaction (Murataliev & Feyereisen, 1996). The fact that NADH cannot support as fast a turnover as NADPH suggests that some step(s) of the catalytic cycle is sensitive to the nature of the bound nucleotide.

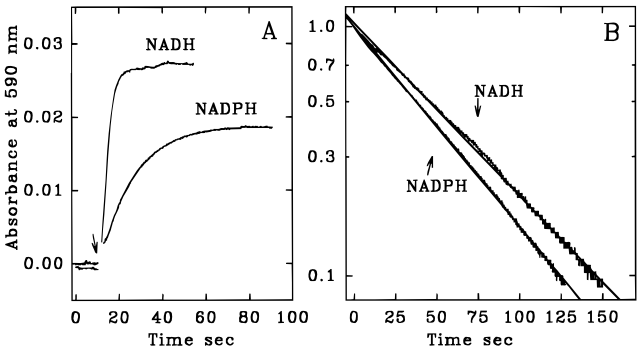

FIGURE 8: Kinetics of FAD semiquinone formation and oxidation by G570D mutant P450BM3. (A) Flavin semiquinone formation. P450BM3 was incubated at a concentration of 8.3 μM in a reaction mixture containing 25 mM pyruvate. 100 μM NADPH or 500 μM NADH was then added to reduce the enzyme, and the reaction was monitored at 590 nm. (B) Semilogarithmic plot of flavin semiquinone oxidation. As the absorbance reached maximum, lactate dehydrogenase was added to final concentrations of 105 and 15 U/mL with NADPH and NADH, respectively. Data were collected at 10/s.

Flavin Semiquinone Formation by FMN-Deficient P450BM3 Mutants. Munro et al. (1996) reported recently that FMN-free mutant P450BM3 did not form flavin semiquinone in the presence of NADPH. On the other hand, the isolated FAD domain of P450BM3 does form a neutral flavin semiquinone in the presence of NADPH (Black, 1994; Sevrioukova et al., 1996b). We re-examined this question using the G570D and W574G FMN-free mutants (Klein & Fulco, 1993). These mutants readily formed a neutral flavin semiquinone under aerobic conditions in the presence of NADPH, as judged from absorbance and absorbance difference spectra (data not shown). EPR spectroscopy confirmed formation of flavin semiquinone species in these P450BM3 mutants (Table 1, experiments 6 and 7). The line widths of EPR spectra of 18.9 and 19.1 G for W574G and G570D, respectively, are characteristic for the neutral flavin semiquinone.

The experiments presented above suggested that nucleotide binding to P450BM3 may control electron transfer properties of the flavin cofactors. Because FAD is the cofactor that accepts electrons from NADPH (Vermilion et al., 1981; Kurzban & Strobel, 1986; Klein & Fulco, 1993), we studied FAD semiquinone formation and oxidation in FMN-deficient mutants using NADPH and NADH as electron donors. The mutant enzymes were reduced in the spectrophotometer cell, and lactate dehydrogenase was then added to oxidize the excess of NAD(P)H. When the nucleotide was added, the absorbance at 590 nm increased, reflecting formation of the neutral flavin semiquinone (Figure 8A). Oxidation of

Table 4: FAD Semiquinone Formation and Oxidation in FMN-Free P450BM3 Mutants in the Presence of NADPH and NADH[a]

		rate constant min^{-1}		
mutant	nucleotide	k_{app}	k_{sq}	k_{full}
W574G	NADPH	3.3 ± 0.4	1.2 ± 0.1	2.1
	NADH	16.4 ± 3.0	1.3 ± 0.3	15.1
G570D	NADPH	3.9 ± 0.6	0.9 ± 0.2	3.0
	NADH	17.8 ± 2.1	1.1 ± 0.3	16.7

[a] See legend to Figure 8 for experimental details. The values are mean ± SD of three separate measurements. The difference between the rate constants for W574G and G570D enzymes are not significant. k_{app}, rate constant of flavin semiquinone formation; k_{sq}, rate constant of flavin semiquinone oxidation; k_{full}, rate constant of one-electron oxidation of fully reduced FAD. k_{app} and k_{sq} were measured as shown in Figure 8. k_{full} was calculated as $k_{app} - k_{sq}$.

