Functional Interactions in Cytochrome P450BM3. Fatty Acid Substrate Binding Alters Electron-Transfer Properties of the Flavoprotein Domain[†]

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Received July 9, 1996; Revised Manuscript Received September 13, 1996[⊗]

ABSTRACT: P450BM3 is a bacterial fusion protein between a cytochrome P450 fatty acid hydroxylase (CYP102) and an FAD- and FMN-containing flavoprotein homologous to NADPH:cytochrome P450 reductase. It has been shown that incubation of P450BM3 with NADPH in the absence of a fatty acid substrate results in inhibition of hydroxylase activity [Narhi, L. O., & Fulco, A. J. (1986) *J. Biol. Chem. 261*, 7160−7169]. We show that laurate-dependent oxidation of NADPH and oxygen consumption are also inhibited under those conditions. The inhibited enzyme is unable to transfer electrons to the heme iron, but reduces artificial electron acceptors such as cytochrome *c*, 2,6-dichlorophenolindophenol, or ferricyanide. Incubation with these acceptors rapidly restores hydroxylase activity of P450BM3. The active enzyme is able to catalyze the reduction of cytochrome *c* and hydroxylation of laurate simultaneously. Cytochrome *c* has no effect on the K_m and V_{max} of laurate hydroxylation. Laurate and other substrates stimulate cytochrome *c* reduction by 50−70%. Carbon monoxide inhibits hydroxylase activity, but stimulates cytochrome *c* reduction 3−4 fold and has no effect on the K_m for cytochrome *c*. This stimulation requires binding of a substrate at the heme catalytic site. Laurate binding induces conformational changes in the flavoprotein domain as shown by a 2-fold increase of the flavin fluorescence. Inactivation of P450BM3 by NADPH abolishes the stimulation of cytochrome *c* reduction by laurate and CO. Complete inhibition of hydroxylase activity correlates with complete lack of stimulation of cytochrome *c* reduction. The results suggest that a specific conformation of the two domains is maintained in the active P450BM3, ensuring high hydroxylase activity. Cytochrome *c* reductase and hydroxylase activities of P450BM3 involve different sites of interaction with the flavoprotein domain, different catalytic intermediates, and different rate-limiting steps.

P450BM3[1] is a self-sufficient cytochrome P450 enzyme from *Bacillus megaterium* catalyzing hydroxylation/epoxidation of long-chain fatty acids preferentially at the ω-2 position (Ho & Fulco, 1976; Miura & Fulco, 1974; Boddupalli et al., 1990). Unlike other cytochromes P450, P450BM3 is a P450 protein fused in a single polypeptide chain with an FAD- and FMN-containing flavoprotein, which provides electrons required for P450 catalysis (Narhi & Fulco, 1986, 1987; Wen & Fulco, 1987). The heme and the reductase domains of P450BM3 share significant homology with cytochrome P450 enzymes of the CYP4 family and microsomal NADPH-dependent cytochrome P450 reductase, respectively (Nelson et al., 1996; Porter, 1991), suggesting that P450BM3 is the product of an ancient gene fusion event. The reductase domain transfers electrons from NADPH to the P450 domain in a tightly coupled reaction, as the stoichiometry of fatty acid hydroxylation to NADPH consumed is close to 1 (Matson et al., 1977; Boddupalli et al., 1990). The turnover rate of P450BM3 is 100−1000 times higher than the rates of most P450 enzymes depending on O_2 and NADPH (Narhi & Fulco, 1986). Thus, the electron transfer channels of the two domains seem to be in close contact in the interface between the flavin and heme domains, ensuring a fast and efficient electron transfer.

P450BM3 is also able to reduce cytochrome *c* with a rate comparable to the rate of fatty acid hydroxylation (Narhi & Fulco, 1986; Narhi et al., 1988). Remarkably, cytochrome *c* does not inhibit myristate hydroxylation catalyzed by P450BM3, and in the presence of low myristate concentrations, it even stimulates hydroxylase activity (Klein & Fulco, 1994). Cytochrome *c* reductase activity in turn is stimulated in the presence of myristate (Klein & Fulco, 1994). Another interesting observation has been reported by Narhi and Fulco (1986), who demonstrated that hydroxylase and cytochrome *c* reductase activities of P450BM3 are differentially sensitive to incubation of the enzyme with NADPH in the absence of a fatty acid substrate. Fatty acid hydroxylase activity is reversibly lost while cytochrome *c* reductase is unaffected under these conditions. These results (Narhi & Fulco, 1986; Klein & Fulco, 1994) imply that the two domains can functionally interact in intact P450BM3, affecting each others properties. Myristate binding facilitates cytochrome *c* reductase activity of the flavoprotein, and flavin reduction in the absence of a fatty acid substrate decreases the rate of catalysis by the heme domain.

A number of structural studies of P450BM3 have suggested that fatty acid substrate binding to the heme catalytic site is associated with conformational changes. Ravichandran et al. (1993) observed heterogeneity in the substrate binding pocket and suggested that substrate binding can

[†] This work was supported by National Institutes of Health Grants GM39014 and ES06694.

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[⊗] Abstract published in *Advance ACS Abstracts,* November 1, 1996.

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

S0006-2960(96)01667-4 CCC: $12.00

change the conformation of the heme domain. Energy minimization of the crystal structure (Li & Poulos, 1995) and molecular dynamics simulations (Paulsen & Ornstein, 1995) also suggested that large conformational motions of the heme domain are likely to take place upon substrate binding. In addition to conformational changes of the heme domain of P450BM3, specific conformational interactions of the two domains might be expected.

In this paper, we present evidence for functional and structural interactions of the flavin and heme domains of P450BM3, and we characterize changes in catalytic properties of the enzyme associated with such interactions. We found that the enzyme reduced by NADPH in the absence of a fatty acid substrate is unable to transfer electrons to the P450 domain and therefore is inactive as hydroxylase. Oxidation of the reduced P450BM3 by cytochrome *c* or DCPIP rapidly restores electron transfer and hydroxylase activity. Electron transfer from the flavoprotein of P450BM3 to cytochrome *c* is stimulated by laurate bound at the heme domain, and inhibition of electron transfer by CO further stimulates cytochrome *c* reduction. Our results indicate that fatty acid substrate binding to the heme domain of P450BM3 alters the catalytic properties of the flavoprotein domain, possibly as a result of induced conformational changes. Cytochrome *c* reductase and fatty acid hydroxylase activities involve different catalytic intermediates and different sites for electron transfer.

