Structure and Redox Properties of the Haem Centre in the C357M Mutant of Cytochrome P450cam

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The effects of site-specific mutation of the axial cysteine (C357M) to a methionine residue in cytochrome P450cam on the enzyme's coordination geometry and redox potential have been investigated. The absorption spectra of the haem centre in the C357M mutant of the enzyme showed close similarity to those of cytochrome c both in the oxidised and reduced forms. A well-defined absorption peak at 695 nm, similar to that seen in the case of cytochrome c and characteristic of methionine ligation to the ferric haem, was observed. The results indicated that the haem of C357M cytochrome P450cam is possibly axially coordinated to a methionine and a histidine, analogously to cytochrome c. The circular dichroism spectra in the visible and the far-UV regions suggested that the tertiary structure of the haem cavity in the C357M mutant cytochrome P450cam was distinctly different from that in the wild-type enzyme or in cytochrome c, although the secondary structure of the mutant remained identical to that of the wild-type cytochrome P450cam. Comparison of the natures of the CD spectra in the 400 nm and 695 nm regions of the C357M mutant of cytochrome P450cam with those of horse cytochrome c suggested (R) chirality at the sulfur atom of the iron-bound methionine residue in the mutant. The redox potential of the haem centre, estimated by redox titration of the C357M mutant, was found to be +260 mV, which is much higher than that in the wild-type enzyme and similar to the redox potential of cytochrome c. This supported the concept that axial ligation of the haem plays the major role in tuning the redox potential of the haem centre in haem proteins.

Introduction

The cytochrome P450 forms the family of haem-containing monooxygenases characterised by strong Soret absorption at $\approx$450 nm in the carbon monoxide complex of the ferrous haem centre in the enzyme.[1–3] These enzymes are involved in monooxygenation of a variety of hydrophobic substrates, including steroid precursors in the biosynthesis of steroid hormones.[1,4] They also play key roles in drug metabolism and detoxification against xenobiotics.[5–7] Although the reactive centre is the haem prosthetic group, the substrate does not directly bind to the metal catalytic centre of this enzyme; rather the substrate-binding site resides close to the haem active site, buried deep inside the protein cavity.[8–10] Cytochrome P450 (Cyt P450cam, E.C.: 1.14.15.1), obtained from the soil bacterium *Pseudomonas putida*, is one well-studied member of this family.[8–13] Cyt P450cam catalyses site-specific hydroxylation of camphor at the 5-*exo* position of the substrate during its metabolic cycle to utilise camphor as its energy source.[10,14] In the absence of camphor, the ferric ion in the haem of Cyt P450cam[8–10] exists in a six-coordinated low-spin state with the fifth axial ligation to the sulfur of Cys357, and a water molecule (actually a cluster of six water molecules, one of which is coordinated to the haem) at the sixth coordination position of the metal ion. The proximal amino acid (Cys357) of Cyt P450cam is located in the region called the "Cys pocket", which is a part of the β-loop.[9,15] The conformation of the Cys357 suitable for binding the haem is stabilized by hydrogen bonding with the NH groups of the adjacent amino acids.[15,16] Moreover, the entire β-loop is stabilized by the hydrogen-bonding network of the histidines forming the β-bulge.[8,15,16] Breaking down of this hydrogen-bonding network of the β-loop can result in formation of "misfolded" haem sites, such as the cytochrome P420 species, with axial histidine coordination to the metal centre in the enzyme.[12,17,18]

The monooxygenase activity of Cyt P450 enzymes has largely been attributed to the presence of the proximal thiolate haem ligand provided by the deprotonated cysteine residue.[10,19,20] Similar types of ligand are found only in a few other enzymes, such as chloroperoxidase[21–23] and nitric acid synthase.[19,24] The majority of haem-containing enzymes, on the other hand, contain a proximal histidine as the ligand to the iron centre in haem.[25,26]

The role of the axial ligand on the structure and biological activity of the haem in different haem proteins and enzymes has been addressed by several authors.[10,15,16,18,21,22,25–30] Replacement of the proximal thiolate ligand of chloroperoxidase (Cys29) with a histidine residue was shown[28] to preserve most of the chlorination, peroxidation, epoxidation and catalase activities of the metal centre in the enzyme. Conversely, the proximal histidine ligand has been replaced with a cysteine in sev-

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eral haem enzymes,[25,26,31] and in most cases such mutations cause complete loss of or severe reduction in the biological activity of the enzyme.[25] Mutagenic replacement of axial histidine with a cysteine in myoglobin, a normally noncatalytic haem protein, however, produced a significant increase in the monooxygenase activity of the mutant protein.[26,32] Mutagenesis of the active-site cysteine with serine in cytochrome P450 2B4 (Cyt P450 2B4:C436S) was shown[30] to convert the enzyme into an NADPH oxidase with negligible oxygenase activity. Replacement of the proximal cysteine of cytochrome P450cam with histidine (C357H) has been reported by two groups,[18,27,29] and the mutant protein showed a much lower oxygenase activity than the wild-type enzyme. In particular, the rate of uncoupling and the K_m for substrate binding were shown to be greatly increased by the C357H mutation, whereas the rate of electron transfer was dramatically decreased.[27] The absorption spectral characteristics of the C357H mutant were shown to resemble those of the cytochrome P420-type species, in which the axial coordination was argued to be with a histidine. It was further proposed, from resonance Raman and other studies, that the C357H mutant probably has a bis-histidine ligation to the haem;[15,18,27] this suggests conformational flexibility of the β-loop.