NAD(P)H led to a decrease in absorbance corresponding to the complete oxidation of FAD (Figure 8B). Because similar results were obtained with both mutant enzymes, Figure 8 shows only the results obtained with G570D mutant, while Table 4 summarizes the data for both mutants.

Semiquinone formation and oxidation followed first-order kinetics, thus allowing rate constants for the two reactions to be calculated. The apparent first-order rate constant of semiquinone formation (k_{app}) under these conditions is a sum of the two rate constants, oxidation of fully reduced FAD (k_{full}) and oxidation of semiquinone (k_{sq}). The rate of absorbance decrease after addition of lactate dehydrogenase is the rate of semiquinone oxidation, k_{sq}. Measuring rates of flavin semiquinone formation (k_{app}) and oxidation (k_{sq}) allows calculation of k_{full}. Neither of the rate constants is a true first-order rate constant, since the reactions involve oxygen as a second reactant, the concentration of which was considered constant in our assay. The results summarized in Table 4 allow two conclusions.

First, fully reduced FAD is less stable than FAD semiquinone. Semiquinone formation is faster than its oxidation, and in the presence of an excess NAD(P)H, a steady-state concentration of semiquinone is maintained. Second, the stability of fully reduced FAD depends on the nature of the nucleotide used to reduce the flavoprotein. The rate of oxidation of fully reduced FAD (k_{full}) with NADH is 5−7 times higher than with NADPH, indicating that FAD hydroquinone is more stable when NADP(H) is bound. The rate of semiquinone oxidation after removal of NAD(P)H was the same for both nucleotides. These results provide strong evidence that in FMN-free mutants bound NADP(H) stabilizes fully reduced FAD, thus decreasing its reactivity toward oxygen. Taken together with the fact that NADPH is a more effective electron donor for the wild-type P450BM3 catalytic turnover (Table 4), these results provide strong evidence that NADP(H) binding plays a role in electron transfer from the flavoprotein to the heme domain in addition to its role as electron donor to flavin cofactors.

DISCUSSION

Intact P450BM3 can undergo an active−inactive transition (Narhi & Fulco, 1986; Murataliev & Feyereisen, 1996). Our approach exploited this active−inactive transition of intact P450BM3 to detect and characterize semiquinone forms that emerge during interaction with NADPH and to identify enzyme species that can or cannot emerge during a normal catalytic cycle. The inactivation of P450BM3 by NADPH is a result of reduction of the flavin cofactors (Murataliev & Feyereisen, 1996). Direct measurements of the flavoprotein reduction state (Table 2) now shows that the inactive enzyme has a three-electron reduced flavoprotein domain. This is likely the semiquinone form described recently by Munro et al. (1996). A three-electron reduced enzyme is not formed during steady-state catalytic turnover even with a poor substrate, tetradecanol (Figure 2). The active, reduced enzyme is a two-electron reduced flavoprotein containing both anionic and neutral semiquinones. The same double semiquinone intermediate is formed during steady-state hydroxylation (Figures 2 and 4; Table 1). It is clear that the flavin cofactors can only be less reduced with faster substrates, and this may explain the failure to detect a flavin semiquinone with laurate as a substrate (Munro et al., 1996).

We describe both absorbance and EPR characteristics of the anionic flavin semiquinone of the intact active P450BM3. This semiquinone form of P450BM3 has spectral properties similar to those described for the anionic semiquinone of FAD-containing L-amino acid oxidase (Massey & Palmer, 1966) and NADH:cytochrome b_5 reductase (Iyanagi, 1977): a high absorbance at about 380 nm and a low absorbance at 600 nm (Figure 3). In addition, this intermediate has distinct EPR properties (Figure 4; Table 1): a line width and saturation pattern characteristic for anionic but not for neutral flavin semiquinone (Palmer, 1969). We cannot determine experimentally which of the flavin cofactors is present as the anionic semiquinone. Sevrioukova et al. (1996a) suggested that anionic FMN semiquinone is formed in P450BM3. However, the recent flavin fluorescence studies demonstrated that FMN is accessible to the solvent while FAD is completely occluded (Sevrioukova & Peterson, 1996). It is reasonable to expect that FMN anionic semiquinone will be rapidly protonated, as the p*K* is about 8.5 (Eherenberg et al., 1967; Draper & Ingraham, 1968). Additionally, the spectral properties described in this work (Figures 3 and 4B; Table 1) are similar to the spectra of the FAD anionic semiquinone in other proteins (Massey & Palmer, 1966; Iyanagi, 1977). Also, NAD^+ binding is required for formation and stabilization of the anionic FAD semiquinone of cytochrome b_5 reductase (Iyanagi, 1977). We conclude that FAD is the cofactor forming anionic semiquinone in P450BM3 and suggest that ionic interactions with the positively charged nicotinamide ring of $NADP^+$ contribute to the overall stabilization of the complex.

