Fatty Acid-Induced Alteration of the Porphyrin Macrocycle of Cytochrome P450 BM3

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ABSTRACT Surface-enhanced resonance Raman scattering (SERRS) of substrate-free and substrate-bound forms of the P450 domain of cytochrome P450 BM3 are reported and assigned. Substrate-free P450 yields mixed spin heme species in which the pentacoordinate high-spin arrangement is dominant. The addition of laurate or palmitate leads to an increase in high spin content and to an allosteric activation of heme mode ν_{29}, which is sensitive to peripheral heme/protein interactions. Differences between laurate and palmitate binding are observed in the relative intensities of a number of bands and the splitting of the heme vinyl modes. Laurate binding to P450 results in different protein environments being experienced by each vinyl mode, whereas palmitate binding produces a smaller difference. The results demonstrate the ability of SERRS to probe substrate/prosthetic group interactions within an active site, at low protein concentrations.

INTRODUCTION

The cytochrome P450 monooxygenase (P450) superfamily of hemoprotein enzymes catalyzes the oxidation of a wide variety of organic molecules in both prokaryotic and eukaryotic organisms (Porter and Coon, 1991; Ortiz de Montellano, 1986). P450s have numerous physiological roles, including the metabolism of steroids and fatty acids (Ortiz de Montellano, 1986). Cytochrome P450 BM3 (CYP102) from *Bacillus megaterium* holds a unique position in the P450 superfamily. It is the only known prokaryote or eukaryote P450 system that is catalytically self-sufficient, possessing the heme-containing P450 domain (isolated cytochrome P450-linked monooxygenase domain of P450 BM3) (N-terminal) fused to the flavin-containing reductase domain (C-terminal) in a single 119-kDa polypeptide (Narhi and Fulco, 1986). It is also the only known bacterial P450 that utilizes a single NADPH-dependent reductase system similar to microsomal systems.

The *cyp*102 gene and its subgenes encoding the separate domains have been cloned, sequenced (Narhi and Fulco, 1986, 1987), and overexpressed in *Escherichia coli* (Narhi and Fulco, 1987; Miles et al., 1992). All proteins have been purified to homogeneity. The high primary structure homology with mammalian P450 fatty acid hydroxlases and the availability of the crystal structure of the P450 domain (Ravichandran et al., 1993) make P450 BM3 the most appropriate model for eukaryotic microsomal P450 systems (Ravichandran et al., 1993; Hasemann et al., 1995).

The heme is ligated on the proximal face by a cysteine (Cys^{400}) and on the distal face by a weakly bound water molecule (Ravichandran et al., 1993; Hasemann et al., 1995), creating a low-spin hexacoordinated ferric center. Distal face access can only occur along a long hydrophobic channel that has been shown to be the substrate binding site (Ravichandran et al., 1993). Recent NMR studies on the binding of lauric acid to the P450 domain of P450 BM3 indicate that the substrate is located at an unusually long distance from the heme iron (Modi et al., 1995). NMR of reduced P450 suggests that substrates are positioned 6 Å closer to the ring than oxidized P450 and indicates that substantial structural change occurs upon heme reduction to move the substrate closer to the heme iron (Modi et al., 1996).

The ability to isolate the heme-containing P450 domain of P450 BM3 in high purity opens the way for detailed analysis of the active site. In this paper we have utilized surface-enhanced resonance Raman scattering (SERRS) to analyze the interaction of fatty acid substrates within the heme domain of P450 BM3. SERRS retains the molecular specificity of resonance Raman and increases the scattering efficiency by coupling the oscillating electric field of incident and scattered light with surface waves of electrons on roughened metal (e.g., Au, Ag, or Cu) surfaces (Creighton, 1988; Weitz et al., 1986; Otto et al., 1992). Experimentally observed enhancement factors as large as 10^4 over resonance Raman have been obtained from chromophores adsorbed on silver colloids or electrodes (Hildebrandt and Stockburger, 1986, 1989), enabling more dilute solutions to be probed.

The interpretation of the SERRS data has been aided by normal coordinate analyses of porphyrin model compounds (Abe et al., 1978), labeling studies of heme proteins (Hu et al., 1993), and comparative resonance Raman data from mammalian (Hildebrandt, 1991; Hildebrandt et al., 1989)

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and bacterial cytochromes-P450 (Hildebrandt and Stockburger, 1986; Wells et al., 1992; Uno et al., 1985). In addition to information on the oxidation, spin, and ligation state of the heme, SERRS indicates subtle structural distortion of the heme on substrate binding. These effects have not been detected by circular dichroism or UV/visible spectroscopy (Macdonald et al., 1996).

MATERIALS AND METHODS

Cytochrome P450 BM3 (P450 domain) preparation

Plasmid construction, gene expression, and protein purification have been extensively described in a previous publication (Miles et al., 1992). Bacterial transformations and other molecular techniques used standard methods that are outlined in other publications (Narhi and Fulco, 1986, 1987; Miles et al., 1992).

