Covalent Attachment of an Electroactive Sulfydryl Reagent in the Active Site of Cytochrome $P450_{cam}$ as Revealed by the Crystal Structure of the Modified Protein

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Abstract: A novel electroactive sulfydryl-specific reagent, *N*-(2-ferrocenylethyl)maleimide (Fc-Mi), was used to attach a redox-active reporter group to cytochrome $P450_{cam}$ from *Pseudomonas putida*. The crystal structure of the modified enzyme was determined at 2.2 Å resolution ($R_{cryst} = 0.18$) and compared to the structure of the wild-type enzyme complexed with its natural substrate. The results showed that two molecules of the electroactive modifier were attached to the protein. One of the ferrocenes was linked to Cys85 via the maleimide moiety and occupied the camphor-binding site in the substrate pocket. The other ferrocene was linked to Cys136 on the surface of the protein. Significant conformational changes were observed on the distal side of the heme when camphor was replaced by ferrocene. The shift in the Sorét band from 392 to 417 nm upon modification arose from the binding of a water molecule to the heme iron immediately below the ferrocene in the active site of the modified enzyme. The electrochemistry of the labeled enzyme showed clear signals originating both from the heme and from the covalently linked ferrocenes. The direct current cyclic voltammogram revealed a striking positive shift in the heme redox potential of the ferrocene-containing $P450_{cam}$ from −380 mV for the camphor-bound wild-type protein to −280 mV for the modified protein.

Introduction

Cytochrome $P450_{cam}$ from *Pseudomonas putida* is a soluble, bacterial heme-dependent monooxygenase that, along with its redox partners putidaredoxin and putidaredoxin reductase, catalyzes the stereospecific hydroxylation of camphor to 5-*exo*-hydroxycamphor.[1,2] The biochemical, electrochemical, and spectroscopic properties of this enzyme have been thoroughly investigated,[3] and correlated with the crystal structures of the substrate-free enzyme,[4,5] its complexes with the natural substrate camphor,[6] and other compounds including carbon monoxide[7] and various substrate analogues,[8,9] and inhibitors.[10,11] Information on substrate binding, electron transfer, and other steps in the $P450_{cam}$ catalytic cycle is necessary to underpin similar research on other prokaryotic as well as eukaryotic P450 enzymes.

We have investigated the introduction of electroactive groups to $P450_{cam}$ by covalent attachment of a sulfydryl-specific redox-active reagent to cysteine residues on the surface of the protein. The use of an electroactive label is particularly interesting since it adds novel redox properties to the enzyme. We have reported the covalent modification of $P450_{cam}$ with a ferrocene-containing reagent *N*-(2-ferrocenylethyl)maleimide (Fc-Mi) and described the direct electrochemistry of the modified protein.[12] While short reaction times gave a modified protein which retained 80% of the camphor hydroxylating activity of the wild-type $P450_{cam}$, we also found that prolonged incubation of the modifying reagent with the protein resulted in complete inhibition of all catalytic activity. Sulfydryl reagents such as organomercurials[13] and *N*-ethylmaleimide,[14] have also been reported to inhibit the enzymatic activity of $P450_{cam}$, but the mechanism of the inhibition remains obscure.

Of the many techniques which have been applied to examine the structure−function relationships of $P450_{cam}$,[2,3] inhibitor studies gave early insights into the process by which the

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Table 1. Data Collection and Refinement Statistics

		Data	
cell parameters		observations	78650
a (Å)	61.1	unique reflns	23420
c (Å)	215.4	R_{merge}[a]	0.076
space group	$P3_221$	completeness (%)	95.0
resolution (Å)	30−2.2		
		Refinement	
non-hydrogen atoms	3605 (including 315 waters)	R_{cryst} (all data)[b]	0.193
R_{cryst} (2σ)[b]	0.183	mean temp factor (Å^2)	18.2
reflns used	21942	rmsds from ideal value	
R_{free} (2σ)[c]	0.221	bonds (Å)	0.007
reflns used	912	angles (deg)	1.4
R_{cryst} (no σ cut)[b]	0.192	mean coordinate error (Å)[d]	0.13
reflns used	22484		
R_{free} (no σ cut)[c]	0.229		
reflns used	936		

[a] $R_{merge} = \sum_j\sum_h|\langle I_{h,j} - I_h\rangle|/\sum_j\sum_h I_{h,j}$ where $I_{h,j}$ is the *j*th observation of reflection *h*. [b] $R_{cryst} = \sum||F_{obs} - F_{calc}||/\sum|F_{obs}|$, where F_{obs} and F_{calc} are the observed and calculated structure factor amplitudes, respectively. [c] R_{free} is equivalent to R_{cryst} for a 4% subset of reflections not used in the refinement. [d] Determined by the SIGMAA method.[30]

substrate gains access to the enzyme's active site. These studies have also given indications of regions of the protein which may undergo conformational changes in this process.[15] Several crystal structures of P450$_{cam}$ with reversibly and irreversibly bound inhibitors are now available.[10,11] Each of these inhibitors is aromatic and contains aromatic nitrogen atoms (e.g., nitrogens from pyridine or imidazole), and they are either attached to the heme iron or retained in the active site by hydrophobic interactions with the side chains of apolar amino acid residues and by hydrogen bonds.