The active, two-electron reduced P450BM3 is oxidized by oxygen with a rate constant of 0.2 s^{-1}, in contrast to the formation of a three-electron reduced flavoprotein and inhibition of hydroxylase activity, which are much slower processes, $k \approx 0.01$ s^{-1} (Figure 5). Evidently, reduction of a one-electron reduced enzyme by NADPH to produce a three-electron reduced flavoprotein is several orders of magnitude slower than flavin reduction of oxidized intact P450BM3, which occurs in milliseconds (Munro et al., 1996). Therefore, NADPH can reduce the flavoprotein domain of P450BM3 rapidly only when the flavins are fully oxidized and not when the flavoprotein is in the one- or two-electron reduced states. Consequently, the flavoprotein reduction state never exceeds 2 during steady-state catalysis, preventing enzyme inactivation.

The rate of P450BM3 inactivation by NADPH is independent of the enzyme concentration, and oxidation of the two-electron enzyme by oxygen is much faster than inactiva-

tion. This excludes intermolecular electron transfer as a mechanism for enzyme inactivation. The slow inactivation by NADPH may reflect (i) a low concentration of one-electron reduced P450BM3 with no $NADP^+$ bound, (ii) a slow binding of NADPH by a one-electron reduced enzyme, or (iii) a slow hydride ion transfer from bound NADPH to a partially reduced flavoprotein. No data are available to determine which mechanism of slow inactivation is operative.

It is difficult to identify spectrally which of the two flavin cofactors is present in the inactive enzyme as the semiquinone. However, based on the redox potentials of the $FADH/FADH_2$ and $FMNH/FMNH_2$ couples of −365 and −270 mV, respectively, for microsomal P450 reductase (Iyanagi et al., 1974), it is reasonable to expect that the inactive enzyme has a fully reduced FMN and a neutral FAD semiquinone. This agrees with our finding that in FMN-free mutants fully reduced FAD is less stable than the semiquinone form (Table 4).

We show that P450BM3 is able to catalyze cytochrome *c* reduction and fatty acid hydroxylation at [P450BM3] > [NADPH]. Cytochrome *c* reduction is stoichiometric to the NADPH added, with a rate of reduction as high as that observed in the usual assay at low P450BM3 concentrations and NADPH excess. Even though no accurate measurement of the rate of catalytic turnover for laurate hydroxylation was possible under these conditions (Figure 7), our estimates suggest that catalysis with limiting NADPH is at least comparable to P450BM3 turnover with NADPH in excess and is certainly much faster than the turnover of most microsomal P450 enzymes.

Our results show that the mechanism proposed by Sevrioukova et al. (1996a) includes several reaction steps and catalytic intermediates that do not occur. First, no priming reaction is required for P450BM3 to complete a catalytic cycle (Figure 7). Secondly, no rapid reduction of the enzyme can occur unless the flavoprotein is fully oxidized (Figure 5). Thirdly, the flavoprotein reduction state does not exceed 2 during catalysis (Figure 2). Finally, it should also be noted that the redox potential values of the flavins suggested by Sevrioukova et al. (1996a) will lead to formation of fully reduced FMN within milliseconds and to the inactivation of the flavoprotein.