These studies indicated that replacement of the cysteine with other amino acids may lead to drastic changes in the structure and function of the haem active site in cytochrome P450. Cysteine, being a thiol ligand, is a soft base in comparison with histidine, while methionine and cysteine belong to the same class of sulfur-containing amino acids with soft ligand characteristics. Replacement of cysteine with methionine may thus potentially stabilise a sulfur-ligated haem species at the active site of cytochrome P450cam. On the other hand, the protruding methyl group in methionine may also have a steric effect on the axially coordinated haem; this may give rise to some subtle conformational changes in the haem pocket. In order to investigate the effect of replacement of the axial cysteine by a weaker thiol ligand, we have made the site-specific mutant C357M of cytochrome P450cam. The absorption spectrum of the haem moiety of the mutant showed distinct evidence of axial coordinations of methionine and histidine, similar to the situation in cytochrome c. This provided a new cytochrome c type of coordination geometry engineered in a haem enzyme, with the haem residing deep inside the protein cavity. The redox potential of the haem in the mutant was found to be close to that in cytochrome c, supporting a significant role for axial coordination in tuning the redox potential of the haem centre. The results thus indicated that the flexibility of the β-loop of cytochrome P450cam may provide for the formation of a unique metal coordination geometry inside the hydrophobic pocket of the enzyme.

Experimental Section

Most biochemicals used in the mutagenesis, expression and purification of the enzymes were purchased from Roche Chemicals. Potassium ferricyanide was purchased from Sigma (USA). All other chemicals were of analytical grade. The gene of our interest (*CamC*) located in pCHC1 plasmid (obtained from Dr. L. L. Wong, University of Oxford, UK)[33] had heat-inducible *Lac* promoter and a chloromphenicol resistant (*Chl*+) marker. Induction of overexpression of the protein was done by heat shock, through an increase in the temperature from 30 °C to 37 °C. A double restriction-digestion of original plasmid with HindIII and XbaI could recover the *CamC* gene. Site-directed mutagenesis at the C357 position with methionine was carried out by a Splicing of Overlap Extension method[34] using two sets of primers as follows:

A-(5′)CGATACTCTAGAGTCATATGACGACTGAAACCATACAAA(3′)

B-(5′)AGGTGCTGGCCAAG**CAT**CAGATGGCTGCCG(3′)

C-(5′)CGGCAGCCATCTG**ATG**CTTGGCCAGCACCT(3′)

D-(5′)AGCCTGCACAAGCTTTCAGCTACTTATACCGCTTTGGT(3′)

Here **B** and **C** primers harbour the mutation sites. The (**AD**) mutant fragment was inserted back into the original vector, which was then electroporated into the BL21 (DE3) strain of *E. coli* and then overexpressed.

The harvested cells from a 4 L culture were lysed with a gas-driven homogenizer (Avestin, USA) and centrifuged at 15000 rpm at 4 °C. Thus collected lysate was loaded onto a DEAE Sepharose column (Pharmacia Biotech), preequilibrated with 50 mM potassium phosphate buffer (pH 7.4, containing 100 mM (1*R*)-camphor), and eluted with 0–500 mM linear potassium chloride gradient with the aid of the AKTA FPLC system (Pharmacia Biotech). The red fractions were pooled and desalted by passage through a Sephadex-G25 column and were then concentrated by ultrafiltration. The final purification was carried out with a Resource-Q (Pharmacia Biotech) column. SDS-PAGE was used to check the purity of the protein (≈10 μg of protein per lane was loaded). The protein concentration was estimated by the Bradford method.[35] The camphor-free protein was obtained by passing the protein through a Sephadex G-25 column, preequilibrated with Tris buffer (50 mM, pH 7.4) at 4 °C. The electronic absorption spectrum of the mutant protein (≈10 μM) was recorded on a Shimadzu UV-2100 spectrophotometer in a cuvette of path length 1 cm. The spectrum of the carbon monoxide complex of the protein was taken after passing CO gas through the protein solution under reduced conditions (in the presence of sodium dithionite). The UV and visible circular dichroism spectra were recorded with a J810 spectropolarimeter (JASCO Ltd.) with 0.1 cm and 1 cm pathlengths and protein concentrations of ≈3 μM and ≈25 μM, respectively, and the data were averaged over 10–20 scans. The steady state fluorescence spectrum was taken with a SPEX spectrofluorimeter, with an excitation wavelength of 280 nm, a pathlength of 1 cm, and a protein concentration of ≈5 μM. All the spectral measurements were carried out at 298 K.