Spectrophotometric protein and enzyme assays

The UV/visible spectra were obtained on a Shimadzu 2101 spectrophotometer (Shimadzu Corporation, Kyoto, Japan). Protein concentrations were determined using the methods of Bradford (1976) and by the BCA technique (Smith et al., 1985), with bovine serum albumin as standard. Cytochrome P450 concentrations were estimated by the method of Omura and Sato (1964), using $\epsilon = 91\ mM^{-1}\ cm^{-1}$ at 450 nm for the ferrous/CO heme adduct.

Colloid preparation

Colloidal silver was prepared according to a modified Lee and Meisel method (Lee and Meisel, 1982). The procedure has been extensively described in a recent publication (Macdonald and Smith, 1996). The prepared suspensions of small colloidal particles (typical diameter 30–35 nm) were stable for a minimum of 3 months.

Sample protocol

Fresh samples of the P450 domain of CYP102 were prepared in 100 μl of 100 mM sodium phosphate (Fisons, Analar grade) buffer (pH 7.5), giving a protein concentration of 20–40 μM. Ten to twenty microliters of freshly prepared protein solution was then added to 290–280 μl of ice-cold 100 mM phosphate buffer (pH 5.8), and the solution left was on ice for 15 min. This solution was added to 2 ml of cooled silver colloid and incubated on ice. Thirty-five microliters of a fresh 1% w/v L-ascorbic acid (>99%; Sigma) solution was added, initiating aggregation of the colloid. The final sample concentration of P450 domain was maintained at $\sim 1.7 \times 10^{-7}$ M. Insertion into a compartment with circulating coolant (iced water) maintained the sample temperature at 2–6°C throughout the Raman measurement process.

Sample: fatty acid preparation

Cytochrome P450 BM3 substrates palmitic acid (>99%; Sigma) and sodium laurate (>99%; Sigma) were routinely prepared as 75 mM and 150 mM methanolic solutions. Substrate addition was performed by two different methods. One was simple addition of cooled methanolic (substrate) solution to the aggregated protein sample, prepared as described above. The other procedure involved the addition of 15 μl of the fatty acid solutions to protein dissolved in 300 μl of mixed 100 mM phosphate buffers (see above). This solution was added to 2 ml of cooled Ag colloid and aggregated with 45 μl of 1% w/v L-ascorbic acid.

Raman collection

Spectra-Physics argon ion (2020/2045) continuous-wave lasers provided 50 mW or 40 mW of 457.9-nm and 514.5-nm excitation. Data acquisition and analysis equipment have been described elsewhere (Macdonald and Smith, 1996). A scanning rate of 1 $cm^{-1}\ s^{-1}$ was used, and slit widths of 5 cm^{-1} (457.9 nm) and 3.6 cm^{-1} (514.5 nm), leading to a resolution of 2 cm^{-1}, were set unless indicated otherwise. Unless stated otherwise, SERRS spectra were recorded 30 min after initial aggregation.

RESULTS

Substrate-free P450 domain

SERRS of the substrate-free P450 domain were recorded using 457.9-nm (Fig. 1 *A*) and 514.5-nm (Fig. 1 *B*) excitation. Assignments and peak positions for the frequency region 1300–1700 cm^{-1} are presented in Table 1. Spin state marker bands (ν_{10}, ν_3, ν_2, ν_{11}) indicate the presence of mixed spin species for the heme protein on the colloidal surface. A band at 1493 cm^{-1} (Fig. 1 *A*) assigned to ν_3 is consistent with a five-coordinate, high-spin (5chs) species. The breadth of the peak and the high energy shoulder at 1502 cm^{-1} indicate the presence of some six-coordinate, low-spin (6cls) species, but the dominant surface species is pentacoordinate. This is confirmed by the relatively low energy recorded for the ν_2 mode at 1572 cm^{-1}. The ferric nature of the porphyrin macrocycle is confirmed by ν_4 at 1372 cm^{-1}. With Q-band excitation (514.5 nm), appreciable SERRS enhancement of nontotally symmetrical modes occurs. The appearance of a B_{2g} mode, ν_{29} at 1400 cm^{-1} (Hildebrandt and Stockburger, 1986), is a notable feature (see Table 1).

Substrate-bound P450 domain

The addition of laurate or palmitate to the P450 domain adsorbed on the surface was followed by measuring the ν_{29}/ν_4 ratio with time. There was gradual change over a period of 2 h. The results are presented for 514.5-nm excitation in Table 2. The SERRS intensity of ν_{29} increased relative to ν_4 with 514.5-nm excitation until the ν_{29} band intensity eventually obtains an intensity similar to that of ν_4. An equilibrium was achieved after ~2 h. Thereafter, the ratio remains constant. Photoinduced decomposition is ruled out, as the sample was removed after each scan (514.5 nm; 40 mW), and similar results were obtained with different illumination times. The frequencies of other bands for this time-dependent study supported further conversion to 5chs, consistent with substrate binding within the channel. SERRS spectra were recorded 30 min and 60 min after initial aggregation following the addition of substrate to the P450 domain in solution, and adsorption of the P450/substrate complex on the surface. They were found to be identical and similar to the final results from the previous experiment. This indicates that the kinetics are slow on the surface, but the final effect of adsorbed laurate and palmitate is to alter the ν_{29}/ν_4 ratio. Because a much faster