Here we report the kinetic, spectroscopic, and electrochemical properties and the crystal structure of an irreversibly inhibited Fc-Mi modified P450$_{cam}$. The modification reaction was carried out on a P450$_{cam}$ mutant in which an exposed cysteine (Cys334) on the surface of the protein was replaced by alanine. This C334A mutation not only prevents dimerization, and therefore improves the handling characteristics of the enzyme,[16] but also facilitates crystallization of P450$_{cam}$.[17] The C334A mutant will be referred to as native P450$_{cam}$ to distinguish it from the wild-type enzyme which has cysteine at the 334 position.

Experimental Section

General. Chemicals and solvents were reagent grade and used without further purification. Water for all solutions was purified using a Milli-Q water purification system (Millipore, U.K.). Cytochrome P450$_{cam}$,[18] putidaredoxin, and putidaredoxin reductase[19,20] were expressed in *Escherichia coli* and purified according to published methods.

Modification of P450$_{cam}$ with Fc-Mi. To 10 mL of cytochrome P450$_{cam}$ (20 μM in 40 mM potassium phosphate, pH 7.4, 1 mM camphor) was added 120 μL of a 20 mM stock of Fc-Mi in ethanol (molar ratio of enzyme:modifier of 1:12), and the reaction mixture was left at 4 °C for 2 h. Unreacted Fc-Mi was removed by ultrafiltration (Amicon), and the enzyme was purified in two steps by FPLC. The first purification step was carried out by anion exchange chromatography on a 6 mL Resource Q column (Pharmacia). A linear salt gradient of 0−300 mM KCl was developed in 40 mM phosphate buffer, pH 7.4, 1 mM camphor, with the modified protein eluting between 100 and 130 mM KCl. The combined fractions were further purified on a Phenyl-Superose HR 5/5 column by developing a gradient from 600 to 0 mM ammonium sulfate in 40 mM phosphate buffer, pH 7.4, 1 mM camphor. Protein fractions eluting between 360 and 260 mM ammonium sulfate were collected and used directly in the electrochemical studies. Prior to crystallization, the buffer was exchanged to 40 mM phosphate, pH 7.4, 100 mM KCl, 5 mM dithiothreitol, and the protein concentrated to 5 mM by ultrafiltration.

Cytochrome P450$_{cam}$ Activity Assay. The NADH turnover activity of the native and modified P450$_{cam}$ was measured in the fully reconstituted system.[1] Incubation mixtures (1.5 mL) contained 0.05 μM P450$_{cam}$, 16 μM putidaredoxin, 0.5 μM putidaredoxin reductase, 200 mM KCl, and 500 μM camphor. After the mixture was equilibrated at 25 °C for 5 min, NADH was added to 250 μM and its absorbance at 340 nm was monitored spectrophotometrically. The NADH turnover rates were calculated using $\epsilon_{340nm} = 6.22$ mM^{-1} cm^{-1}.

Electrochemistry. Direct-current (dc) cyclic voltammetry experiments were carried out under argon at room temperature in a two-compartment glass cell with a working volume of 0.5 mL. The working compartment accommodated the platinum gauze counter electrode in addition to the edge plane pyrolytic graphite working electrode. A saturated calomel electrode (SCE) was used as the reference in a sidearm which was connected to the working compartment via a Luggin capillary. All potentials were referenced to the SCE. An AUTOLAB R potentiostat by Eco Chemie B.V. was used to record and control the potential of the working electrode. All measurements were made in 40 mM potassium phosphate, pH 7.4, buffer containing 1 mM camphor.

Crystallization and Data Collection. Crystals were obtained by the hanging-drop vapor diffusion method. The mother liquor in the Linbro tray was 1.6−1.9 M ammonium sulfate, 100 mM KCl, and 100 mM HEPES, pH 7.5. The protein solution (5 mM P450$_{cam}$ in 40 mM phosphate pH 7.4, 100 mM KCl, 5 mM dithiothreitol) was mixed with this solution in a 1:1 ratio and left to equilibrate with the reservoir solution at 18 °C. Red crystals appeared within 1 day as hexagonal plates. The largest crystals (0.2 × 0.3 × 0.3 mm) grew for a period of approximately 3 days.

