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Roles of two surface residues near the access channel in the substrate recognition by cytochrome P450cam

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ABSTRACT

Detailed stopped-flow kinetics of binding of 1R-camphor to cytochrome P450cam has been studied at different temperatures for the wild type as well as for two site specific mutants T192E and S190D of the enzyme, where the surface exposed Threonine and Serine residues were mutated by acidic amino acids. The near-UV and visible circular dichroism spectra as well as the intrinsic fluorescence spectra of the WT and mutant enzymes showed that the mutation of the enzyme did not affect the tertiary structure of the enzyme over the temperature range 4–30 °C. The S190D mutation did not show any significant change in the rate constants of the substrate association while they were much lower in the T192E mutant compared to the WT enzyme. The activation energies for substrate association and dissociation processes were determined from the analysis of temperature dependence of the rate constants by the Arrhenius equation over the temperature range 4–19 °C. The activation energy for the substrate association was found to be significantly higher in the T192E mutant compared to the S190D mutant or the WT enzyme. The results showed that the Threonine 192 that resides on the F–G loop and directed towards the putative substrate access channel of the enzyme, plays an important role in recognition of the substrate at the surface of the enzyme. These results showed that though the active site of the enzyme resides deep inside the protein matrix, the substrate is recognized at the surface of the enzyme and directed towards the active site through the access channel.

1. Introduction

Cytochrome P450s form a ubiquitous protein family with functions including the biosynthesis of many physiologically important compounds and the degradation of xenobiotics [1,2]. Cytochrome P450cam (EC 1.14.15.1, P450cam) from *Pseudomonas putida* stereo- and regio-specifically hydroxylates camphor to 5-*exo* hydroxy camphor. The crystal structure of P450cam has been solved at high resolution in the substrate-free form as well as that with the substrate and substrate analogues bound to the enzyme [3–8]. These structures have provided the basis for the structural understanding of the cytochrome P450s, and explained many questions related to the structure–function relationship in these enzymes. The heme moiety is found to be buried ~20 Å deep inside the protein matrix in P450cam as in most other P450s and thus the heme is completely isolated from the surrounding solvent [9].

The crystal structure of P450cam [5,6] indicated the existence of at least two regions in the protein structure, which could form the substrate entry and product exit channels in P450cam. The channels that are predominantly hydrophobic in nature were suggested as the possible access pathways for the hydrophobic substrate. One of these channels is located between the B' helix and the F/G loop (Fig. 1A). The hydrophilic channel located near the heme propionic groups and filled with ordered water molecules was suggested as a possible exit channel for the product, 5-hydroxy camphor [10]. The crystal structures of P450cam with a large inhibitor (1-N-imidazoyl-2-hydroxy-2-(2,3-dichlorophenyl)) and large substrate analogues (i.e. ruthenium sensitizer-linked admantane) show that the substrate might access the heme active site via a path involving movement of F,G,H and I helices [3,10]. Molecular dynamics simulation studies [1,11–13] suggested that the most likely channel for substrate entry is lined by aromatic residues (Tyr96, Phe87, Tyr29, and Phe193). Site directed mutagenesis and stopped-flow kinetics and other studies [1,14] indicated that Tyr29, Phe87 (in B/B' loop) and Phe193 (in F/G loop) participate during substrate access. Salt links involving Lys178, Asp182, Arg186 and Asp251 (tethering F–G loop to the I helix) have also been shown [1,15,16] to play important roles in the access of camphor to the active site of the enzyme.

Although there are several reports [15–17] on the identification of the residues that form the access channel for the substrate to the active site of the enzyme, there has been no studies on whether there

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Abbreviations: WT: Wild type enzyme, P450cam: Cytochrome P450cam (CYP101) from *Pseudomonas putida*, which catalyzes the hydroxylation of 1R-camphor; P450f: Cytochrome P450 camphor-free form; P450b: Cytochrome P450 camphor-bound form; k_{on}: Camphor association constant; k_{off}: Camphor dissociation constant, K_d: Equilibrium dissociation constant of camphor; $E_{a(on)}$: Activation energies for camphor association, $E_{a(off)}$: Activation energies for camphor dissociation.