MATERIALS AND METHODS

P450BM3 Purification. The *Escherichia coli* strain pbsBM-3 with a plasmid carrying the P450BM3 gene was a generous gift of Prof. Armand Fulco. The cells were cultured in LB medium for 24 h essentially as described (Klein & Fulco, 1993), harvested by centrifugation, and washed with 0.1 M potassium phosphate buffer, pH 7.6. Cells were resuspended in 50 mM Tris-HCl buffer, pH 7.7, containing 0.5 mM EDTA, and lysate was prepared by ultrasonic disruption. The membrane fraction was removed by centrifugation at 100000*g* for 30 min. The cytoplasmic fraction was loaded on a 2.5 × 25 cm DEAE-agarose column, and P450BM3 was eluted with a 400 mL linear gradient of 0−400 mM NaCl in a 40 mM Tris-HCl buffer, pH 7.7, containing 0.5 mM EDTA. Colored fractions containing cytochrome *c* reductase activity were pooled and concentrated, and P450BM3 was purified on a 2′,5′-ADP−agarose column (Pharmacia) essentially as described (Li et al., 1991; Black et al., 1994). The final P450BM3 preparation was extensively dialyzed against 50 mM potassium phosphate buffer, pH 7.6, to remove 2′-AMP and concentrated by ultrafiltration, glycerol was added to a final concentration of 50%, and the enzyme was stored at −80 °C at 80−130 μM. The enzyme was homogeneous as judged by SDS−PAGE. The purified P450BM3 preparation contained FAD/FMN/heme in a 0.92:1.08:0.90 molar ratio per mole of enzyme determined by protein assay.

Activity Assay. All enzyme incubations and activity assays were carried out at 25 °C in 0.1 M potassium phosphate buffer, pH 7.6. Where indicated, an NADPH-regenerating system consisting of 2.0 mM glucose 6-phosphate and 4.0 units/mL glucose-6-phosphate dehydrogenase was included in the reaction mixture. Cytochrome *c* reductase activity was measured by the absorbance increase at 550 nm in the presence of 100 μM NADPH, regenerating system, and 50 μM cytochrome *c* using an extinction coefficient of 21 mM^{-1} cm^{-1}. NADPH oxidation was measured in the presence of 100 μM nucleotide and 500 μM laurate by the decrease in absorbance at 340 nm using an extinction coefficient of 6.22 mM^{-1} cm^{-1}. The final volume of the samples for cytochrome *c* reduction and NADPH oxidation assay was 0.75 mL. Laurate hydroxylation was measured in the presence of 100 μM NADPH, regenerating system, and 500 μM [^{14}C]laurate, specific radioactivity of 130 dpm/nmol, in a final volume of 0.5 mL. The samples were incubated for 2 min, the reaction was stopped by addition of 50 μL of 5 N HCl and laurate, and the oxidation products were extracted with 1.0 mL of ethyl ether. The ether phase was taken into a separate tube and evaporated, and the residue was dissolved in 40 μL of methanol and applied on Silica TLC plates. The plates were developed in hexane/ethyl ether/acetic acid (60:19:1); the spots containing radioactivity were identified by autoradiography, cut out, and counted. The amount of product formed was calculated as a fraction of the total (substrate plus product) radioactivity. The turnover number for cytochrome *c* reduction measured in the presence of 50 μM cytochrome *c* and 100 μM NADPH was about 3200 min^{-1}, and that for hydroxylase reaction in the presence of 100 μM NADPH and 500 μM laurate was 1200−1300 min^{-1}. In good agreement with published results (Boddupalli et al., 1990), laurate hydroxylation by our P450BM3 preparation was tightly coupled to NADPH oxidation and oxygen consumption.

Oxygen Consumption. An apparatus from Cameron Instruments was used. The data were collected with an IBM-compatible computer through an analog-to-digital conversion card. The software for collecting data and calculation of the results was written in Microsoft QBASIC and kindly provided by Dr. José Ribeiro. The reactions were run at 25 °C in a final volume of 1.5 mL. The initial oxygen concentration was taken as 240 μM. Beef heart submitochondrial particles respiring on NADH were used for instrument calibration.

Other Procedures. Absorption spectra were recorded on a Perkin Elmer Lambda 19 spectrophotometer, and fluorescence emission spectra were recorded on a Photon Technology International spectrofluorometer. A CO-saturated buffer was prepared by bubbling gaseous carbon monoxide through the 0.1 M potassium phosphate buffer, pH 7.6, for at least 1 h before experiments. The concentration of CO in a saturated solution was calculated from its solubility as 800 μM at 25 °C. A reaction mixture with the required CO concentration was prepared by diluting CO-saturated buffer with air-saturated buffer. Protein concentration was determined by the Lowry procedure (Lowry et al., 1951) using bovine serum albumin as a standard.

RESULTS

Inactivation of P450BM3 by NADPH. Narhi and Fulco (1986) reported that hydroxylase activity is inhibited when intact P450BM3 is incubated with NADPH in the absence of substrates, while cytochrome *c* reductase does not change under these conditions. This was confirmed by the results shown in Figure 1. Incubation of P450BM3 in the presence of 100 μM NADPH and an NADPH-regenerating system without fatty acid substrates resulted in a loss of 80−90%

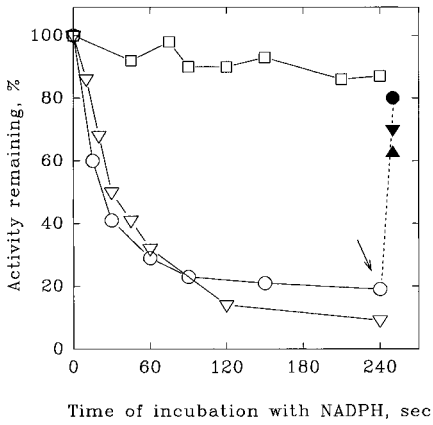

FIGURE 1: Effect of incubation with NADPH on laurate hydroxylation and cytochrome *c* reduction by P450BM3. P450BM3 was incubated in 0.1 M potassium phosphate buffer, pH 7.6, in the presence of 100 μM NADPH and an NADPH-regenerating system. The rate of [^{14}C]laurate hydroxylation (○), laurate-dependent NADPH oxidation (▽), or cytochrome *c* reduction (□) was measured. After 4 min incubation with NADPH, 50 μM cytochrome *c* (▼), 50 μM DCPIP (●), or 100 μM ferricyanide (▲) was added (indicated by the arrow). Hydroxylation was measured by adding [^{14}C]laurate 10 s later.