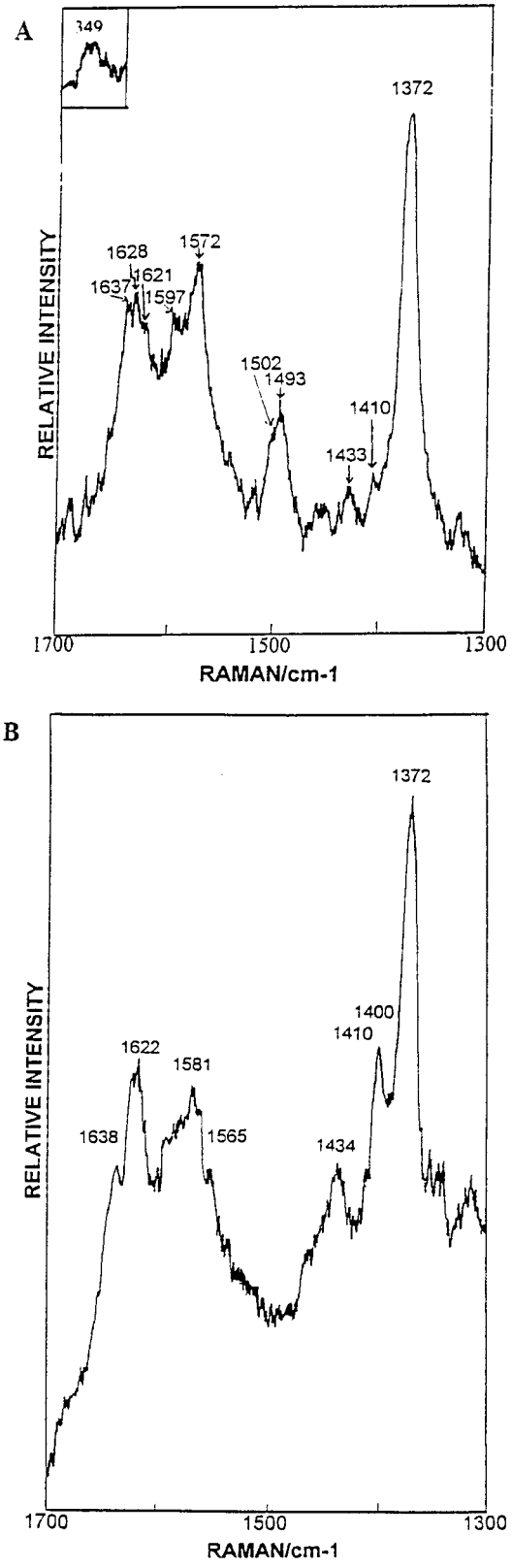

FIGURE 1 SERRS of P450 domain. (*A*) Recorded using 457.9-nm excitation (50 mW) at a final protein concentration of 3.12×10^{-7} M. (*B*) Recorded using 514.5-nm excitation (50 mW) at 3.05×10^{-7} M. Both were recorded 30 min after ascorbic acid-induced aggregation.

equilibrium occurs when the P450/substrate complex is preformed in solution and then added to the surface, all other spectra were taken by this approach (Figs. 2 and 3). They were recorded 30 min after aggregation to ensure that the aggregation process was stable. The spectra represent the final equilibrium state for the P450/substrate complex.

SERRS for laurate- and palmitate-bound P450 complexes prepared in solution and the complex adsorbed on the surface are presented in Fig. 2. For laurate-bound protein (Fig. 2 *A*), the absence of a high-energy shoulder for ν_3 and the disappearance of the 1637 cm^{-1} (ν_{10}) band confirm a further shift in the 6cls-5chs equilibrium toward the high-spin species upon laurate binding. The oxidation state marker band ν_4 remains at 1372 cm^{-1}. Poorly resolved vinyl scissoring (1410 and 1433 cm^{-1}) and stretching modes (1620–1630 cm^{-1}) are apparent, as is a formally Raman forbidden (E_u) mode, with ν_{38} present as a shoulder at ~1592 cm^{-1} (Abe et al., 1978).

The palmitate-bound P450 domain (Fig. 2 *B*) gave SERRS that were similar but not identical to those of the laurate-bound form. All of the major in-plane bands (ν_3, ν_2, ν_{10}) have frequencies identical to that of laurate and are illustrative of a 5chs heme species. The frequency of ν_4 remains unaltered at 1372 cm^{-1}.