To estimate the redox potential, the reduced mutant protein (≈10 μM) was titrated with potassium ferricyanide ($K_3[Fe(CN)_6]$) from a 100 μM stock and the corresponding absorption spectra due to oxidation of the mutant cytochrome P450cam were recorded. The spectral cross sections at the Soret and the visible absorption peaks were used for further calculations after dilution and background corrections had been performed. The temperature of the cell was maintained at 298 K during the titration. The equation based on the following simplified model was used to fit the absorbance data for cytochrome c and the C357M mutant of cytochrome P450cam:

$$P{-}Fe^{2+} + F{-}Fe^{3+} \underset{K'}{\overset{K}{\rightleftharpoons}} P{-}Fe^{3+} + F{-}Fe^{2+} \quad (1)$$

Here P denotes the protein and F denotes the ferricyanide, with the redox states of the iron centre shown explicitly. K and K' denote the overall equilibrium constants expressed in forward (K) and reverse (K') directions, respectively. The standard redox potential (ΔE^0_P) of the protein was obtained from the equilibrium[1] by use of the Nernst equation as:

$$\Delta E^0_P = \Delta E^0_F - \frac{RT}{n\Phi} \ln K \quad (2)$$

Here ΔE^0_F is the redox potential of $K_3[Fe(CN)_6]$ (Fe^{3+}/Fe^{2+}) in the reaction mixture, determined by cyclic voltammetry with platinum electrodes in the reaction mixture with an Ag/AgCl reference electrode. The value of ΔE^0_F was found to be $+(259 \pm 10)$ mV. Φ is the Faraday constant and n, the number of electrons involved in the redox equilibrium, is 1.

In order to determine the redox potential of the wild-type Cyt P450cam, the oxidised protein ($\approx$13 μM) was titrated with potassium ferrocyanide ($K_4[Fe(CN)_6]$; 10 μM) and the reduction of the protein was followed spectrophotometrically. The standard redox potential (ΔE^0_P) of the wild-type protein was given as:

$$\Delta D^0_P = \Delta E^0_F + \frac{RT}{n\Phi} \ln K' \quad (3)$$

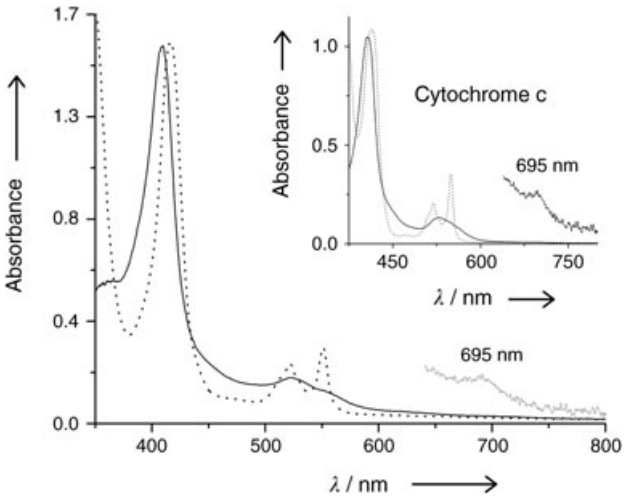

Figure 2. Absorption spectra of ($\approx$10 μM) C357M mutant Cyt P450cam, where the solid curve is the oxidised form (peak at 409 nm) and the dotted curve is the reduced form (peak at 414 nm). The broad and weak peak at 695 nm of the oxidised C357M mutant enzyme is shown by expanding the absorbance scale (10 times) inside the box. Inset: absorption spectra of $\approx$13 μM horse cytochrome c in the oxidised (——) and reduced (·····) forms. The 695 nm band of the oxidised protein is shown by expanding the absorbance scale (10 times) above the spectra.

Results

UV-visible absorption spectra of the wild-type and the C357M mutant of cytochrome P450cam

The cysteine at the C357 position of Cyt P450cam was replaced by methionine through splicing of overlap extension method of site-directed mutagenesis procedure.[34] Figure 1 shows the

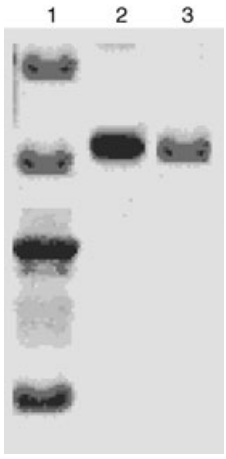

Figure 1. SDS PAGE of C357M mutant along with the wild-type Cyt P450cam ($\approx$10 μg protein per lane). Lanes 1) SDS marker, 2) C357M mutant Cyt P450cam, 3) wild-type Cyt P450cam.