X-ray data were collected at 100 K. Prior to data collection, crystals were soaked in a cryoprotectant solution (1.9 M ammonium sulfate, 15% glycerol, 100 mM KCl, and 100 mM HEPES, pH 7.5) for 5 min before mounting, using the method of Teng.[21] A data set to 2.2 Å resolution was collected on a 30 cm MAR Research imaging plate detector using CuKα radiation ($\lambda = 1.5418$ Å) from a Rigaku rotating anode X-ray generator operating at 60 kV, 70 mA. Data were processed using DENZO and its companion program SCALEPACK.[22] Structure factors were derived from intensities using the program TRUNCATE

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from the CCP4 package.[23] Statistics of the data set used in the refinement are given in Table 1.

The structure was solved by molecular replacement using the program AMoRe.[24] The search model was the camphor-bound wild-type $P450_{cam}$ [Protein Data Bank code 2CPP], but the camphor was removed from the model prior to all calculations. The rotation function search in the resolution range 20−3 Å produced a clear solution with peak height of 12.8 σ. The translation function indicated that the correct space group was $P3_221$ with a correlation coefficient of 0.56 and a crystallographic R value of 0.413, compared to its enantiomorph pair ($P3_121$), which had a correlation coefficient of only 0.243 and an R value of 0.535. The electron density map, based on phases obtained from the molecular replacement solution, was of high quality and proved easy to interpret. The starting structure for the bound Fc-Mi was generated using ideal bond lengths, bond angles, and torsion angles with the program SYBYL. Model building and refinement were pursued with alternate cycles of manual refitting using O[25] and simulated annealing using X-PLOR.[26] A bulk solvent correction allowed all measured data from 30 to 2.2 Å to be used. When the free R factor did not decrease any further, water molecules were added to the atomic model at the positions of large positive peaks ($>3\sigma$) in the difference electron density at places where the resulting water molecule fell into an appropriate hydrogen bonding environment only. At the final stages, restrained isotropic temperature factor refinements were carried out for each individual atom. Introduction of water molecules in each of the refinement steps resulted in a decrease in both conventional and free R values. After the refinement, the structure was analyzed for geometric quality using the PROCHECK package.[27] Most of the residues (89.9%) were in the most favorable regions in the Ramachandran plot with no outliers. The overall measure of structure quality (G factor, as defined in PROCHECK) was 0.3, which is 2.8 standard deviations better than the mean for structures of the same nominal resolution.

The atomic coordinates and structure factors have been deposited in the Protein Data Bank (Chemistry Department, Brookhaven National Laboratory, Upton, NY 11973).[28]

Results

Spectroscopy. The addition of a 12-fold molar excess of the sulfydryl-specific Fc-Mi-modifying reagent to low-spin substrate-free cytochrome $P450_{cam}$ did not result in any changes in the electronic spectrum; only the 417 nm Sorét band was observed. On the other hand, addition of a similar excess of the reagent to camphor-bound $P450_{cam}$ in a buffered solution containing 1 mM camphor resulted immediately in a shoulder at 417 nm on the characteristic high-spin heme Sorét band at 392 nm. The time-dependent spectrum of the enzyme taken during modification is shown in Figure 1. An isosbestic point was observed at 408 nm. The intensity of the 417 nm Sorét band increased with time at the expense of the 392 nm band, and the conversion was complete after *ca.* 2 h. The 417 nm Sorét band is characteristic of substrate-free $P450_{cam}$ and suggests the coordination of a water molecule or an exogenous ligand to the heme iron. Reduction of the modified protein in the presence of carbon monoxide gave the 448 nm Sorét band characteristic of the ferrous carbonmonoxy form of P450 enzymes. The activity of the modified enzyme was compared to that of the untreated $P450_{cam}$ by measuring the NADH consumption rate in the fully reconstituted system with camphor as substrate.[1] No activity was found for the Fc-Mi-modified $P450_{cam}$.