NADH was regarded as a poor electron donor for P450BM3 (Klein & Fulco, 1994). Our results show that, at saturating concentrations, NADH can support catalysis rates as fast as 22 s^{-1} for cytochrome *c* reduction and 13 s^{-1} for fatty acid hydroxylation (Table 3). However, maximal velocities at infinite nucleotide concentration are still severalfold higher with NADPH as an electron donor, suggesting that electron transfer from NADH through FAD and FMN to the heme domain or to cytochrome *c* is slow. We conclude that some step(s) of electron transfer from NAD(P)H to electron acceptors is nucleotide-specific. This agrees with our finding that fully reduced FAD in FMN-deficient mutants is less reactive toward oxygen when NADPH is used as the electron donor rather than NADH (Figure 8A; Table 4). Because the redox potentials of the two nucleotides are very close, the observed effects of NADPH can only be rationalized on the basis of specific interactions of the nucleotide with the flavoprotein. These results (Tables 3 and 4) strongly suggest that NADP(H) binding controls the electron transfer properties of the flavoprotein domain. This may be achieved directly by an effect of the nicotinamide moiety on the redox potential of the $FAD/FADH_2$ couple or indirectly through induced conformational changes.

Nucleotide binding is known to influence the properties of flavin cofactors in flavoenzymes. Facilitation of electron transfer from adrenodoxin reductase by nucleotide binding has been demonstrated (Lambeth & Kamin, 1977, 1979; Sugiyama et al., 1978). Both the rate and number of electrons transferred to cytochrome *c* from fully reduced adrenodoxin reductase−adrenodoxin complex depend on NADP(H) binding. NAD^+ binding to cytochrome b_5 reductase results in formation and stabilization of an anionic FAD semiquinone (Iyanagi, 1977). Stabilization of FAD semiquinone by $NADP^+$ was also shown with adrenodoxin reductase (Sugiyama et al., 1978, 1979). A third observed effect of nucleotide binding is to shift the redox potential of flavins. With adrenodoxin reductase (Lambeth & Kamin, 1976) and cytochrome b_5 reductase (Iyanagi, 1977), the midpoint potential of FAD shifts by about +100 mV when $NAD(P)^+$ is bound. One of the possible ways to shift redox potential is through an interaction of the anionic semiquinone of FAD with the positively charged nicotinamide ring of $NAD(P)^+$. This interaction, leading to a tighter binding of $NAD(P)^+$, may be a driving force for nucleotide oxidation and for the first electron transfer from FAD hydroquinone to a secondary acceptor (such as ferredoxin, cytochrome b_5, or FMN in P450 reductase).

To date, the role of NADPH in catalysis by P450BM3 has implicitly been limited to the supply of electrons. However, our results and those obtained with other flavoproteins suggest that, in addition to providing reducing equivalents, NADP(H) binding may also control the electron transfer properties of the flavin cofactors. We suggest that, in the P450BM3 mechanism, NADP(H) binding may have specific functions: (1) $NADP^+$ stabilizes the FAD anionic semiquinone. This may shift the equilibrium of hydride ion transfer from NADPH to FAD and may facilitate transfer of one reducing equivalent from $FADH^-$ to FMN. (2) NADPH can only reduce FAD with high rates when one electron is rapidly transferred to FMN. NADPH cannot rapidly reduce FAD until FMN is oxidized because transfer of one reducing equivalent is required for fast and efficient hydride ion transfer. This is essential to prevent formation of the inactive three-electron reduced flavoprotein. (3) NADP(H) binding may be essential to assure fast electron transfer from FMN to the heme iron.

Mechanism of Electron Transfer by the Flavoprotein Domain of P450BM3. Identification of the active semiquinone intermediates and our findings (i) that NADPH is unable to reduce P450BM3 rapidly unless flavoprotein domain is fully oxidized, (ii) that NADP(H) binding controls electron-transfer properties of the flavin cofactors, and (iii) that P450BM3 is able to catalyze hydroxylase reaction in the presence of substoichiometric amounts of NADPH lead us to propose the following mechanism of electron transfer by the P450BM3 flavoprotein domain (Figure 9). This mechanism does not require any priming reaction to support fast rates of catalysis, it implies an essential role of nucleotide binding in splitting a pair of reducing equivalents, and it allows an easy completion of the catalytic cycle with regeneration of the oxidized enzyme.