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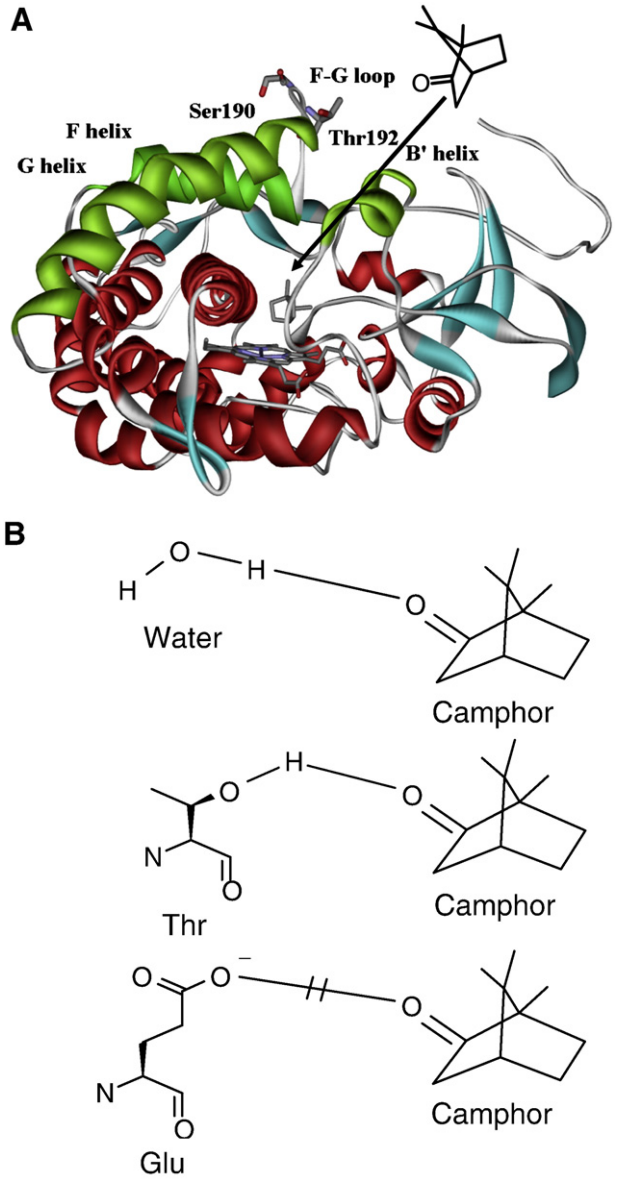

Fig. 1. A Schematic structure of camphor-bound cytochrome P450cam generated from its crystal structure (PDB code: 1DZ4). F, G and B' helices are shown in green, which were found to be part of one important proposed substrate access channel. Serine190 and Threonine 192 are in F–G loop. Hydroxyl group of Serine190 is located just above the F–G loop, pointing away from access channel and that of Threonine192 is close to the entry of the access channel. B. Schematics of hydrogen bond formation between Threonine and camphor. Figure also shows that such hydrogen cannot form between Glutamic acid and camphor.

were any specific recognition site for the substrate at the protein surface that directs the substrate to the access channel. The high rate of camphor binding to the enzyme indeed indicates that there might be a well defined recognition site for the substrate at the enzyme surface, which would facilitate substrate to enter into the protein matrix and pass through the access channel to the active site of the enzyme. Crystal structure and other studies suggested that the F/G loop (Fig. 1) of P450cam is flexible and acts like a gate to the substrate channel of the enzyme. The Ser190 and Thr192 are the two polar residues residing at the surface exposed part of the F/G loop that can potentially act as the initial recognition sites of the substrate by forming hydrogen bond to the carbonyl (C=O) of camphor through the hydroxyl group of these residues (Fig. 1B). In order to evaluate the role of these residues in recognition of the substrate by the enzyme, we have carried out detailed kinetic and spectroscopic studies on substrate binding to the S190D and T192E mutants of P450cam. The alcoholic –OH of Ser190 and Thr192 at the surface of the protein would be solvated in water and mutation of these residues by polar residues (Asp and Glu) would not drastically affect the degree of solvation of the protein surface. It is important to note that the alcoholic –OH (in Ser and Thr) is a better H-bond donor compared to the –OH of water hence the hydrogen bond strength of the carbonyl of camphor (acceptor) with water (donor) would be weaker compared to that with an alcoholic group (such as Thr). On the other hand, the carboxylates of Asp or Glu residues are H-bond acceptors, thus they would not form any specific hydrogen bond with the camphor. The camphor molecules would also not form any preferential hydrogen bond with the water molecules that are associated to the Glu or Asp residues, compared to the bulk water. The carbonyl group of the camphor would not thus experience any preferential affinity towards the Glu or Asp residues and would possibly remain hydrogen bonded to the solvent (water) molecules. The study of the effects of these mutations on the interaction of the substrate with the enzyme would thus aid to understand any role of the Thr 192 or Ser 190 on the recognition of the substrate in the enzyme. The results have been discussed in the light of understanding roles of these residues as possible recognition sites for the substrate to move to the access channel of the active site of the enzyme.