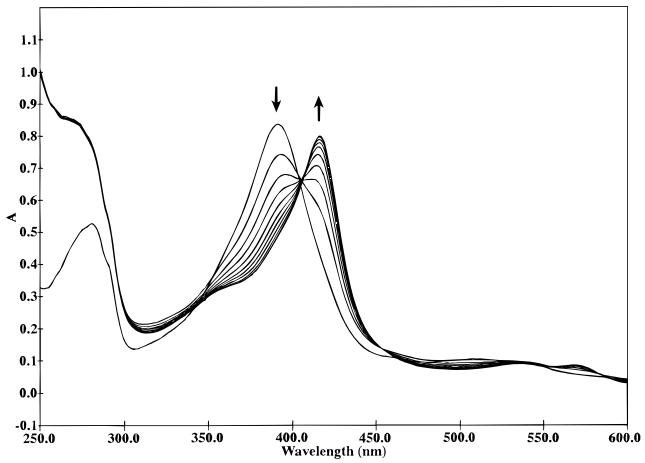

Figure 1. Time-dependent UV−visible absorption spectrum of the camphor-bound $P450_{cam}$ (0.85 μM in 40 mM potassium phosphate buffer, pH 7.4, containing 1 mM camphor) obtained on the modification of the enzyme with Fc-Mi in a 2-h period.

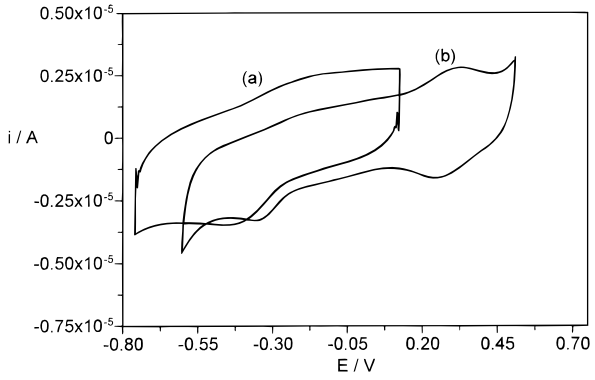

Figure 2. Direct-current cyclic voltammograms of (a) camphor-bound $P450_{cam}$ and (b) Fc-Mi-modified $P450_{cam}$ taken on an edge−plane graphite electrode in deaerated 40 mM phosphate buffer, pH 7.4, containing 1 mM camphor. The concentration of the enzymes were 12 μM. Potential scan rate: 50 mV s^{-1}.

Electrochemistry. The dc cyclic voltammogram of the Fc-Mi-modified cytochrome $P450_{cam}$ on a bare edge plane graphite electrode is shown in Figure 2b. Three reversible redox couples were observed. The first, at −280 mV (all potentials vs SCE), corresponded to the heme redox potential of the modified $P450_{cam}$. The second and third couples, which were not very distinct at this resolution, were observed at +220 and +260 mV, which were characteristic of ferrocene moieties. The redox potentials of substrate-free and camphor-bound $P450_{cam}$ determined by dc cyclic voltammetry are −530 and −390 mV, respectively.[29] Thus the heme reduction potential of the Fc-Mi-modified $P450_{cam}$ is 110 mV higher than that of unmodified camphor-bound $P450_{cam}$ (Figure 2a).

Crystallography. To ascertain the mode of inhibition of $P450_{cam}$ by the Fc-Mi modifier, we have determined the crystal structure of the modified $P450_{cam}$ protein at 2.2 Å resolution. The refined atomic model of the inhibited $P450_{cam}$ in the vicinity of the heme active site, together with the final $2F_o - F_c$ electron

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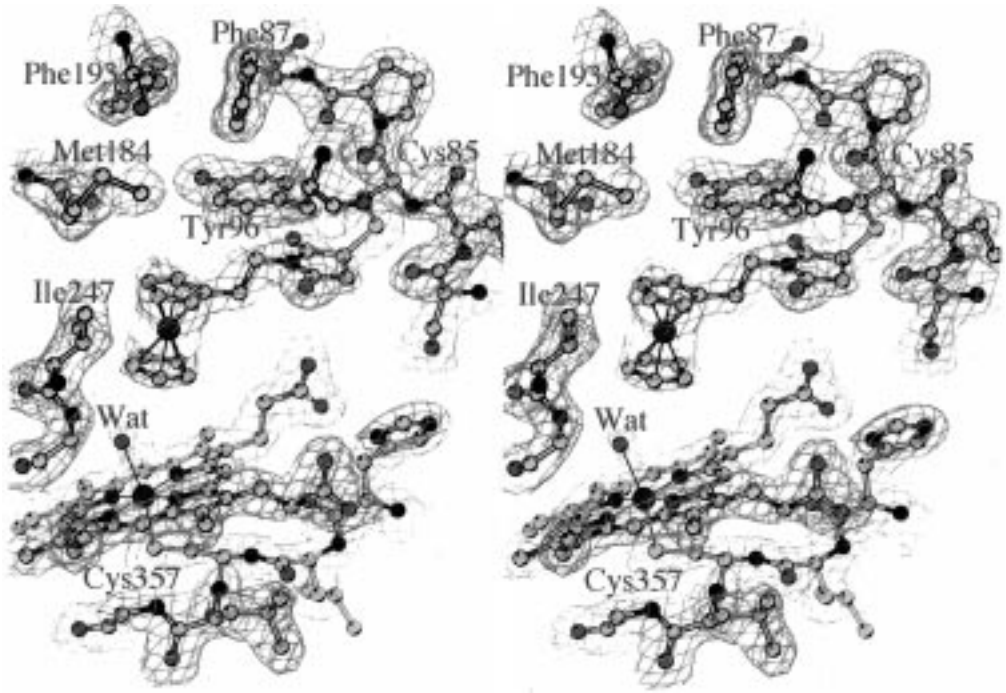