SDS-PAGE of the purified C357M mutant of Cyt P450cam along with the wild-type enzyme, showing the single band characteristic of pure mutant protein. The absorption spectrum of the C357M mutant Cyt P450cam is shown in Figure 2. The oxidized C357M Cyt P450cam showed a Soret peak at $\approx$409 nm ($\varepsilon_{409} = \approx 140 \pm 10$ mM^{-1}cm^{-1}) and visible bands at 526 nm and $\approx$695 nm (weak band shown in Figure 2 with the absorbance scale $\approx$10 times expanded), whereas the reduced protein showed the Soret absorption at $\approx$414 nm ($\varepsilon_{414} = \approx 145 \pm 15$ mM^{-1}cm^{-1}) and visible peaks at $\approx$522 nm and $\approx$552 nm. On the other hand, the wild-type ferric Cyt P450cam (data not shown) showed peaks at $\approx$417 nm, $\approx$537 nm and $\approx$570 nm in the absence of camphor and peaks at $\approx$392 nm, $\approx$509 nm and $\approx$532 nm in the presence of camphor, as reported earlier[17] and distinctly different from those shown by the C357M mutant. The absorption spectral peaks of the C357M Cyt P450cam are very similar to those of cytochrome c[36,37] in both the oxidised and the reduced forms (inset of Figure 2). Moreover, the weak absorption band at 695 nm in oxidised cytochrome c (shown in the inset of Figure 2 with the absorbance scale $\approx$10 times expanded), characteristic of the iron–methionine bond in the ferric haem,[36,37] was also observed in the case of the oxidised C357M Cyt P450cam mutant. The absorption spectral details of the wild-type and C357M mutant cytochrome P450cam, along with those of horse cytochrome c, are summarized in Table 1. The close similarity of the absorption spectra of the haem centre of the mutant enzyme and those of cytochrome c suggests that the electronic structures of the active sites in these two systems might be similar to each other. The small difference in the observed absorption maxima might arise due to the fact that cytochrome c contains haem c covalently linked to the protein while cytochrome P450cam mutant C357M had haem b at the active site. Cytochrome c consists of a six-coordinated low-spin haem with the fifth axial position of the metal ion occupied by a histidine residue and the sixth position by a methionine residue.[36–38] This

Table 1. Summary of the absorption spectral characteristics of the wild-type cytochrome P450cam in the absence of the camphor substrate, the C357M mutant of cytochrome P450cam and horse cytochrome c in different oxidation states of the metal ion.

Wild-type Cyt P450cam in absence of camphor [nm]		C357M mutant of Cyt P450cam [nm]		Cytochrome c [nm]	
oxidized	reduced	oxidized	reduced	oxidized	reduced
417, 537, 570	410, 552	409, 526, 695	416, 522, 552	407, 530, 695	414, 520, 550

result thus indicates that the C357M mutant of Cyt P450cam has an axial ligation of methionine 357, with the other axial position occupied by one of the histidines, possibly from the β-loop of the enzyme.

Unlike in the case of the wild-type enzyme,[10,14,33] addition of camphor to the C357M mutant of Cyt P450cam did not produce any change in the absorption spectrum of the haem centre, indicating either that the camphor does not bind to the mutant enzyme at all or that binding of the substrate does not have any effect on the electronic structure of the metal prosthetic group. Passing carbon monoxide gas through a solution of the C357M mutant of Cyt P450cam under reducing conditions (in the presence of sodium dithionite) did not produce any change in the UV-visible spectrum of the reduced mutant enzyme, indicating that carbon monoxide does not bind to the reduced haem in the C357M mutant. Passing carbon monoxide into a reduced solution of cytochrome c also does not affect the absorption spectrum of cytochrome c, while a similar experiment with cytochrome P450 results in the observation of a distinct band, characteristic of the formation of the CO complex of ferrous haem with axial cysteine, at 450 nm.[2,3,12] This further supports the conjecture that the coordination geometry of the haem centre in the C357M mutant of Cyt P450cam is analogous to that in cytochrome c, with the axial coordination positions of the haem being occupied by a histidine and a methionine residue.

Secondary-structure determination by far-UV circular dichroism spectra

The far-UV circular dichroism (CD) spectra of the wild-type and the C357M mutant Cyt P450cam are shown in Figure 3a. The CD spectra of the proteins in the far-UV region give a measure of the secondary structure content.[36] The far-UV CD spectrum of Cyt P450cam was unchanged after mutation of Cys357 to methionine (Figure 3a), with prominent peaks at ≈222 nm and ≈208 nm, indicating that the secondary structures of the wild type and of the mutant remain almost identical to each other. Analyses of the far-UV CD spectra with the aid of the CDNN[39] neural network program showed almost identical secondary structures for the wild-type and mutant proteins, with ≈45% helix, 28% beta sheets, consistent with earlier reports[39] and with the known crystal structure of the wild-type protein.[8,9,41,42]

Steady-state fluorescence of mutant and wild-type cytochrome P450cam

The steady-state intrinsic fluorescence of the proteins, shown in Figure 3b, shows that the fluorescence spectra of Cyt P450cam, with the emission maximum at ≈335 nm, remained unchanged after mutation of the Cys357 residue to methionine. Cytochrome P450cam contains five tryptophan residues (W42, W55, W63, W374 and W406), with W42 lying close to the haem centre (≈17 Å) while the others are further away (>24 Å) from the haem active site.[8,9,43] The quantum yield and emission peak position of the tryptophan residue depends on the environment around the fluorophore. Earlier studies by our group[43] have shown that the contribution of W42 to the steady-state fluorescence is very small, owing to its fast fluorescence life-