of hydroxylase activity as measured by NADPH oxidation or [^{14}C]laurate hydroxylation. These experimental conditions also resulted in the inhibition of laurate-dependent oxygen uptake catalyzed by P450BM3 (see below). The slightly higher (by ~10%) residual activity of the inhibited P450BM3 measured by [^{14}C]laurate hydroxylation compared to the measurements by NADPH oxidation (Figure 1) can be explained by the differences in experimental conditions. Initial reaction rates were measured for NADPH oxidation while [^{14}C]laurate hydroxylation was an end point assay requiring a 2 min incubation time. It is likely that a small fraction of the inactive P450BM3 slowly reactivated during the [^{14}C]laurate assay, thus giving higher activities. The cytochrome *c* reductase activity of BM3 was not significantly affected by incubation with NADPH (Figure 1).

We hypothesized that if the loss of hydroxylase activity of P450BM3 resulted from reduction of the reductase domain, reactivation of the enzyme upon oxidation by artificial electron acceptors would occur. Addition of cytochrome *c*, DCPIP, or ferricyanide to the inhibited P450BM3 10 s prior to [^{14}C]laurate addition indeed restored most of the hydroxylase activity (Figure 1). These electron acceptors are readily reduced when added to the reduced P450BM3, and this triggers a rapid reactivation of the hydroxylase activity.

A similar inhibition of hydroxylase activity was observed when laurate-dependent oxygen uptake was measured (Figure 2). A 2 min incubation of P450BM3 in the presence of NADPH and absence of fatty acid substrate decreased the subsequent laurate-dependent oxygen uptake rate by 85%. Addition of 50 μM cytochrome *c* 5−10 s prior to laurate rapidly restored ~70% of the oxygen consumption rate. Thus, the reduction of P450BM3 by NADPH inhibited oxygen uptake as well as NADPH oxidation and [^{14}C]laurate hydroxylation, and, therefore, inactivation is not a result of uncoupling. The ability of electron acceptors like cytochrome *c*, DCPIP, or ferricyanide to reactivate the enzyme shows that a specific reduction state of the reductase domain of P450BM3 determines whether the enzyme is active or not as a fatty acid hydroxylase. Visible absorption spectroscopy showed that inhibited P450BM3 has decreased absorbance in the 350−500 nm range corresponding to flavin reduction, and elevated absorbance in the 500−650 nm range characteristic for the neutral flavin semiquinone. Thus, inhibited P450BM3 appears to be a three-electron reduced enzyme.

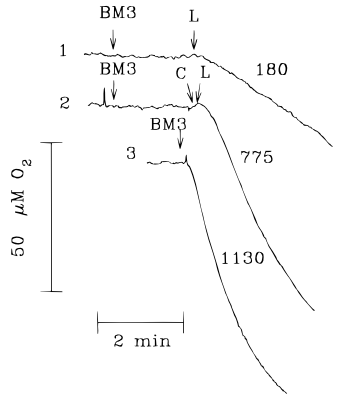

FIGURE 2: Inhibition of the laurate-dependent O_2 uptake catalyzed by P450BM3 by incubation with NADPH. The reactions were carried out in 0.1 M potassium phosphate buffer, pH 7.6, in the presence of 100 μM NADPH and an NADPH-regenerating system and other reagents as indicated. 1, The reaction mixture contained NADPH, and arrows indicate additions of P450BM3 (BM3) and laurate (L). 2, The reaction mixture contained NADPH, and arrows indicate addition of P450BM3 (BM3), cytochrome *c* (C), and laurate (L), respectively. 3, The reaction mixture contained NADPH and laurate, and the arrow indicates addition of P450BM3 (BM3). The final concentrations of NADPH, laurate, cytochrome *c*, and P450BM3 were 100, 500, 100, and 0.04 μM, respectively. The numbers on the curves show the reaction rates expressed as moles of O_2 per mole of P450BM3 per minute.

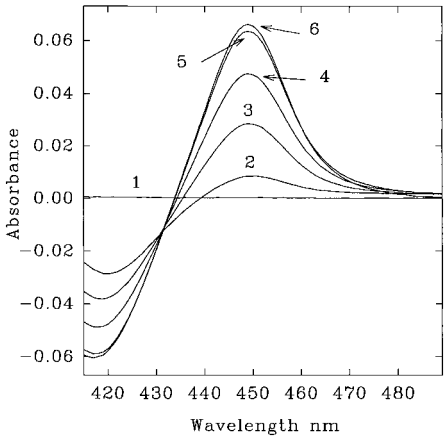

FIGURE 3: CO complex formation by P450BM3 reduced by NADPH in the absence of laurate. P450BM3 (0.8 μM) was incubated in a CO-saturated 0.1 M potassium phosphate buffer, pH 7.6, containing 67 μM NADPH, 1.4 mM glucose 6-phosphate, and 3.0 units/mL glucose-6-phosphate dehydrogenase for 5 min, and spectrum 1 was recorded. Laurate was added then to a final concentration of 500 μM, and spectra were recorded 20 s (2), 4 min (3), 8 min (4), and 12 min (5) after laurate addition, respectively. Spectrum 6 was obtained 20 s after addition of 200 μM DCPIP to the reduced P450BM3 (spectrum 1), immediately followed by laurate.