Figure 3. Stereoview of the final electron density map around the active site of cytochrome P450$_{cam}$. The Fc-Mi inhibitor is covalently linked to Cys85, and an axial water molecule is coordinated to the heme iron. SIGMAA[30] weighted $2mF_o - \Delta F_c$ electron density using phases from the final model is contoured at 1σ level, where σ represents the rms electron density for the unit cell. Contours more than 1.4 Å from any of the displayed atoms have been removed for clarity. Figure was drawn with MOLSCRIPT modified by Robert Esnouf (Oxford).[31]

density map, is shown in Figure 3. The electron density was very well defined in this region and clearly showed the presence of the Fc-Mi modifier in the substrate pocket. An isolated lobe of positive density linked to the iron atom established the presence of a coordinated water molecule (Fe−O distance 2.48 Å). The map also revealed that the sulfur atom of Cys85 was covalently linked to the maleimide moiety of the modifier, while the ferrocene moiety was oriented toward the heme and occupied the camphor binding site.

The superposition of the active site of camphor-bound wild-type P450$_{cam}$ and the Fc-Mi-inhibited enzyme is shown in Figure 4. The structure of the camphor-bound enzyme is represented in blue and the modified enzyme in red. Replacement of camphor by the ferrocene induced rearrangement of the main chain and side chains of the enzyme around the active site. The highly apolar ferrocene moiety was bound in the hydrophobic pocket of the active site at a position overlapping with, but slightly shifted from, the position previously occupied by camphor. The plane of the unsubstituted cyclopentadienyl ring of the ferrocene moiety was canted away from being parallel with the heme by 29°. The shortest distance from this ring to the heme iron was 5.3 Å, which was 1.1 Å further away than the closest approach of camphor in the wild-type structure, and the closest approach to the porphyrin was 4.3 Å from the *meso*-carbon between the two heme propionate groups. The two oxygens of the maleimide moiety were held by hydrogen bonds to protein side chains. A strong hydrogen bond was observed between one of these oxygens and the O$^{\gamma}$ of Ser102 (O−O distance 2.76 Å), while the other oxygen formed a weaker H-bond with one of the carboxylate oxygens of Asp297 (O−O distance 3.11 Å). In the structure of the camphor-bound wild-type enzyme (Figure 4, blue) there is no water present in the active site. The heme iron is 5-coordinate and displaced from the porphyrin core toward the proximal Cys357 by about 0.40 Å. When ferrocene replaced camphor in the active site (Figure 4, red), a water molecule entered the distal heme pocket and coordinated to the heme iron. In this structure, the iron is in the plane of the porphyrin and the concomitant movement of the side chain of Cys357 is clearly visible.

Dramatic changes were observed in the position of the main chain as well as the side chains between residues Cys85 and Tyr96. The short helical structure between residues 89 and 96 was completely disrupted (Figure 4), and the largest shift in the position of α-carbons was about 10 Å at residue Gly93. These movements destroyed the putative cation binding site proposed by Poulos.[6] The six ligands which define the cation binding site are the four backbone carbonyl oxygens of Glu84, Gly93, Glu94, and Tyr96, and two water molecules, one of which was hydrogen-bonded to the side chain of Glu84. All of these residues were affected by the modification at Cys85. In the camphor-bound structure, Cys85 (located between the α-helical and the β-sheet regions of the protein) occupied a position close to the surface of the protein. After modification with Fc-Mi, the side chain of Cys85 rotated by 180° and was pulled toward the active site of the enzyme. Closer inspection of the two structures shown in Figure 4 revealed that the side chain of Tyr96 had flipped by 90° to accommodate the substituted cyclopentadienyl ring of the modifying agent. This movement would have brought this side chain into contact with Phe87 in the camphor-bound structure, and accordingly, the side chain of Phe87 was twisted by 90° from its original position, moving toward the surface of the protein. Even though the side chain of Phe193 would not have come into contact with the Fc-Mi molecule had it remained in its position in the camphor-bound structure, it nevertheless moved toward the surface of the protein to a position where it was parallel to, and in van der Waals contact with, the side chain of Phe87 at its new location.