Analysis of the 1000–1300 cm^{-1} region

SERRS was recorded for the substrate-free (Fig. 3 *A*), laurate-bound (Fig. 3 *B*), and palmitate-bound (Fig. 3 *C*) P450 domain in the frequency region 1000–1300 cm^{-1}. Two bands at 1170 cm^{-1} (ν_{30}) and 1132 cm^{-1} (ν_{22}) were observed for adsorbed substrate-free P450. In addition to these two modes, SERRS of laurate-bound P450 gives bands at 1064 cm^{-1} (a ring methyl mode), 1087 cm^{-1} (vinyl rocking deformation), and a split vinyl deformation or "*cis*" CH wag (1010 cm^{-1}; 1020 cm^{-1}). Palmitate-bound protein gives the "*cis*" CH wag vinyl mode at 1008 cm^{-1} and the methyl mode at 1065 cm^{-1}. Assigned bands are tabulated in Table 1.

UV/visible spectroscopy

Spectra of substrate-free and palmitate-bound P450 domain are presented in Fig. 4. The native protein has a large Soret absorbance band at 419 nm and μQ bands at 570 nm (Q_0) and 535 nm (Q_1). Upon substrate binding, the Soret band shifts to 390 nm, characteristic of a high-spin protein, and the Q bands merge, creating a single band at ~550 nm. A dissociation constant of ~1 μM has been reported for this fatty acid (Miles et al., 1992).

DISCUSSION

The surface-enhanced Raman scattering (SERS)-active substrate has to be carefully prepared to maintain protein integrity (Rospendowski et al., 1991). The colloid surface is

TABLE 1 Vibrational assignments between 1000 and 1700 cm^{-1} for the substrate-free P450 domain, with 514.5-nm and 457.9-nm excitation (50 mW), and the laurate-bound and palmitate-bound P450 domain with 457.9-nm excitation (50 mW)

	514.5-nm excitation	457.9-nm excitation		
Vibrational nomenclature*	Substrate-free P450 domain/cm^{-1}	Palmitate-bound P450 domain/cm^{-1}	Laurate-bound P450 domain/cm^{-1}	
ν_{10}	1638 [6cls]	1637 [6cls]		
$\nu_{C=C}$ [#]	1622; 1628sh	1628; 1621sh	1628; 1621	1628; 1621sh
ν_{37}		1597	1591	1592
ν_{19}	1581 [6cls]; 1572sh [5chs]			
ν_2		1572 [5chs]	1570 [5chs]	1570 [5chs]
ν_{11}	1565 [6cls]			
ν_{38}			1534	
ν_3		1502 [6cls]; 1493 [5chs]	1493 [5chs]	1493 [5chs]
			1465	
$\delta_{=CH2}$	1434; 1410sh	1433; 1410	1435	
$\nu_{29}/(\nu_{20})$ [§]	1400		1400	1400
ν_4	1372	1372	1372	1372
ν_{21}	1307			
ν_{13}		1230	—	—
ν_{30}		1170	1171	1170
ν_{22}		1132	1129	1129
$\delta_{as=CH2}$; δ_{CH3}		~1090	1089	1087
δ_{CH3}		—	1065	1064
ν_{23}		—	1038	—
$\delta_{as=CH2}$		—	1008	1020; 1010

5chs, Five-coordinate, high-spin hemoprotein; 6cls, Six-coordinate, low-spin hemoprotein.
* Adopted from Abe et al. (1978).
[#] 5chs contribution of ν_{10} expected at ~1625 cm^{-1} hidden under vinyl stretching region.
[§] Band at 514.5 nm is attributed to ν_{29}.

coated with a polymeric citrate layer, leaving residual uncomplexed carboxylate groups that provide a negatively charged surface for protein adsorption (Munro et al., 1995). Computer modeling of the protein based upon the crystal structure coordinates indicates two positively charged surface areas at physiological pH. The region most likely to be involved in surface binding from an electrostatic viewpoint has nine lysine and histidine residues. These are His^{38}, Lys^{59}, Lys^{94}, Lys^{97}, His^{100}, Lys^{113}, His^{236}, Lys^{306}, and His^{408}. The other region, which appears less favorable, involves Lys^{9}, Glu^{13}, Lys^{15}, Lys^{24}, Lys^{41}, Glu^{43}, Lys^{76}, Arg^{79}, Asp^{80}, and Lys^{187}. However, if the latter area were adsorbed, some residues on the surface of the protein at the opening of the substrate binding pocket would be involved in the adsorption to the colloid, preventing substrate binding. The fact that substrates do bind to the adsorbed protein supports the modeling predictions and suggests that the former region is bound to the surface. It should be noted that, as a degree of protein unfolding occurred at submonolayer coverage for cytochrome *c* (Macdonald and Smith, 1996), the protein/surface coverage of the P450 was maintained at or above monolayer coverage. Under these conditions, van der Waals forces from protein/surface and protein/protein contacts will also be important in determining the structure of the assembly of adsorbed proteins and the kinetics of substrate binding.

TABLE 2 Monitoring substrate-induced relative enhancement of ν_{29} with respect to ν_4 over time, using 3.12 × 10^{-7}M P450, which was preadsorbed before substrate addition to the silver surface

Time (min)	ν_{29}/ν_4 SERRS Intensity ratio
30*	0.43
60[#]	0.62
90[§]	0.68
120[¶]	0.97

* With no substrate 30 min after aggregation.
[#] With addition of palmitic acid (1.44×10^{-3}M final concentration) 30 min thereafter.
[§] After a further 30 min.
[¶] Two hours after aggregation.
Exciting line 514.5 nm; 40 mW. The signal was stable after ~2 h, and spectra were virtually identical to those obtained from P450/substrate complexes preaggregated in solution.