To determine which catalytic step is inhibited during P450BM3 incubation with NADPH, the experiment presented in Figure 3 was carried out. P450BM3 was incubated in a CO-saturated buffer in the presence of 67 μM NADPH and an NADPH-regenerating system for 5 min. No ferrous P450BM3−CO complex was formed during this time, showing that the heme domain remained oxidized in the

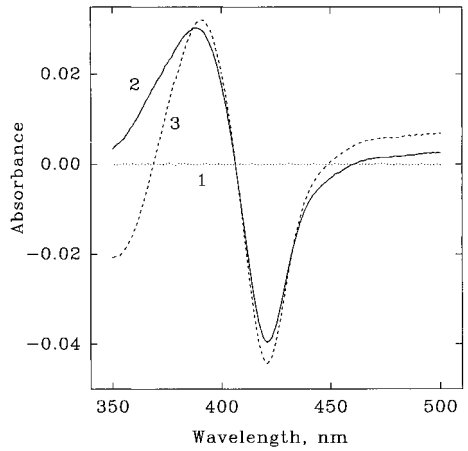

FIGURE 4: Laurate binding by oxidized and NADPH-reduced P450BM3. Spectrum 1 (dotted line), background. Spectrum 2 (solid line), oxidized P450BM3. The concentration of P450BM3 was 2.0 μM. The difference spectrum was recorded 20 s after addition of 1.0 mM laurate. Spectrum 3 (dashed line), reduced P450BM3. The enzyme was incubated at 2.0 μM concentration in a spectrophotometer cell in the presence of 50 μM NADPH, 2.0 mM glucose 6-phosphate, and 2.0 units/mL glucose-6-phosphate dehydrogenase for 5 min, and the difference spectrum was recorded 20 s after the addition of 1.0 mM laurate.

inactivated enzyme (Figure 3, spectrum 1). This is in agreement with previous reports (Li et al., 1991; Klein & Fulco, 1994) that P450BM3 is unable to form a ferrous CO complex in the absence of a fatty acid substrate. If the flavin domain of the reduced P450BM3 is capable of fast electron transfer, one would expect rapid ferrous CO complex formation upon laurate addition. However, the formation of the ferrous P450BM3−CO complex was very slow upon addition of 500 μM laurate to the reduced P450BM3, as shown by difference spectra recorded at different times (Figure 3, spectra 2−5). Only about 50% of the P450BM3 was reduced after a 4 min incubation. In contrast, addition of 200 μM DCPIP to the reduced P450BM3 5 s before the addition of laurate caused a rapid reduction of all the P450BM3 and conversion to the ferrous CO complex within 20 s, the shortest time required for mixing and recording (Figure 3, spectrum 6). All the added DCPIP was reduced within the mixing time. DCPIP was chosen in this experiment because it does not interfere with the spectral assay.

Slow formation of the ferrous CO complex by inhibited P450BM3 after laurate addition can result from slow binding of the fatty acid to the reduced enzyme, or from inhibited electron transfer from the flavin domain to the heme iron. We measured binding of laurate by the oxidized and reduced P450BM3 by type I spectral changes, and the results are shown in Figure 4. P450BM3 incubated with NADPH for 5 min showed absorbance changes at 390 and 420 nm upon the addition of 1.0 mM laurate that were similar to those observed with the control, oxidized enzyme. P450BM3 inhibited by incubation with NADPH retains a low residual hydroxylase activity. Therefore, the decrease in absorbance below 370 nm with the reduced enzyme can be explained by oxidation of NADPH present in the sample. The difference spectra presented in Figure 4 were obtained about 20 s after addition of laurate to the enzyme, the shortest time required for adding, mixing, and recording the spectra. We attempted to measure the rate of laurate binding by stopped-flow spectrophotometry. However, with both oxidized and reduced P450BM3 preparations, the absorbance decrease at 420 nm upon addition of 1.0 mM laurate was completed within the dead time of our instrument (15 ms), and no further changes at 420 nm was observed within the next 20 s (data not shown). We conclude that binding of 1.0 mM laurate by both enzyme forms is a rapid process. Thus, the reduced P450BM3 is able to bind laurate as fast as the oxidized enzyme does, and, therefore, slow formation of the ferrous−CO complex by the reduced enzyme is a direct result of the inhibited electron transfer from the flavoprotein domain to the heme iron.

P450BM3 incubated with NADPH retains cytochrome *c* reductase activity and is also able to reduce DCPIP and ferricyanide. This implies that during reduction by NADPH a specific interaction between the two domains of P450BM3 required for fast electron transfer to the heme iron is altered, while the electron-transfer properties of the flavin domain with respect to the artificial acceptors remain essentially unaffected. The results of Figures 3 and 4 demonstrate that the reductase domain of P450BM3 reduced by NADPH in the absence of a fatty acid substrate is unable to transfer the first electron to the P450 domain at a high rate, even upon binding of a fatty acid substrate, thus inhibiting all the functions associated with the heme domain. Reoxidation of the inactive enzyme by artificial electron acceptors such as ferricyanide, DCPIP, or cytochrome *c* produces a catalytically competent form of the enzyme capable of fast transfer of the first electron to the heme iron and of high hydroxylase activity.

Effect of Carbon Monoxide on Cytochrome c Reduction and Laurate-Dependent NADPH Oxidation. Fatty acid hydroxylation catalyzed by P450BM3 is inhibited by CO (Hare & Fulco, 1975; Ho & Fulco, 1976), but nothing is known of the effect of CO on the function of the flavoprotein domain. We studied the effect of CO on the cytochrome *c* reductase activity of P450BM3. Surprisingly, 400 μM carbon monoxide stimulated the cytochrome *c* reductase activity of P450BM3 about 3.5-fold. We verified that CO does not change the spectral properties of cytochrome *c*. Thus, the increased rate of cytochrome *c* reduction in the presence of CO results from a higher activity of P450BM3 rather than from an increased absorbance of reduced cytochrome *c* in the presence of CO. In the absence of laurate, reduction of cytochrome *c* was clearly biphasic at low carbon monoxide concentrations. At concentrations of 10−150 μM, CO stimulated the initial rate of cytochrome *c* reduction, but the reaction rate then slowed down to the rate observed in the absence of carbon monoxide. The higher the concentrations of carbon monoxide, the longer the cytochrome *c* reduction rate was stimulated, and at CO concentrations of 300 μM or higher no detectable decrease in stimulation was observed. In the presence of 500 μM laurate, the cytochrome *c* reductase activity of P450BM3 was also stimulated by carbon monoxide. However, cytochrome *c* reduction in the presence of the fatty acid substrate was always monophasic and did not slow down even at low CO concentrations. Initial rates of cytochrome *c* reduction in the presence of carbon monoxide were the same in the presence or absence of laurate.