Reduced P450BM3 binds laurate as fast as the oxidized enzyme (Murataliev & Feyereisen, 1996), and a number of studies with the isolated hemoprotein domain of P450BM3

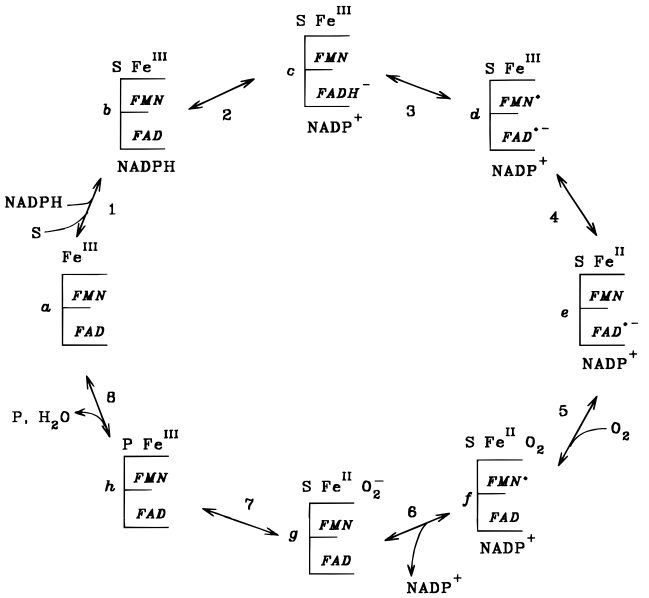

FIGURE 9: Suggested mechanism of electron transfer by the flavoprotein domain of intact P450BM3. Oxidized and reduced heme iron are designated as Fe^{III} and Fe^{II}, respectively. $FADH^-$, two-electron reduced anionic FAD; $FAD^{\bullet -}$, anionic semiquinone; $FMN^{\bullet}$, neutral semiquinone; S, fatty acid substrate; P, hydroxylation product. Catalytic conversion of the substrate at the heme catalytic site is shown as step 7. The cycle represents a single catalytic turnover. Under steady-state catalysis conditions, NADPH can bind to complexes g and h, and after product release and substrate binding, one of the complexes b, c, or d can emerge. See text for details.

Table 5: Kinetic Parameters of P450BM3-Catalyzed Reactions

parameter	value	source
$K_{m,NADPH}$	$1-1.5\ \mu M$	present work
$K_{m,Laur}$	$180\ \mu M$	*a*
$k_{1(NADPH)}$	$3.5-15 \times 10^7\ M^{-1}\ s^{-1}$	present work
$k_{1(Laurate)}$	$1.8 \times 10^5\ M^{-1}\ s^{-1}$	*a*
k_2, k_3, k_5[d]	$\geq 400\ s^{-1}$	*a*
k_4	$130-223\ s^{-1}$	*b*
$k_{6(NADP^+)}$[e]	$\geq 200\ s^{-1}$	*a*
k_6, electron transfer	$150\ s^{-1}$	*c*
k_7 and k_8	$150\ s^{-1}$	*c*

[a] Murataliev and Feyereisen (1996). [b] Munro et al. (1996), rate of heme iron reduction is substrate-dependent. [c] Black et al. (1994), the rate of tetradecyltrimethyl ammonium bromide hydroxylation. [d] Maximal rate of cytochrome *c* reduction. [e] Because NADPH is a two-electron donor and cytochrome *c* is a one-electron acceptor, $NADP^+$ release step is taken as half of the maximal rate of cytochrome *c* reduction.

also showed that fatty acid binding is not altered when compared to the intact enzyme (Li et al., 1991; Boddupalli et al., 1992a; Miles et al., 1992). Thus, to simplify the scheme, we show fatty acid substrate to bind at step 1, although binding at steps 2 and 3 is also possible. Protonation/deprotonation steps are not shown because these processes are usually very fast. The term "electron transfer" refers to either electron or hydrogen atom transfer. The scheme (Figure 9) essentially represents a single catalytic turnover. Modifications to the mechanism that may be required to account for steady-state catalysis will be discussed below. Some kinetic constants of P450BM3 catalysis obtained with intact, active P450BM3 and their source are summarized in Table 5. Conformational changes induced by fatty acid binding are not shown because in the presence of substrate the enzyme rapidly assumes the conformation optimal for fast electron transfer to the heme moiety.