The overall structure of the Fc-Mi-modified P450$_{cam}$ revealed that a second Fc-Mi molecule was attached to Cys136 in the enzyme (Figure 5). At this position the electroactive sulfydryl reagent is exposed on the surface. Since Cys334 had been substituted by alanine and Cys357 is the heme proximal ligand,

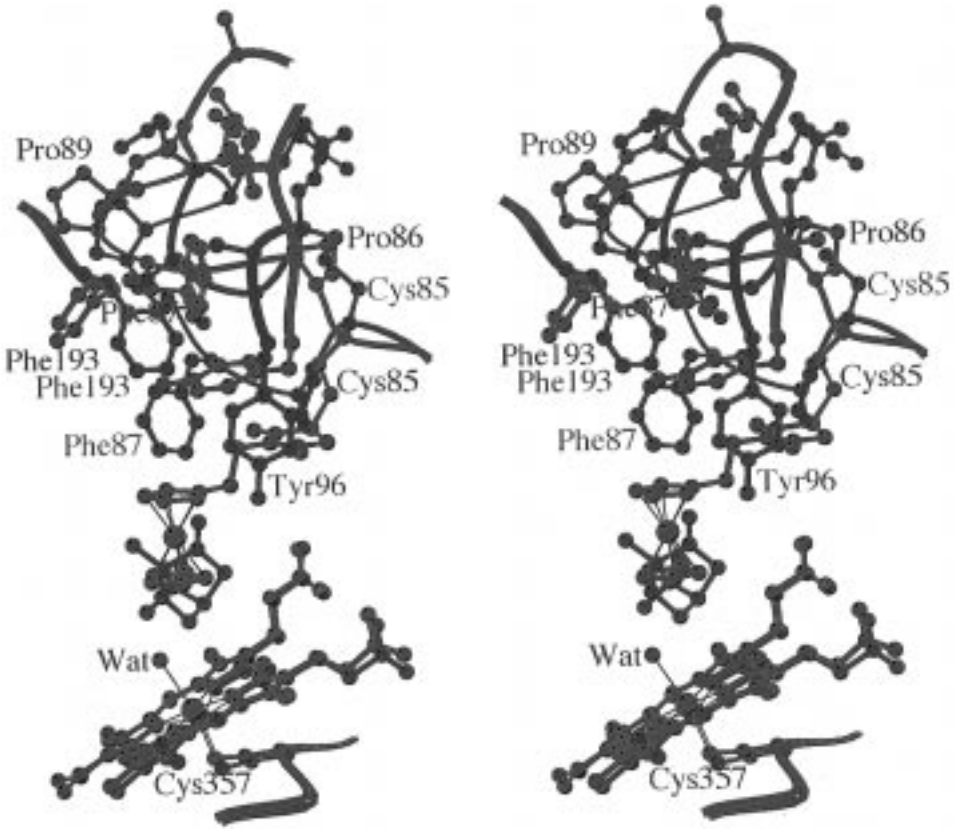

Figure 4. Stereo-superposition of the active site of camphor-bound $P450_{cam}$ (blue) and with the Fc-Mi covalently coupled to the Cys85 (red). There is a major structural reorganization between residues 85 and 96 both on the main chain and the side-chains of the protein. The ferrocene moiety of the inhibitor overlaps with the camphor in the binding pocket of the enzyme. The iron is pulled back into the porphyrin plane. Figure was drawn with MOLSCRIPT.[31]

there are four more cysteine residues in this $P450_{cam}$ mutant, at positions 58, 148, 242, and 285, which could potentially be alkylated. However, none was modified under the reaction conditions.

Discussion

We have shown that prolonged incubation of $P450_{cam}$ with the sulfydryl-specific modifying reagent *N*-(2-ferrocenylethyl)-maleimide (Fc-Mi) abolished the camphor oxidation activity of the enzyme. However, the ferrous carbonmonoxy form showed the 448 nm Sorét band characteristic of P450 enzymes, indicating that the immediate environment of the heme had not been drastically altered even though the modified protein was catalytically inactive. The red shift in the Sorét absorption band of the enzyme (Figure 1) revealed a change in the coordination state of the heme iron upon modification. The pentacoordinated, high-spin, substrate-bound form of $P450_{cam}$ (Sorét at 392 nm) became hexacoordinated (Sorét at 417 nm) in the Fc-Mi-modified enzyme and, as the crystal structure of the modified enzyme revealed, a water ligand became axially coordinated to the iron atom on the distal side of the heme.