We studied the effect of the order of laurate and carbon monoxide addition on cytochrome *c* reduction by P450BM3 to understand the different effect of CO on the kinetics of cytochrome *c* reduction in the presence and absence of laurate (Figure 5). When cytochrome *c* is present as the only

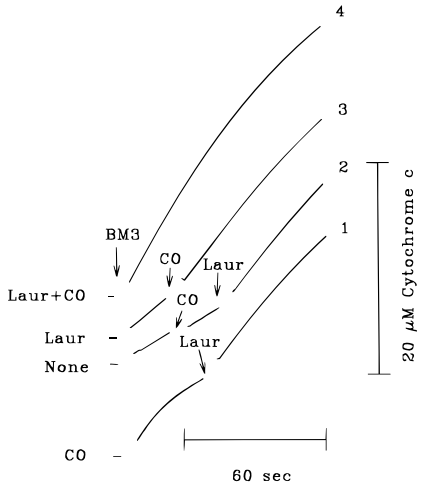

FIGURE 5: Effect of laurate and carbon monoxide on the time course of cytochrome *c* reduction by P450BM3. Final concentration of P450BM3 in the reaction mixture was 1.5 nM. The reaction mixture before P450BM3 addition contained 100 μM NADPH, 50 μM cytochrome *c*, and additionally 500 μM laurate and/or 27 μM CO as indicated. Where indicated by arrows, carbon monoxide (CO) and laurate (Laur) were added in the course of the reaction to the final concentrations of 27 and 500 μM, respectively. CO was added as a saturated solution. The reaction was started by the addition of the enzyme (BM3) and followed by the absorbance change at 550 nm.

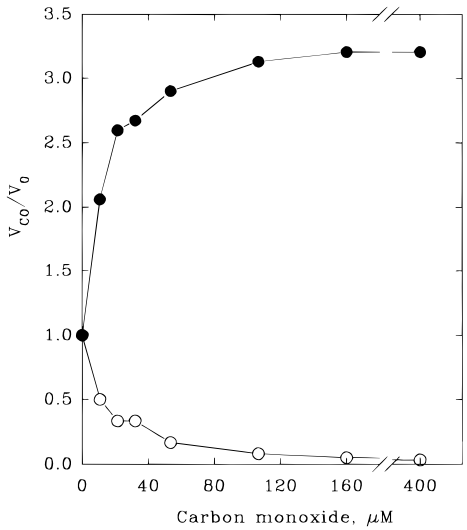

FIGURE 6: Effect of carbon monoxide concentration on cytochrome *c* reduction and laurate-dependent NADPH oxidation catalyzed by P450BM3. The experimental conditions were as described in the legend to Figure 5. NADPH oxidation (○) and cytochrome *c* reduction (●) were measured in the presence of 500 μM laurate. The *y* axis shows the ratio of the activities with CO to the activities with no CO added. The activity without CO was 1092 min^{-1} for laurate-dependent NADPH oxidation and 3531 min^{-1} for cytochrome *c* reduction.

substrate (trace 1), a low CO concentration (27 μM) stimulates the initial activity which decreases with time to reach a value close to the reaction rates in the absence of carbon monoxide. If carbon monoxide is added into the reaction mixture 20 s after P450BM3, no activation is observed (trace 2). Addition of laurate 20 s later results in a stimulation of cytochrome *c* reductase activity. Laurate alone stimulates the cytochrome *c* reductase activity of P450BM3 by 50−70%, and addition of CO in the course of the reaction immediately increases cytochrome *c* reductase activity even further (trace 3). The presence of both laurate and carbon monoxide in the reaction mixture produces a 3−4-fold stimulation of the rate of cytochrome *c* reduction (trace 4). These experiments showed that cytochrome *c* reductase activity is stimulated by carbon monoxide only when fatty acid is present in the reaction mixture. The activating effect of CO on the initial rate of cytochrome *c* reduction with no laurate added (Figure 5, trace 1) likely reflects the presence of a ligand/substrate at the P450BM3 catalytic site of the purified, active enzyme.

Figure 6 shows the effect of carbon monoxide concentration on cytochrome *c* reductase and laurate-dependent NADPH oxidase activities of P450BM3. Cytochrome *c* reductase activity was stimulated and laurate-dependent NADPH oxidation was inhibited at increasing CO concentrations. The half-maximal effect of CO on both activities was observed at about 10 μM carbon monoxide, suggesting that the two processes, stimulation of cytochrome *c* reduction and inhibition of hydroxylase activity, result from CO association to one binding site, likely heme iron. Cytochrome *c* reductase activity of P450BM3 is stimulated by laurate alone (Figure 5), and to a larger extent by laurate plus carbon monoxide (Figures 5 and 6). However, neither laurate nor laurate plus carbon monoxide affected the K_m for cytochrome c (Figure 7), demonstrating that the activation is not a result of a facilitated binding of cytochrome *c*. Thus, the stimulation of cytochrome *c* reductase activity by CO in the presence of

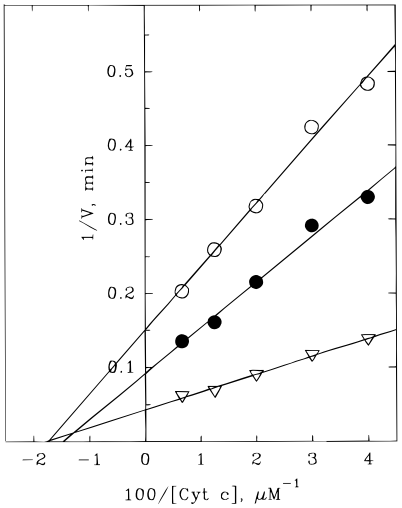

FIGURE 7: Effect of laurate and carbon monoxide on the K_m for cytochrome *c* reduction. The reaction was measured in the presence of 100 μM NADPH and cytochrome *c* concentrations varied from 20 to 150 μM. (○) Control; (●) 500 μM laurate; (▽) 500 μM laurate and 480 μM carbon monoxide.

laurate reflects an increased rate of electron transfer to cytochrome *c*. The latter in turn can result from either a higher rate constant of electron transfer or an increased steady-state concentration of the specific enzyme form that catalyzes cytochrome *c* reduction. However, it is clear that fatty acid binding causes conformational changes in the protein which are transferred to the flavoprotein domain and change its electron-transfer properties.