Substrate-free P450 domain

The relatively low frequencies of spin-state-sensitive modes such as ν_2 (1572 cm^{-1}) and ν_3 (1493 cm^{-1}) are typical of tetrapyrrole expansion (Partharasathi et al., 1987) and are consistent with the pattern of a dominant high-spin pentacoordinate heme adsorbed on the surface. UV/visible absorption studies (see Fig. 4) indicate a 6cls configuration for the protein in solution (Miles et al., 1992). Thus surface adsorption of the protein has altered the 6cls-5chs equilibrium compared to that of the protein in suspension in buffer. This change is easily effected in this protein, which exists in solution as a spin equilibrium in any case, and it does not

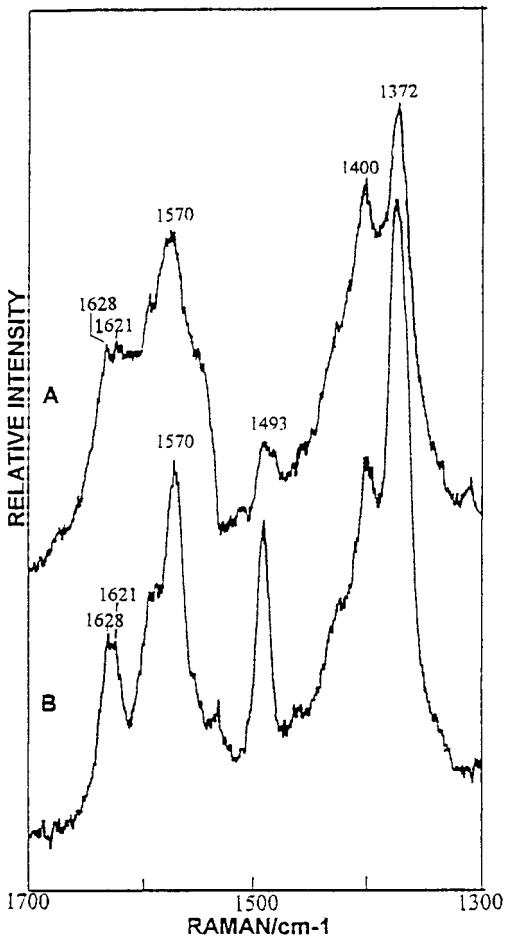

FIGURE 2 SERRS spectra of substrate P450 domain. The complex was formed before adsorption on the surface. (*A*) 2.99×10^{-7} M P450 in the presence of 9.71×10^{-4} M sodium laurate. (*B*) 1.19×10^{-7} M P450 in the presence of 4.04×10^{-4} M palmitic acid. SERRS spectra were recorded 30 min after aggregation. 457.9-nm excitation (50 mW).

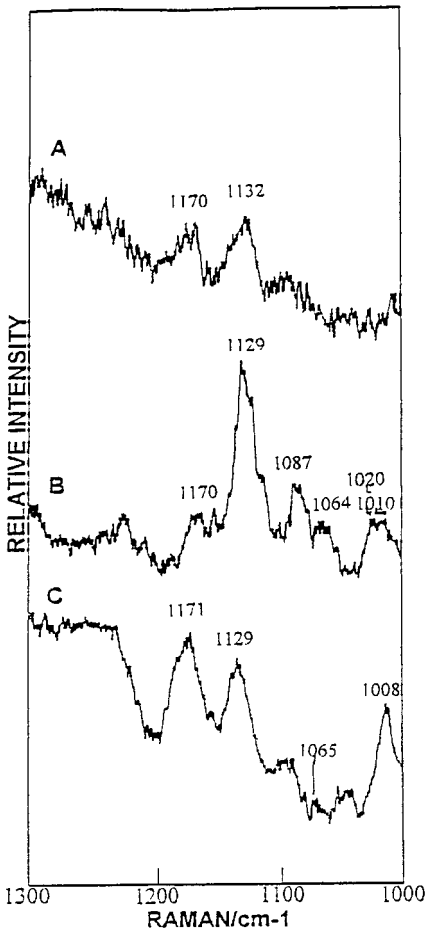

FIGURE 3 SERRS spectra (1000–1300 cm^{-1}) of P450 domain (*A*) substrate free (3.12×10^{-7} M), (*B*) with sodium laurate (2.99×10^{-7} M protein: 9.71×10^{-4} M laurate), and (*C*) with palmitic acid (1.19×10^{-7} M P450: 4.04×10^{-4} M substrate). All spectra were recorded 30 min after aggregation. 457.9-nm excitation (50 mW).

imply any extensive denaturation. The change is probably attributable to the influence of packing and the electrical double layer.