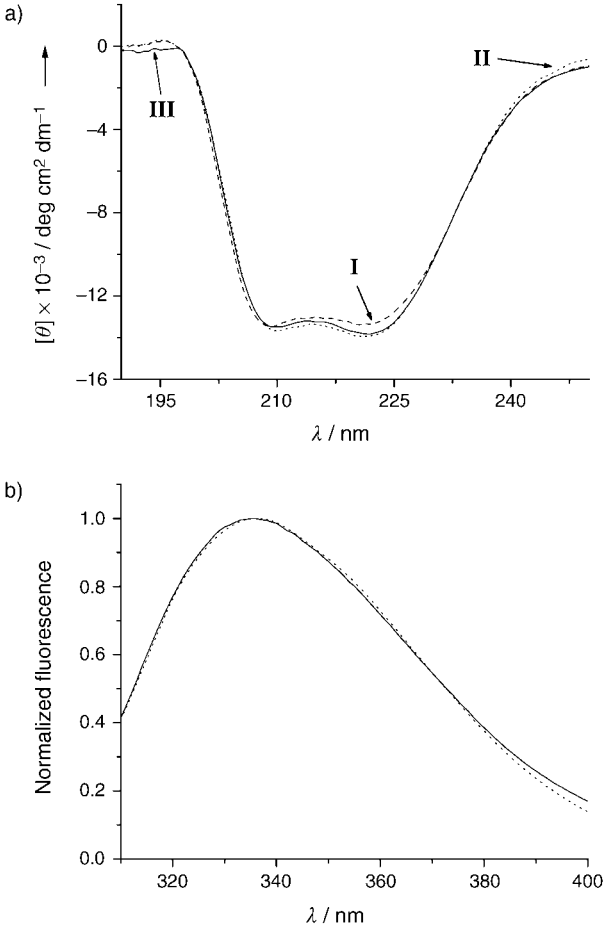

Figure 3. a) Far-UV CD of the C357M mutant Cyt P450cam, along with that of the wild-type Cyt P450cam. The protein concentration is ≈3 μM. Curve I: wild-type Cyt P450cam (oxidised). Curve II: C357M mutant Cyt P450cam (oxidised). Curve III: C357M mutant Cyt P450cam (reduced). b) Steady-state fluorescence spectrum of C357M mutant (oxidised form, dotted line) along with that of the wild-type Cyt P450cam (oxidised form, solid line) clearly indicating the similarity (peak at ≈333 nm). The protein concentration is ≈5 μM.

time due to energy transfer to the haem. The other four tryptophan residues are too far from the haem to experience efficient fluorescence energy transfer to the prosthetic group and so should not be affected by changes in the structure of the haem. The observation of identical steady-state fluorescence spectra of the wild-type and the C357M mutant of Cyt P450cam thus indicates that the average environment around the four distant tryptophan residues in the enzyme was not affected on mutation of the Cys357 residue of the enzyme. This further supports the conjecture that both the secondary and a significant amount of the tertiary structures of the enzyme possibly remain the same in the wild-type and in the C357M mutant of Cyt P450cam. These steady-state fluorescence spectra, however, would not be able to detect any change in the W42 residue, which lies very close to the haem and so would make very little contribution to the overall steady-state intrinsic fluorescence of the protein.

Probing tertiary structure around the haem by circular dichroism spectra in the visible region

The circular dichroism spectra of the haem proteins in the visible region provide important information on the tertiary structure around the haem centre in the protein.[36] Figure 4a shows the CD spectra of the wild-type and the C357M mutant of Cyt P450cam in the visible region, along with those of cytochrome c. The visible CD of the C357M mutant of Cyt P450cam showed a prominent peak at 403 nm in the oxidized state and at 406 nm in the reduced state (Figure 4a, upper panel). The wild-type enzyme showed distinctly different CD spectra in the visible spectral region, with bands at ≈347 nm and ≈412 nm in the absence of camphor and at ≈386 nm and ≈538 nm in the presence of the substrate (Figure 4a, lower panel). The C357M mutant did not show any change in its haem CD spectra on addition of camphor. Although the absorption spectra of C357M Cyt P450 mutant and cytochrome c are very similar, their haem CD spectra are distinctly different from each other. The haem CD spectra of cytochrome c in the oxidised (≈403 nm) and reduced (≈425 nm) forms are shown in Figure 4a, middle panel, for comparison.[36] The results thus suggest that although the overall secondary structure of the enzyme remains almost unchanged on mutation, the tertiary structure of the haem pocket is drastically changed on replacement of the Cys357 by methionine in Cyt P450cam. The haem pocket of the mutant is also distinctly different from that of cytochrome c, although the coordination geometry of the metal ion is the same in the C357M mutant and in cytochrome c.