The presence of laurate stimulates the cytochrome *c* reductase activity of P450BM3, and inhibition of electron utilization by CO increased cytochrome *c* reduction rates even further (Figure 7). On the other hand, reduction of P450BM3 by NADPH in the absence of fatty acid substrate inhibited electron transfer from the reductase domain to the heme iron (Figure 3). It would be reasonable to expect that

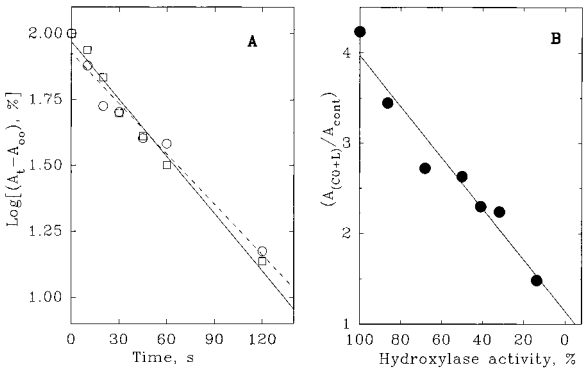

FIGURE 8: Correlation of inhibition of hydroxylase activity and loss of stimulation of cytochrome *c* reduction by laurate and CO during incubation of P450BM3 with NADPH. (A) The enzyme was incubated with 50 μM NADPH in the presence of an NADPH-regenerating system, and NADPH−oxidase activity in the presence of 500 μM laurate (□, solid line) and cytochrome *c* reductase activity in the presence of 500 μM laurate and 400 μM CO (○, dashed line) were measured at the times indicated. The semilogarithmic plot shows the inhibition of the two activities as a function of time, and the lines are linear regressions. The rate constants are 1.0 min^{-1} for hydroxylase and 0.9 min^{-1} for cytochrome *c* reductase activities. (B) Replot of the data shown in (A). The ordinate shows the ratio of cytochrome *c* reductase activity measured in the presence of laurate and CO to the activity measured in their absence (control). The abscissa shows the percentage of NADPH−oxidase activity remaining.

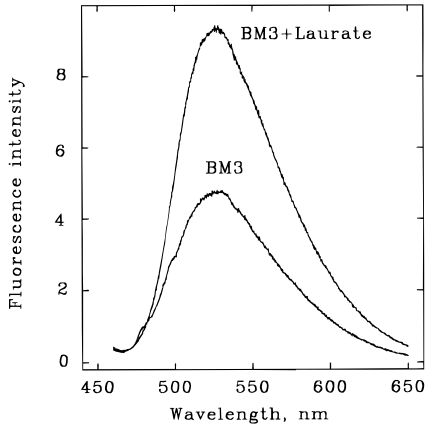

FIGURE 9: Effect of laurate binding on P450BM3 flavin fluorescence. P450BM3 (2.5 mM) was incubated in 100 mM Tris-HCl, pH 7.7, buffer at room temperature. Fluorescence emission spectra from 460 to 650 nm (excitation wavelength of 450 nm) were recorded before and after addition of laurate to a final concentration of 1 mM.

if electron transfer to laurate is required for activation of cytochrome *c* reduction, incubation of P450BM3 with NADPH would abolish the activation. The results of Figure 8 show that this is the case. Incubation with NADPH caused an inhibition of hydroxylase activity and a decrease in the stimulation of cytochrome *c* reduction by laurate and CO, both proceeding with the same rate constant (Figure 8A). Correlation of the inhibition of hydroxylase activity with the residual stimulation of cytochrome *c* reduction gave a straight line, with complete inactivation corresponding to the complete lack of stimulation (Figure 8B). As shown above, reduced P450BM3 binds laurate, but is unable to increase cytochrome *c* reductase activity in the presence of CO. Thus, a catalytic intermediate of electron transfer from FMN to the heme iron is involved in the stimulation of the cytochrome *c* reductase activity of P450BM3.

It should be noted here that laurate and CO also stimulated DCPIP reduction about 3-fold, but had very little if any effect on ferricyanide reduction (data not shown). It is well recognized that cytochrome *c* and DCPIP reduction involves FMN as an electron donor, while ferricyanide accepts electrons from FAD (Vermilion et al., 1981; Kurzban & Strobel, 1986; Klein & Fulco, 1993). It appears that laurate, or laurate and CO together, only affects electron-transfer reactions associated with the FMN cofactor. The stimulation of cytochrome *c* reduction is also observed with a number of other substrates, such as palmitate, tetradecanol, and hexadecanol (data not shown).

Effect of Laurate Binding on Flavin Fluorescence. A severalfold stimulation of cytochrome *c* reduction by laurate is observed under experimental conditions in which no electron flow to the heme iron occurs. This suggests that binding of the fatty acid substrate at the heme catalytic site alters electron-transfer properties of the flavoprotein by induced conformational changes. We studied the effect of laurate on the flavin fluorescence of intact active P450BM3 to test this hypothesis. Fluorescence emission spectra of P450BM3 were recorded before and after addition of 1 mM laurate in the range of 460−650 nm, at the excitation wavelength of flavins of 450 nm. Figure 9 shows the spectra obtained. Addition of laurate resulted in an almost doubling fluoresence intensity of the flavin cofactors of P450BM3, indicating that fatty acid substrate binding induces conformational changes in the flavoprotein domain. This observation is in agreement with the finding that laurate stimulates cytochrome *c* reduction severalfold in the presence of CO. Because this stimulation does not require electron transfer to the heme iron, it must be a result of the conformational changes of the flavoprotein domain induced by the fatty acid binding to the catalytic site. As mentioned above, laurate and CO only stimulated activities that involve electron transfer through FMN. It is tempting to suggest that enhancement of flavin fluorescence (Figure 9) reflects mostly an alteration of the environment of FMN induced by laurate binding.