Confirmation of the presence of undenatured P450 from UV/visible absorption spectroscopy of the protein adsorbed to colloid cannot be obtained because the high extinction coefficient of the small metal particles (Mulvaney, 1996) dominates the spectrum, even for multiple layers of adsorbed protein. However, the SERRS evidence is strong. The broad band at 349 cm^{-1}, assigned to the thioether Fe-S stretch (Champion et al., 1982) and ν_8, indicates that ligation of the distal face by cysteine (Cys^{400}) is maintained upon surface adsorption of the protein. The band is weak because with 457.9-nm or 514.5-nm excitation, it is preresonant with a charge transfer transition rather than with the porphyrin ring transitions. The presence of the Fe-Cys^{400} stretch effectively rules out extraction of the heme from the protein matrix. The possibility of formation of the inactive mode of P450 (P420) is ruled out by the alteration of the frequency of ν_3 upon substrate or inhibitor binding. Furthermore, the 6cls-to-5chs conversion induced by fatty acid binding noted for the P450 domain of P450 BM3 is consistent with native and active P450. Champion and coworkers (see Hildebrandt, 1991) found that the spin-state-sensitive marker bands ν_2 and ν_3 of P420 CAM, as opposed to active P450 CAM, were unaltered upon substrate binding. A 6cls form of the P450 domain has recently been reported for the binding of metyrapone (an inhibitor) in the absence or presence of palmitate (Macdonald et al., 1996). Further SERRS studies have shown the sensitivity of the broad proximal Fe-Cys^{400} ν_8 stretch to the electron donating properties of different inhibitors and a large downshift in the frequency of ν_4 for reduced protein (unpublished results). This is indicative of reduced P450, not reduced P420 adsorbed on the colloidal substrate (Hildebrandt et al., 1988). All of the above evidence supports the presence of native P450 domain adsorbed on the colloidal surface, ruling out major distortion of the substrate binding pocket containing the active site.

Evidence for two structurally different vinyl groups for substrate-free P450 is indicated by the splitting patterns for

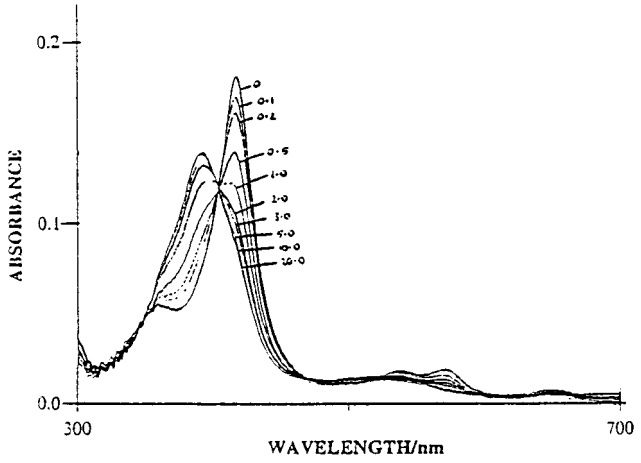

FIGURE 4 UV/visible absorption spectra after the addition of sodium palmitate (0–20 μM) to the heme domain of cytochrome P450 BM3 (1 μM).

vinyl modes (Fig. 1). The vinyl in-plane symmetrical CH deformation or scissoring motion (Choi et al., 1982a,b) is a doublet at 1433 cm^{-1} and 1410 cm^{-1}, and the poorly resolved vinyl stretching 1620–1630 cm^{-1} region may also suggest the presence of two bands, arising from one vinyl substituent coplanar with the heme ring (1621 cm^{-1}) and one tilted with respect to the ring (1628 cm^{-1}). The resolution is poor, but this spectrum has been obtained on a number of occasions, and the features are reproducible.