Binding of NADPH to oxidized P450BM3 (step 1) in a bimolecular reaction produces a short-living complex b. The monomolecular rate constant of hydride-ion transfer from NADPH to FAD (step 2) is at least $400\ s^{-1}$. This step is readily reversible because the redox potentials of NADPH/$NADP^+$ and FAD/$FADH_2$ pairs are close (Iyanagi et al., 1974). However, the equilibrium of step 2 is shifted forward due to the fast transfer of one reducing equivalent to FMN (step 3). Electron transfer to FMN (step 3) results in formation of a double semiquinone intermediate (complex d) that is stabilized by ionic interactions of $NADP^+$ with the FAD anionic semiquinone.

Heme iron reduction in step 4 can be as fast as $223\ s^{-1}$ with some substrates and depends on the nature of the fatty acid substrate bound (Munro et al., 1996). Transfer of the first electron to the heme iron produces a one-electron reduced flavoprotein intermediate, complex e, with FAD anionic semiquinone stabilized by $NADP^+$. Neutral FMN semiquinone is not present when P450BM3 catalyses hydroxylation of laurate (Munro et al., 1996), because the rate of electron transfer to the P450 domain is very high. With a poor substrate, such as tetradecanol, FMN semiquinone is detectable, but in lesser amount than in the absence of the substrate (Figure 2).

As the first electron is transferred to the heme iron (step 4), the second electron is now able to move from FAD anionic semiquinone to oxidized FMN. This electron transfer (step 5) is driven by the large redox potential difference (-270 and -110 mV) and decreases the affinity of the enzyme to $NADP^+$, facilitating oxidized nucleotide release in the next step. Oxygen binds at step 5, and the second electron is transferred (step 6) to the P450 domain, $NADP^+$ is released, and the fully oxidized flavoprotein is regenerated, ready to accept hydride ion from the next NADPH that would bind under steady-state conditions. Since the mechanism of catalysis at the heme catalytic site is not clear, substrate hydroxylation is shown as a single step 7. Hydroxylated product is released (step 8), regenerating fully oxidized enzyme and completing the catalytic cycle.

With respect to NADPH and fatty acid binding, the kinetic mechanism of P450BM3 should be random bi-bi because (i) NADPH can bind and reduce P450BM3 with or without fatty acid substrate bound at the heme catalytic site and (ii) both oxidized and reduced P450BM3 can bind a fatty acid substrate. Therefore, under steady-state conditions with excess NADPH present, the nucleotide can bind to complexes g and h, and reduction of the flavoprotein may occur before hydroxylated product (P) release. Catalysis in this case will proceed through the same cycle as soon as the reaction product is released and the next substrate molecule is bound. Substrate binding at steady state to a binary NADPH-P450BM3 complex will produce one of the complexes b, c, or d.

P450BM3 aggregation at relatively high concentrations has been reported (Black & Martin, 1994). Ruettinger and Fulco (1981) showed that P450BM3 hydroxylase activity did not depend on the protein concentration. We found that hydroxylase activity of the purified P450BM3 did not change in the range of 10−250 nM, and cytochrome *c* is reduced with the same rate at 5 nM or 7.5 μM enzyme concentration. The results of Figure 7B show that turnover of the enzyme−NADP(H) complex does not depend on the protein concen-

tration in the range of 0.25−2.0 μM. These results suggest that P450BM3 oligomerization is not required for catalytic activity.

We provide evidence that NADP(H) has functions in P450BM3 catalysis in addition to the supply of reducing equivalents. Nucleotide binding affects stability of the reduced flavin cofactors as well as the rate of electron transfer from the flavoprotein to the electron acceptor. Similar effects have been observed with a variety of flavoproteins. We are not aware of any results that would suggest a similar effect of nucleotides in the mechanism of microsomal P450 reductase. Because microsomal P450 reductase and the flavoprotein domain of P450BM3 have a common evolutionary origin (Porter, 1991), we need to ascertain whether sequence divergence has been accompanied by changes in the mechanism of each flavoprotein. This study is currently underway.

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