Effect of Cytochrome c on Laurate Hydroxylation. Cytochrome *c* reduction by P450BM3 was stimulated in the presence of 500 μM laurate (Figure 5). We studied the effect of cytochrome *c* on laurate hydroxylase activity, and found that 100 μM cytochrome *c* had very little if any effect on the laurate hydroxylation rate when the fatty acid concentration was varied in the range of 80−500 μM. Both in the absence and in the presence of cytochrome *c*, the K_m for laurate was about 180 μM, and the V_{max} of hydroxylation was 1990 and 1870 min^{-1} in the absence and presence of 100 μM cytochrome *c*, respectively. Thus, the stimulation of cytochrome *c* reduction by laurate occurs with no effect on laurate hydroxylation, and electron transfer to cytochrome *c* during laurate hydroxylation has no effect on electron transfer to the heme iron. Therefore, P450BM3 is able to catalyze the conversion of both substrates simultaneously with rates equal or higher than the reaction rates measured with a single substrate.

DISCUSSION

Klein and Fulco (1994) reported that cytochrome *c* reduction by P450BM3 is stimulated by a fatty acid substrate, myristate. Our results show that this stimulation is not a

specific feature of the myristate hydroxylase activity, but is also observed with laurate (Figures 5 and 7), as well as a number of other substrates. When two substrates, cytochrome *c* and fatty acid, are present in the reaction mixture simultaneously, hydroxylase activity does not change even though cytochrome *c* reduction is stimulated (Klein & Fulco, 1994; see also above). Moreover, each reaction occurs in the presence of an alternative substrate with no changes of K_m for the substrates. The ability of P450BM3 to catalyze simultaneously fatty acid hydroxylation and cytochrome *c* reduction, with no inhibition by the alternative substrate, suggests that cytochrome *c* binding and reduction do not interfere with functioning of the P450 domain. In other words, cytochrome *c* accepts electrons from a site different from the one utilized by the P450 domain, and such electron "draining" to cytochrome *c* does not slow down hydroxylation. In P450BM3, cytochrome *c* reductase activity represents a side reaction involving catalytic step(s) that are not part of the normal hydroxylase catalytic cycle. This conclusion is in agreement with the results of Klein and Fulco (1993), who found that conservative substitutions of Trp574 by aromatic amino acids resulted in an 80% loss of hydroxylase activity while cytochrome *c* reductase activity was not significantly changed. The amino acid Trp574 participates in fast electron transfer to the heme domain of P450BM3 but not to cytochrome *c* (Klein & Fulco, 1993).

Our observation of the unusual activation of cytochrome *c* reductase activity by carbon monoxide (Figures 5−7) is novel. It appears that upon binding of a fatty acid substrate, the concentration of a certain intermediate of the catalytic cycle with elevated cytochrome *c* reductase activity increases. This is consistent with the fact that activation does not change the K_m for cytochrome *c* (Figure 7). The concentration of this intermediate is not limiting hydroxylase activity since cytochrome *c* has no inhibitory effect on fatty acid hydroxylation, but determines the rate of cytochrome *c* reduction. Further activation by CO can then be explained as follows. Binding of carbon monoxide to the P450BM3−substrate complex with elevated cytochrome *c* reductase activity prevents electron utilization for hydroxylation, thus increasing electron flow from the highly active intermediate to cytochrome *c*. Since CO activates cytochrome *c* reduction only when an endogenous or added substrate is bound (Figure 5), substrate binding seems to be required for this activation. Thus, binding of a fatty acid substrate to the heme domain induces conformational changes that alter properties of the flavoprotein domain. Two conformations of the substrate binding pocket of the heme protein domain of P450BM3 have been detected by X-ray crystallography (Ravichandran et al., 1993; Li & Poulos, 1995). Energy minimization of the X-ray structure of the heme domain of P450BM3 (Li & Poulos, 1995) as well as molecular dynamics simulations (Paulsen & Ornstein, 1995) suggested that large conformational changes of the heme domain are associated with fatty acid binding. Recent results of Modi et al. (1996) showed that hydroxylation involves substantial structural changes of the protein. The results presented here provide evidence that major conformational changes do occur when laurate binds to the catalytic site of the heme domain. These conformational changes also alter properties of the flavoprotein domain leading to enhancement of the flavin fluorescence intensity and changes in electron transfer properties of the FMN, but not FAD, of the enzyme.

As shown in Figure 6, carbon monoxide alone produced a transient activation of the initial rate of cytochrome *c* reduction. This stimulation can be explained by invoking the presence of endogenous bound substrate in P450BM3. Upon addition into the reaction mixture containing carbon monoxide, P450BM3 with the bound substrate rapidly binds CO and forms an inactive complex with high cytochrome *c* reductase activity. CO is a reversible inhibitor, and a fraction of the enzyme not inhibited by CO will oxidize bound substrate and no longer be stimulated. The lower the CO concentration, the larger fraction of the CO-free enzyme and the faster bound substrate will be oxidized and released; thus, a shorter transient stimulation of cytochrome *c* reductase activity will be observed. With no CO present, the enzyme loses bound substrate within the first catalytic turnover, and no activation of cytochrome *c* reduction can be detected. A lag in [^{14}C]palmitate hydroxylation by P450BM3 was observed by Matson et al. (1977), and this lag was abolished by incubation of the enzyme with palmitate prior to the start of the reaction with NADPH. This old observation is consistent with the idea that native P450BM3 contains a slowly-metabolized bound substrate. Incubation with palmitic acid leads to the exchange of this endogenous substrate with rapidly metabolized palmitate thus abolishing the lag phase.

The ability of P450BM3 to turn over in the presence of two substrates (laurate and cytochrome *c*) with higher rates than in the presence of only one substrate implies that the reductase domain of P450BM3 has a much higher catalytic capacity than that measured in the presence of a single substrate. The stimulating effect of carbon monoxide on cytochrome *c* reduction reported here (Figures 5−7) constitutes strong evidence that this is indeed the case. In the presence of laurate and carbon monoxide, P450BM3 is able to catalyze cytochrome *c* reduction with maximal rates of about 25 000 min^{-1}, or approximately 400 s^{-1} (Figure 7). Since cytochrome *c* accepts electrons from FMN of P450BM3 (Klein & Fulco, 1993) or microsomal cytochrome P450 reductase (Vermilion et al., 1981; Kurzban & Strobel, 1986), one can conclude that the rate of 400 s^{-1} is the minimal rate of electron transfer from NADPH through FAD to FMN in P450BM3.