Substrate-bound P450 domain

Enhancement of the ν_{29}/ν_{20} band

An increase in the relative SERRS intensity of the 1400 cm^{-1} band assigned to ν_{29}/ν_{20} modes was observed for both laurate-bound and palmitate-bound P450 (Fig. 2 *B*). This is attributed to substrate-induced effects on the porphyrin and not to the symmetrical carboxylate stretch of the fatty acid substrates at 1401 cm^{-1}. There are two reasons for our assignment. A previous publication has analyzed SERS data from palmitic acid (Suh, 1992). Only after employing harsh chemical conditions was the author able to achieve partial adsorption of palmitate to bare and unprotected (Creighton) silver colloid (Suh, 1992). For the colloid used in our study, it has been shown that a negatively charged polymeric matrix of citrate moieties coats the silver surface (Munro et al., 1995). This adsorbed polymeric layer is stable and is not likely to be replaced by a negatively charged long-chain fatty acid anion, nor is such an anion likely to bind. No SERS of palmitic acid was observed when palmitic acid was added in the absence of protein, under otherwise identical experimental conditions. In addition, palmitic acid adsorbed to bare silver has additional bands, including strong bands at 928 cm^{-1} and 288 cm^{-1}, attributable to a C-COO^- stretch and an "accordion mode" overtone of the hydrophobic chain, respectively, which are not observed. The selection rules for SERS (Creighton, 1988; Weitz et al., 1986) indicate that, as well as the 1401 cm^{-1} mode, at least some of these modes must have SERS intensity, irrespective of the orientation of palmitic acid. None was observed in this case. Moreover, the enhancement due to SERRS is much greater than that due to SERS, with the result that none of the groups on the protein surface that adsorb to the silver give any appreciable SERS activity. The sensitivity of this mode to changes in the environment has been observed previously for cytochrome *c* in the absence of substrate (Macdonald et al., 1996). Thus it is very likely that the assignment made is correct, and the band at 1400 cm^{-1} is attributable to the heme vibrations and is not due to SERS from silver surface bound substrate. Both ν_{29} and ν_{20} involve displacements of peripheral methylene substituents as well as Fe-pyrrolic nitrogens, and are therefore particularly sensitive to alterations of the periphery of the heme ring close to the vinyl environment (Choi et al., 1982b). Highly homologous P450s (97% sequence homology) exhibit markedly different intensities for the 1400 cm^{-1} band (Wolf et al., 1988). This effect was attributed to slightly different protein alignments inducing a subtle change in the electronic structure of the heme. Fatty acid substrates for the P450 domain of P450 BM3 are positioned against the rigid β-sheet and stabilized by favorable van der Waals forces from hydrophobic residues (Li and Poulos, 1997). An allosteric mechanism involving protein structural realignments relative to the heme periphery upon substrate binding seems the most likely reason for the enhancement of the 1400 cm^{-1} band. This is is supported by the recently published crystal structure of substrate (palmitoleate)-bound P450 domain (Li and Poulos, 1997). This indicates movement of the amino acid residues in the I helix near the heme to accommodate a proposed oxygen-binding site. Thus, because the 5chs configuration of the heme ring predominates in the substrate-free protein adsorbed to the colloidal surface, the enhancement of the 1400 cm^{-1} mode is an excellent marker band for protein structure changes that influence the heme in the binding pocket.

After addition of the substrate to a suspension of colloid coated with adsorbed protein, the ν_{29}/ν_4 ratio obtained from SERRS with 514.5-nm excitation (see Table 2) changed slowly over time. Thus substrate binding to P450 preabsorbed on the surface occurs releatively slowly. This is consistent with reduced substrate accessibility to the substrate binding channel, because of packing and binding to the silver. However, the same final time-stable intensity ratio is obtained either from substrate addition to adsorbed protein, or from substrate addition to protein solution followed by surface adsorption. The effect is similar in magnitude for both palmitate- and laurate-bound protein.

The broadening in the ν_2 region on laurate addition to the P450 domain is consistent with water expulsion from the active site upon substrate binding. The width of the band at 1570 cm^{-1} assigned as ν_2 is increased by ~45% after the addition of the substrate. An explanation for broadening

based on this type of behavior has been detailed in a previous publication (Hildebrandt et al., 1989).

Palmitate-bound P450 domain

For palmitate-bound P450, notable differences from laurate-bound protein are seen in the sharpening of bands and in the changes in relative intensity. The sharp and intense ν_3 band indicates the presence of only the high-spin form, and because this band has significant displacements on the outer heme ring, the sharpness suggests a relatively strain-free environment. The intensity ratio of the bands at 1400 cm^{-1} and 1372 cm^{-1} lies between those of the substrate-free and laurate-bound forms, indicating a different, less strained configuration compared to laurate. Other relative intensity differences between palmitate and either substrate-free or laurate-bound P450, including E_u modes (Abe et al., 1978), occur throughout the 1000–1700 cm^{-1} region. For example, there are clear changes in ν_3 relative intensity and in the bands in the 1600 cm^{-1} region. These reflect subtle structural alteration to the heme pocket, causing a change in the heme scattering through the allosteric effect and/or slight orientational realignment of the protein on the surface.

Analysis of 1000–1300 cm^{-1} region

Most of the bands in the 1000–1300 cm^{-1} region originate from porphyrin modes and include large contributions from substituent groups (Choi et al., 1982a,b; Abe et al., 1978; Hu et al., 1993). Indeed, previous studies have suggested that this region may provide a "fingerprint" for specific heme-protein interactions (Hildebrandt, 1991; Wolf et al., 1988; Rospendowski et al., 1991). The vibrational frequencies and relative intensities of vinyl in-plane and out-of-plane deformations (Choi et al., 1982a,b; Abe et al., 1978; Hu et al., 1993) are particularly sensitive to subtle structural distortion of the heme environment, mirroring substrate-induced protein interactions within the hydrophobic channel. In the presence of substrate, marked changes occur in this region of the SERRS spectrum.

For laurate-bound P450 (Fig. 3 *B*), the "*cis*" CH wag is broad and has two poorly resolved maxima (1010 cm^{-1}, 1020 cm^{-1}), suggesting two structurally different protein environments for vinyl substituents. The substrate forms van der Waals contacts with hydrophobic residues in the channel (e.g., Leu^{437}, Met^{185}), inducing an allosteric mechanism to affect the immediate protein environment around each vinyl group. The palmitate-bound P450 (Fig. 3 *C*) 1000–1300 cm^{-1} region shows a sharper vinyl band at 1008 cm^{-1}. This indicates that the two vinyl groups experience a similar environment. These and previous observations indicate greater vinyl asymmetry of the laurate-bound P450 domain than in the case for the palmitate-bound domain.