The cyclic voltammogram of the modified $P450_{cam}$ (Figure 2b) showed the incorporation of ferrocenes into the protein as two reversible redox couples at 220 and 260 mV. The difference in the potential of the ferrocene couple is probably due to the different electrostatic environments around the attached electroactive labels, and we note that the singly modified protein had a potential of 260 mV.[12] The electrochemical scans of the modified and native $P450_{cam}$ revealed a striking shift in the heme reduction potential (Figure 2a,b). The

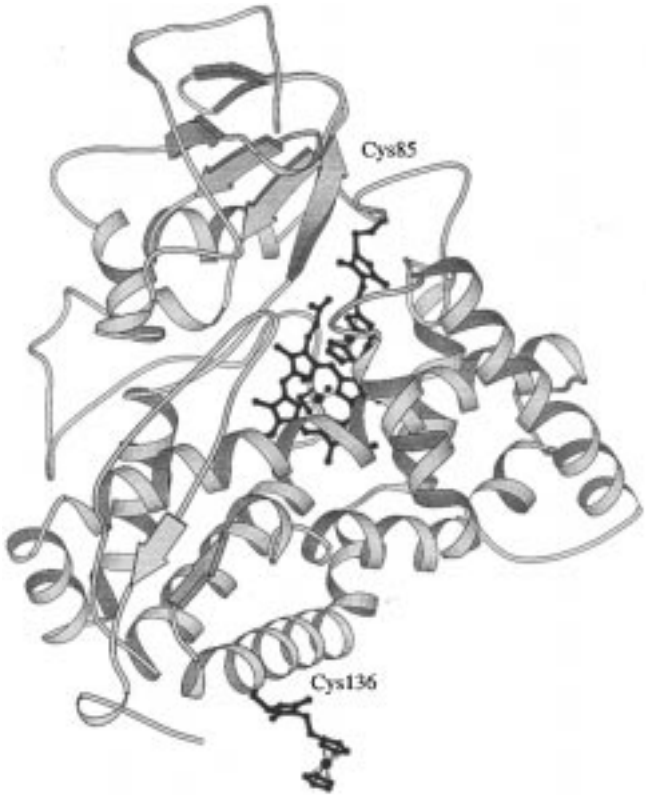

Figure 5. Overall structure of the FcMi-modified $P450_{cam}$ reveals two ferrocenes attached to the molecule. The Cys85 and the Cys136 were alkylated by the electroactive sulfydryl reagent. Figure was drawn with MOLSCRIPT.[31]

modification by Fc-Mi produced a 250 mV increase in the heme redox potential of the modified enzyme compared to substrate-free P450$_{cam}$ and, indeed, a 110 mV increase compared to the camphor-bound form. These data suggest that putidaredoxin, in the presence of NADH and putidaredoxin reductase, could reduce the 6-coordinate, low-spin ferric form of the heme in this ferrocene-containing P450$_{cam}$. The reduced form of the enzyme could then bind molecular oxygen, and it is conceivable that a certain degree of hydroxylation could occur on the irreversibly linked inhibitor found in the crystal structure. However, since this reaction is stoichiometric rather than catalytic, only the total inhibiton of the enzyme would be detected when the catalytic NADH turnover by the fully reconstituted system was assayed.

The crystal structure of the modified P450$_{cam}$ allowed a more detailed interpretation of the effect of protein modification. Significant conformational changes accompanied the conversion of camphor-bound to inhibitor-bound P450$_{cam}$. The side chain of Cys85 rotated by 180° to form the covalent link to the maleimide, and the short α-helical structure between residues 85 and 96, which also forms the putative cation binding site of the enzyme,[6] was consequently disrupted. Many of the active site residues underwent major repositioning. The unusually large shift in the positions of α-carbons, as much as 10 Å at Gly93, suggests that there is a large degree of flexibility in the structure of P450$_{cam}$ and that the enzyme can tolerate significant changes on the distal side of the heme. However, the crystallographic thermal parameters of the main chain for residues 85−96 are not higher than other parts of the camphor-bound wild-type P450$_{cam}$, and therefore, the structure in this region is not very mobile, which may have a bearing on the rate of the conversion to the low-spin Fc-Mi-modified form.