The Soret CD spectra of oxidised cytochrome c and of the C357M mutant of Cyt P450cam show distinct negative Cotton effects at ≈416 nm. Negative Cotton effects at ≈416 nm in Soret CD spectra of cytochrome c have previously been shown to be characteristic of the iron–methionine bond,[44] so the Soret CD spectrum of the mutant also supports the assignment of a "cytochrome c-type" coordination geometry in the mutant.

Figure 4b shows visible CD spectra of the C357M mutant of Cyt P450cam and of horse heart ferri-cytochrome c in the 600–700 nm range. The distinct negative Cotton effect at 695 nm in the CD spectrum of cytochrome c was previously shown to be characteristic of (*R*) chirality of the Fe–methionine bond in the protein.[45] A small but distinct negative Cotton effect at ≈695 nm was also observed in the CD spectrum of the C357M mutant of Cyt P450cam, indicating that the Fe–methionine bond in the mutant also might have (*R*) chirality.

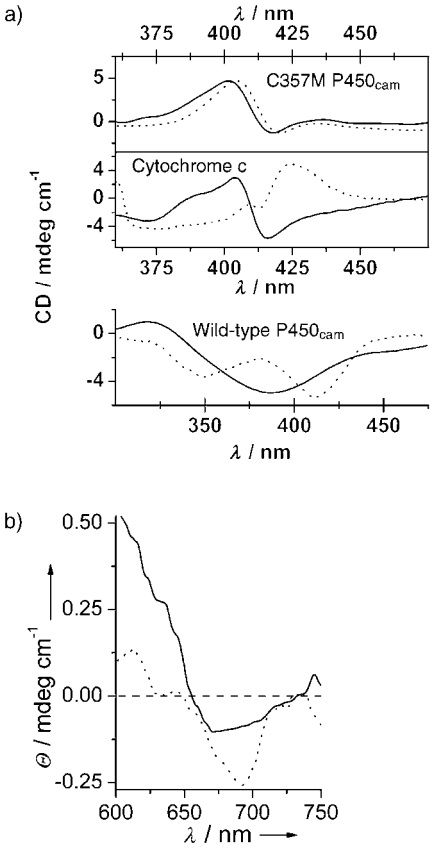

Figure 4. a) Upper panel: Visible CD of C357M mutant Cyt P450cam (····: reduced form, ——: oxidised form). Here the protein concentration is ≈25 μM. Middle panel: haem CD of cytochrome c (····: reduced form, ——: oxidised form). Here the protein concentration is ≈25 μM. Lower panel: haem CD of wild-type cytochrome P450cam in the presence and in the absence of camphor (····: in the absence of camphor, ——: in the presence of camphor). Here the protein concentration is ≈15 μM. b) Visible CD (600–750 nm) of C357M mutant Cyt P450cam (——) in the oxidised form, corresponding to the Fe–methionine bond. ····: corresponding spectrum of cytochrome c. Here the protein concentration is ≈25 μM.

Redox potential of the haem centre

In order to determine the effect of the change in the coordination geometry of the haem on the redox properties of the metal centre in Cyt P450cam, we determined the redox potential of the mutant protein by redox titration with potassium ferricyanide solution.[46] Titration of the protein solution with potassium ferricyanide was followed by optical spectroscopy to determine oxidation of the ferrous haem to ferric haem during the titration. Titration of a ferro cytochrome c solution with potassium ferricyanide was used to check the accuracy of

the method.[46,47] The typical spectral titration of reduced C357M Cyt P450cam with potassium ferricyanide is shown in Figure 5. The spectral data were analysed by using Equation (4):

$$A_\lambda^R = \varepsilon_\lambda^R P_0 - (\varepsilon_\lambda^R - \varepsilon_\lambda^O)\frac{(P_0+F_0^i)-\sqrt{F_0^i-P_0)^2+4F_0^iP_0K}}{2(1-K)} \quad (4)$$

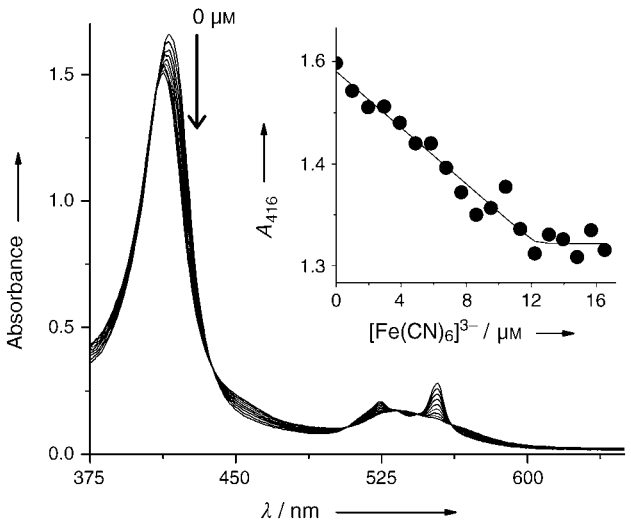

Figure 5. Redox titration of the C357M mutant (reduced, ≈10 μM) Cyt P450cam with ferricyanide. The arrow indicates the direction of change in spectra (from reduced to oxidised form) on addition of ferricyanide solution from 0 μM to ≈15 μM. The inset shows the spectral cross section taken at 416 nm, the solid line being the least squares fitting of absorbance data with Equation (1).