It has been reported that P450BM3 catalyzes hydroxylation of hexadecyltrimethylammonium bromide with rates as high as 150 s^{-1} (Black et al., 1994). This turnover rate is still lower than the catalytic capacity of the reductase domain, implying that the overall catalytic turnover of P450BM3 is limited by events taking place at the catalytic site of the heme domain. This might explain the lack of inhibition of cytochrome *c* reductase activity by a fatty acid substrate or hydroxylase activity by cytochrome *c*: the reductase domain supplies electrons much faster than the combined rates of both reactions. The hydroxylation rate of 150 s^{-1} reported by Black et al. (1994) represents a minimal estimate for the rate constants of the steps common to all substrates: first and second electron transfer from the reductase to the heme domain; proton abstraction, or dioxygen cleavage [see review Mueller et al. (1995)]. Most P450BM3 substrates, however, are hydroxylated with slower rates, indicating that specific substrate-related step(s) must limit enzyme turnover.

Recently Munro et al. (1995) suggested that inhibition of hydroxylase activity during incubation with NADPH results at least partially from the accumulation of $NADP^+$, compet-

ing with NADPH. Our observations that inhibition is developed in the presence of an NADPH-regenerating system and that the inhibited enzyme can be rapidly reactivated by artificial electron acceptors (Figures 1 and 2) show that the state of flavin reduction rather than the accumulation of $NADP^+$ is responsible for the inactivation. Absorption spectroscopy indicated that the inhibited enzyme is a three-electron reduced form (data not shown). As has been shown by Li et al. (1991) and confirmed recently (Sevrioukova & Peterson, 1995; Sevrioukova et al., 1996), reduction of the isolated reductase domain of P450BM3 by NADPH results in formation of a three-electron reduced flavoprotein with FMN being fully reduced. Thus, $FMNH_2$ is unable to provide electrons required for catalysis at the heme catalytic site. It is still unknown why fully reduced FMN is unable to transfer electrons to the heme iron even when laurate binds to the reduced enzyme. Two possible explanations can be advanced. The first one is that the redox potential of FMN in the inactive enzyme is not negative enough to reduce heme iron even in the presence of CO. Such an explanation does not seem plausible since the redox potential of fully reduced FMN is negative enough to be able to reduce heme iron. Another possibility, as suggested by Narhi and Fulco (1986), is that enzyme reduction in the absence of fatty acid substrate induces conformational changes that alter the relative orientation of the two domains and misalign the electron-transfer channels of the flavoprotein and heme domains. Structural alterations in intact P450BM3 upon reduction with NADPH were observed by circular dichroism spectrometry in the near-UV−visible region (Munro et al., 1995), suggesting that some conformational changes in the protein did occur. Because inhibition of hydroxylase activity by reduction of P450BM3 also prevents stimulation of cytochrome *c* reductase activity by laurate and CO (Figure 8), but does not affect cytochrome *c* reduction in their absence, we favor the view that reduction induces conformational changes in the enzyme. The observation that stimulation of cytochrome *c* reduction occurs when electron flow to the heme iron is severely inhibited by CO (Figures 5−7) also supports the existence of conformational interactions between the two domains. Such a stimulation is not observed when the enzyme is inactivated by NADPH (Figure 8), even though no electron flow to the heme iron occurs.

The NADPH-inhibited P450BM3 is rapidly reactivated by artificial electron acceptors (Figure 1) or by prolonged dialysis (Narhi & Fulco, 1986), which likely also leads to the reoxidation of the flavin cofactors. The results of Figure 3 show that the heme domain of P450BM3 with laurate bound can only be reduced slowly once the flavoprotein domain has been reduced in the absence of a fatty acid substrate. The conformational changes associated with P450BM3 inactivation do not inhibit reduction of the artificial electron acceptors by the flavoprotein domain, but prevent electron transfer to the heme domain of P450BM3. Thus, there is at least one form of the reductase domain that is able to catalyze cytochrome *c*, DCPIP, or ferricyanide reduction, but is inactive as an electron donor for the heme domain, even when fatty acid substrate is bound. The results presented in this paper and by Klein and Fulco (1993, 1994) show that hydroxylase and cytochrome *c* reductase activities of P450BM3 have different rate-limiting steps, and also involve different elementary step(s) and enzyme intermediate(s).

The two domains in intact P450BM3 appear to specifically influence each others conformations, and this is essential for efficient catalysis. This hypothesis would explain the failure to reconstitute significant hydroxylase activity from individual domains of P450BM3 (Narhi & Fulco, 1987; Li et al., 1991; Miles et al., 1992; Boddupalli et al., 1992). If specific functional interactions of the two domains are necessary for high catalytic rates, separation of the domains would prevent fast electron transfer from FMN to heme iron. This is in agreement with the results of Munro et al. (1994), who showed that near-UV and visible circular dichroism spectra, which assess the environment of aromatic amino acids and cofactors, are different for intact P450BM3 and for an equimolar mixture of the individual domains. These authors concluded that the interaction of the domains in intact P450BM3 involves some structural alterations, which cannot occur in the mixture of the individual domains (Munro et al., 1994). Our results can also explain the absolute requirement of a bound fatty acid for heme reduction by NADPH (Li et al., 1991; Klein & Fulco, 1994). With no substrate bound, an inhibited state of the flavoprotein, which is unable to supply electrons required for the heme reduction and catalysis, is rapidly formed.

In summary, we conclude that two processes, reduction of cytochrome *c* and reduction of the heme domain of P450BM3, involve the following: (a) different electron-transfer site from FMN; (b) different elementary steps of the catalytic cycle; (c) different flavoprotein intermediates; and (d) different rate-limiting steps. Thus, at least with P450BM3, special caution should be taken when projecting results obtained with cytochrome *c* reduction to the mechanism of reduction of the P450 heme iron. Similar caution might also apply to studies of the interactions between microsomal cytochrome P450 reductase and cytochrome P450. In addition, our data and a number of structural and functional data obtained recently with P450BM3 suggest that properties of the two domains in the intact enzyme may differ considerably from the properties of the separated flavoprotein and heme domains.

ACKNOWLEDGMENT

We thank Professor Armand Fulco for his generous gift of the P450BM3 expression plasmid and Dr. V. Guzov for helpful discussions and reading the manuscript. We also thank anonymous reviewers for constructive suggestions.

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BI961667U