When the ferrocene modifier replaces the camphor in the active site, the closest part of the ferrocene is located at a distance of 5.3 Å from the heme iron, 1.1 Å further than camphor in the wild-type structure, so there is sufficient room for a water molecule to coordinate to the heme iron. This situation is analogous to that found by Raag and Poulos in the crystal structure of P450$_{cam}$ with bound norcamphor,[9] adamantane, camphane, and thiocamphor,[8] in which an axial water ligand is bound to the heme in all cases. There was, however, a major structural difference between the Fc-Mi-modified P450$_{cam}$ and the substrate-bound and substrate-free P450$_{cam}$ proteins. The iron is displaced from the porphyrin core toward the proximal Cys357 by *ca.* 0.3 Å in all of these forms of wild-type P450$_{cam}$, whereas in the modified enzyme, the iron is in the plane of the porphyrin ring, similar to the case when CO binds to reduced, wild-type P450$_{cam}$.[7]

In the camphor-bound structure of P450$_{cam}$, there is no obvious channel for the substrate to diffuse from the solvent medium into the protein active site. By detailed analysis of the thermal parameters of the protein side chains in the substrate-free and camphor-bound structures,[4,5] and from the structures of P450$_{cam}$ with bound inhibitors,[10,11] Poulos proposed that a depression on the surface of P450$_{cam}$ which terminates at Tyr96 may form the substrate access channel.[15] Furthermore, the various structures suggest that the side chains of Phe87, Tyr96, and Phe193 may play important roles in substrate access. In particular, it was proposed that the side chain of Phe193 moves from the interior of the protein onto the surface to act as an initial hydrophobic recognition site for camphor and then moves back into the protein interior with the entry of the substrate. The structure of the Fc-Mi-modified P450$_{cam}$ supports these proposals. Thus the large reorganizations observed in the short distal helix between residues 85 and 96 indicate that significant changes (though in all probability less severe) in the protein structure in this region could also occur during normal substrate entry to the P450$_{cam}$ active site without jeopardizing the stability of the overall protein structure. The movement of the Phe193 side chain to the surface is also consistent with the high mobility required for this residue in the proposed model.

The dramatically increased heme redox potential of the modified P450$_{cam}$ is difficult to rationalize. The potential was shifted to −280 mV, 110 mV more positive than the potential of the 5-coordinate high-spin heme of camphor-bound P450$_{cam}$, even though the heme was found to be 6-coordinate, with a water bound to the low-spin heme iron. A potentially important factor revealed by the crystal structure is the position of the iron relative to the plane of the porphyrin ring. The iron is in the plane of the porphyrin in the modified P450$_{cam}$, while in all the other ferric P450$_{cam}$ structures determined to date, it is displaced by 0.3−0.4 Å toward the sulfur atom of the proximal Cys357. Iron−porphyrin σ-bonding should be strengthened when the iron moves into the porphyrin plane, and this should favor the ferric form due to the stronger metal−ligand interaction expected for the higher oxidation state metal center. Although the position of the iron in the ferrous form of the modified protein is not known, we note that, if the iron is out of the porphyrin plane in the ferrous form, the metal−ligand interaction will be reduced, which is not consistent with the increased heme redox potential. We would therefore suggest that the iron is also in the plane of the porphyrin in the ferrous form of the modified P450$_{cam}$ and that as a result π-back donation from the iron 3d orbitals to the porphyrin π^* acceptor orbitals becomes an important factor.

The results of our electrochemical, spectroscopic, kinetic, and crystallographic studies on cytochrome P450$_{cam}$ suggest two possible mechanisms for the binding of the ferrocene moiety at the active site of the enzyme. One of these requires that Fc-Mi binds first, having high affinity for the active site, and thus replacing camphor, and then the maleimide moiety reacts with an available cysteine thiol nearby. The sulfydryl group of Cys85 is not near to this site and, in order to react with the maleimide moiety, a 180° rotation of this side chain must take place to facilitate covalent bond formation. The access route to the active site is possibly the same as that of the camphor. The other mechanism would involve covalent attachment of the Fc-Mi reagent to active cysteines on the surface of the enzyme first, which would then be followed by an "internalization" of the reagent attached to the thiol of Cys85. The driving force for internalization would be the need to reduce the hydrophobic surface area of the protein. Reorganization of the molecule during internalization would lead to the expulsion of camphor from the active site and the binding of the ferrocene moiety of the reagent at a position overlapping with the position of camphor in the pocket. A necessary requirement for such a reorganization is a degree of structural flexibility above the heme pocket of cytochrome P450$_{cam}$. In this respect, both mechanisms have identical requirements regarding the structural dynamics of the enzyme.

In conclusion, the use of a sulfydryl-specific reagent as a handle to introduce covalently specific labels into the active site of cytochrome P450$_{cam}$ unexpectedly opened up further possibilities for the study of the mechanism of this enzyme.

The position of the iron in relation to the plane of the porphyrin ring has been experimentally identified as a possible factor affecting the heme redox potential. Further experiments to clarify the mechanism of the modification may shed new light on the mechanism of substrate entry to the active site of cytochrome $P450_{cam}$.

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