Here A is the absorbance at wavelength λ, P_0 and F_0 are the initial concentrations (the superscript "i" denotes the ith titration) of protein and ferricyanide, respectively (mol L^{-1}), and the ε values are the extinction coefficients of the protein (the superscript "R" denotes the reduced state and "O" denotes the oxidised state) at the wavelength λ (the subscript λ denotes the wavelength).

Analysis of mutant protein versus ferricyanide titration data (i.e., the absorbance of the protein as a function of ferricyanide concentration) in terms of Equations (1) and (2) (below) gave a standard redox potential of $+260\pm20$ mV for the C357M mutant protein. Analogously, analyses of the spectral titration data of the oxidised wild-type Cyt P450cam with potassium ferrocyanide in terms of Equations (1) and (3) (below; with K in Equation (1) replaced by K') gave the standard redox potential of -170 ± 20 mV for the wild-type Cyt P450cam.

The high redox potential of C357M Cyt P450cam was also manifested in the ease of formation of the reduced haem species of the protein in the presence of reducing agent. The electron transfer to the mutant was found to be independent of the presence of the substrate (camphor); this indicates that there is no enzymatic activity of the mutant cytochrome P450cam.

Discussion

The coordination geometry of the haem in cytochrome c has been extensively studied by various techniques.[33,42,44,45,48] Measurement of nOe's between the atoms of axial methionine and the haem group in cytochrome c has been used to identify the conformation of the methionine residue with respect to the haem ring.[45] These NMR studies[45,48] showed that the ε-CH_3 group of the axial methionine in both yeast and mammalian cytochrome c is located on top of the pyrrole ring I and lies near the *meso* protons (α and δ) of the haem moiety.[45] This conformation of the methionine residues was shown to give rise to strong negative Cotton effects in the 695 nm CD band, and *R* chirality was identified at the sulfur atom of the coordinated methionine residue.[45] The 695 nm CD band is generally very weak, while the visible CD band corresponding to the Soret transition of the haem has recently been shown to have characteristic features that can be used to probe the iron–methionine ligation in the cytochromes, whilst the typical negative Cotton effect at 416 nm of the Soret CD[44] in the horse cytochrome c was correlated to the iron–methionine bond. In the current case of the C357M mutant of Cyt P450cam, the oxidised protein showed a typical absorption band characteristic of the iron–methionine bond at 695 nm. The NMR spectrum of the mutant is much broader than that of cytochrome c, mainly because of the high molecular weight (M_w of Cyt P450cam is ≈46 500, while that of cytochrome c is ≈12 400), and no well-resolved NMR spectrum of the protein that might enable assignment of the ligand amino acid signals could be obtained. The CD bands of the mutant in the 695 nm region, although very weak, showed definite indications of negative Cotton effects similar to those reported for mammalian cytochrome c. Analogously to mammalian cytochrome c, the Soret CD of the C357M mutant cytochrome P450cam showed negative Cotton effects at ≈416 nm, which is characteristic of *R* chirality in the methionine residue. Cytochrome c-551 from *P. aeruginosa*, on the other hand, showed positive Cotton effects in the Soret CD[49] as well as the 695 nm CD band,[45,48] characteristic of (*S*) chirality in the methionine residue. From these results it may be argued that the axial coordination of the haem in the C357M mutant of cytochrome P450cam may involve *R* chirality in the axial methionine residue, as observed in the cases of both mammalian and yeast cytochrome c.

The cysteine (C357 in Cyt P450cam) ligation to the haem from the proximal side is considered an important prerequisite for the observed level of Cyt P450 activity.[14–16,19,27,29] The Cys sulfur is stabilized by the "Cys pocket" formed by the hydrogen bonding network of amino acids Cys(357)-Leu-Gly-Gln.[15] Replacement of L358 with a proline reduced the reduction potential by ≈30–40 mV. The presence of proline breaks the hydrogen bonding network, resulting in turn in the negative shift of the Fe^{3+}/Fe^{2+} redox couple. It had been suggested earlier that an increase in the number of NH–S hydrogen bonds can increase the electron-donating ability of the cysteine and lower the redox potential of the haem in cytochrome P450.[15] Replacement of C357 with a histidine moiety not only drastically reduced the catalytic activity[27] but also affected the cam-

phor binding. At the same time, replacement of cysteine with histidine gave rise to an increase in the redox potential from −173 mV (wild Cyt P450cam) to −156 mV.[27,50,51] Surprisingly, the C357M mutant protein showed a much higher (+260 ± 20 mV) redox potential than the wild-type Cyt P450cam.