The differing effects of the vinyl environments in the presence of laurate or palmitate may be related to their differing binding affinities within the pocket. Palmitate has a much lower K_d than laurate (~1–2 μM compared to ~100 μM). Palmitate and laurate are tethered via their carboxylate groups to the amino acid residue Arg^{47} within the binding channel (Ravichandran et al., 1993). If it is assumed that the hydrophobic tail of palmitate has a binding position similar to that identified for laurate by NMR studies (Modi et al., 1995), the remaining four terminal methylene groups for palmitate would adopt a position closer to the heme ring. NMR data have positioned the terminal carbon on the laurate chain 0.76 nm above the ring (Modi et al., 1995). The effect of the additional methylene groups would be to alter the immediate protein environment around the vinyl groups on pyrrole rings B and C. This results in the vinyl group on ring B having a microscopic environment similar to that of the vinyl mode on ring C, yielding sharp SERRS bands. Structural realignment around the vinyl regions is supported by the recently published crystal structure of the substrate-bound P450 domain (Li and Poulos, 1997). Amino acid realignment in helix I in relation to the substrate-free case alters the environment around the heme and creates a postulated oxygen binding site. Fig. 5 gives a diagrammatical representation of the protein environment around the vinyl-containing pyrrole rings B and C. Some amino acids proximal to each vinyl group have been omitted from the illustration to aid clarity. The vinyl group on ring C is

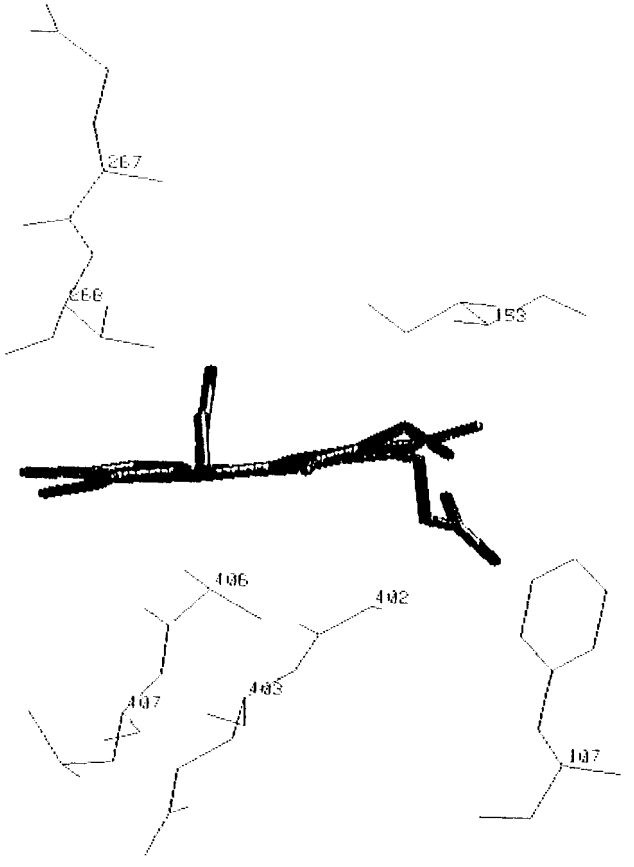

FIGURE 5 Illustration of amino acid environments around each vinyl substituent on the heme macrocycle of P450 domain BM3.

influenced by amino acids such as Phe^{107} and Ile^{153}. The vinyl substituent on ring B has a local environment influenced by proximal amino acids such as Thr^{268}.

CONCLUSIONS

The addition of laurate or palmitate to the heme domain of cytochrome P450 BM3 leads to an allosteric activation of the heme band at 1400 cm^{-1}, which is mostly attributable to ν_{29}, and which is sensitive to peripheral contacts of heme/protein within the substrate binding pocket. Surface-adsorbed substrate-free P450 BM3 is predominantly a five-coordinate heme species with some six-coordinate protein present. Further heme conversion to a pentacoordinate species upon substrate addition coupled with band narrowing confirms the presence of substrate within the binding pocket and the removal of water from the active site. Specific differences between the nature of laurate and palmitate binding have been observed in the splitting patterns of the ring substituent vinyl modes. Laurate binding gives different protein environments for each vinyl group, leading to the observation of vinyl mode doublets. However, palmitate binding to P450 creates similar protein environments for the vinyl groups, which is reflected in a single signal observed for the vinyl vibration at 1008 cm^{-1}. These differences result from the binding characteristics of each fatty acid hydrophobic tail within the substrate binding pocket. These differences have not been observed with circular dichroism or UV/visible spectroscopies (Macdonald et al., 1996), nor have they been reported by researchers using other techniques. This indicates the validity of using SERRS to probe substrate/heme interactions within an active site at low protein concentrations. Vinyl splitting patterns may provide a useful probe for the "neatness of fit" of substrates within the active site of P450 BM3.

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