One of the manifestations of the structural diversity of haem proteins is the variation between them in their redox and electron-transfer properties. The redox potential for the Fe^{3+}/Fe^{2+} reaction of the haem in simple solutions is very small (−450 mV),[52] while that in cytochrome c is highly positive.[53] The standard redox potential primarily reflects the overall thermodynamics of the equilibrium between the redox states.[54] The structural basis for the control of metalloprotein redox potentials has been extensively studied over many years.[38,55] Though the redox potential of a haem protein is mainly controlled by the first coordination sphere of haem,[38,55] many additional factors have also been recognized.[38,47,56–61] Apart from the hydrophobic effects,[55,58] several other contributing factors[47,57,58,61] to explain how the protein matrices control the reduction potential have also been proposed. These include[38,54,57,58] the nonpolar nature of protein matrices, the solvent accessibility of the redox centre, and the net charges and dipole moments of the surrounding amino acids.

The change in the axial ligation of the haem in the Cyt P450 system to a state analogous to that in cytochrome c in the present case provides the first ever known example of the creation of the "cytochrome c-type" coordination geometry in any other protein. The haem in cytochrome c is partially exposed to the solvent,[38] while the haem in cytochrome P450cam—as well as in its mutant—is deeply buried inside the hydrophobic pocket of the protein.[8] Apart from the axial ligation effect, the high redox potential of cytochrome c was shown also to have contributions from several nonbonded interactions within the protein cavity.[54,57,61] The effect of the protein matrix on the redox potential of a buried haem was studied[61] with derivatives of *S. cerevisiae* cytochrome c isoform 1. It was proposed that the nonbonded amino acids in the protein cavity could influence the microscopic dielectric constant around the metal centre, and both the enthalphic and the entropic effects on the dielectric constant were described.[61] A library of cytochrome b562 variants with small variations in the nonbonded amino acids was constructed in order to evaluate the evolution of the redox potential of the haem with constant axial coordination but with variation in the polarizability of the medium.[57,58] Simple considerations of solvent accessibility of the metal centre or the existence of more polar residues in its vicinity were not sufficient to explain the redox potential variations in the library of cytochrome b562 mutants.[57] The specific nature of the interaction of an amino acid with the haem prosthetic group is potentially important in determining its influence on the redox potential of the metal centre. Certain mutations of amino acids in the protein matrix near the haem moiety in cytochrome c (*S. cerevisiae*) were shown to decrease the enthalpy of reduction ($\Delta\Delta H_{red} < 0$) but led to a net decrease in the redox potential ($\Delta\Delta E^0_m < 0$), arguably due to the opposing effect of the simultaneous large decrease in the entropy of reduction[61] ($\Delta\Delta S_{red} < 0$), where

$$\Delta\Delta E^0_m = \frac{-\Delta\Delta H_{red}}{n\Phi} = \frac{\Delta\Delta H_{red} + T\Delta\Delta S_{red}}{n\Phi}$$

These studies thus highlight that the nonbonded amino acids in a haem protein can indeed contribute significantly to the redox potential of the haem.[57] The fact that the redox potentials of the C357M Cyt P450cam mutant and of cytochrome c are very similar to each other suggests that the differences between the two proteins in the solvent environments around the haem, and in other nonbonded interactions with the haem, might not cause any variation in the net free energy of reduction ($\Delta\Delta G_{red} \approx 0$) of the metal centre.

The C357M mutant of Cyt P450cam was completely devoid of any oxygenase activity and the efficiency of reduction of the mutant was independent of camphor. Unlike in the case of the wild-type enzyme, there was no spectroscopic evidence of binding of camphor to mutant protein. The oxygenase activity of cytochrome P450 involves binding of the substrate, electron transfer, oxygen binding to the metal ion and then a series of internal electron/proton transfer steps, which finally lead to oxygen transfer to the substrate. Disruption of any one step in the whole catalytic cycle would affect the activity of the enzyme. Binding of oxygen to the haem constitutes a crucial step of the catalysis by this enzyme. Unlike the wild-type enzyme, the mutant C357M Cyt P450cam does not bind oxygen in the reduced state, so the enzymatic property would be absent in the mutant protein even if the substrate-binding site were not affected on mutation.

Conclusion

Replacement of the axial cysteine of cytochrome P450cam with a methionine by site directed mutagenesis (C357M) produced a great enhancement of the redox potential, from −173 mV to +260 mV. Further spectroscopic studies showed that the haem coordination in mutant C357M Cyt P450cam resembled that in cytochrome c (i.e., Met-His ligation in the haem centre). The methionine residue was found to have *R* chirality analogous to that in mammalian cytochrome c. The six-coordination geometry of the mutant C357M protein was possibly formed by rearrangements in the proximal β-loop of cytochrome P450cam. Unlike the wild-type cytochrome P450cam, the C357M mutant did not show any spectroscopic signature of substrate binding or formation of reduced carbon monoxide complex. The mutant protein was also found to be devoid of any oxygenase activity.

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Keywords: circular dichroism · cytochrome P450cam · haem · redox chemistry · site-directed mutagenesis

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