2. Materials and methods

Restriction enzymes and buffers for molecular biology works were obtained from New England Biolabs. DEAE-Sepharose, Q-Sepharose and Sephadex G-25 columns were from Pharmacia Biotech. General reagents and 1*R*-camphor were from Sigma/Aldrich. UV–Visible optical spectra were recorded on a Shimadzu (UV-2100) spectrophotometer coupled with a thermostated cell holder (TCC-260 temperature controller). Stopped-flow kinetic experiments were carried out using a Hi-Tech SF61MX stopped-flow spectrometer. The sample handling unit was mounted inside a thermostated bath compartment and the temperature of the compartment was maintained (within ± 0.3 °C) using a circulating water bath.

2.1. Mutation, expression and purification

The $pCHC_1$ plasmid, encoding the wild type cytochrome P450cam (C334A mutant of the native enzyme) was a kind gift from Prof. L.-L. Wong (University of Oxford, UK). Site-directed mutagenesis was carried out using Quikchange™ mutagenesis kit (Stratagene). The forward and reverse primers used for T192E mutation were 5′-GATGGCAGCATG *gaaTTC*GCAGAG-3′ and 3′-CTACCGTCGTAC*cttAAG*CGTCTC-5′ respectively. These primers introduced an EcoRI restriction site into 594 bp position of *camC* gene along with T192E mutation (shown in small letters). EcoRI recognition sequences are italized. The forward and reverse primers used for S190D mutations of P450cam were 5′-CCCGTCCaGATGGCgaCATGACCTTC-3′ and 3′-GGGCAGGtCTACCGctG-TACTGGAAG-5′. These primers remove the unique BseAI restriction site of the *camC* gene at 578 bp position (shown in small letters). These primers were designed in such a way that introduction of an extra restriction site in the *camC* gene (in the case of T192E mutation) or removal of a restriction site (in case the of S190D mutation) do not affect the resulting protein sequences. These primers were obtained from GENEI (Bangalore, INDIA). PCR amplification of the mutant gene was carried out on a PTC-2000 Peltier thermocycler (MJ Research). Plasmids were isolated from the colonies obtained upon transforming the PCR product into XL1-blue Super-competent cells (Stratagene). Comparison of the restriction digested mutant plasmid with the restriction digested wild type P450cam plasmid by agarose DNA gel electrophoresis confirmed the mutation [18].

Both WT and mutant (T192E & S190D) P450cam were expressed using *E. coli* BL21 (DE3) cells. The proteins were expressed and

purified using reported method [18,19]. Purified protein, with A390/A280>1.4, was concentrated and stored in small aliquots in 50% (v/v) glycerol at −30 °C.

The camphor-free P450cam was prepared by passing substrate-bound P450cam through a G-25 column equilibrated with 50 mM Tris–HCl buffer (pH 7.4) three to four times to complete removal of camphor before the experiment. The concentration of camphor-free form of P450cam in each sample was determined by measuring absorbance at 417 nm ($\varepsilon_{417\ nm}$ = 111.3 mM^{-1} cm^{-1}) [19].

2.2. Steady-state fluorescence measurements

Temperature dependence of the steady-state intrinsic fluorescence of the enzymes was studied using a Spex Fluorolog-1681T spectrofluorometer fitted with a thermostated cell holder. The fluorescence excitation wavelength was kept at 295 nm and the fluorescence intensity was measured at different temperatures. Temperatures were maintained by a circulating water bath.

2.3. Circular dichroism spectroscopy

Circular dichroism (CD) measurements were carried out on a Jasco J-810 (Jasco Ltd, Tokyo, Japan) spectropolarimeter. The tertiary structure of the enzymes were probed by CD spectra in the near-UV and the visible spectral range (250–650 nm) using 20 μM enzyme in a quartz cuvette of 1 cm path length. The temperature dependence of the tertiary structure was studied by monitoring the CD signal at 389 nm in the presence of 100 mM KCl, with 1 mM (1*R*)-camphor. All of the experiments were done with a freshly prepared sample in 50 mM Tris buffer (pH 7.4). The temperature of the sample was raised from 5 to 75 °C with a heating rate of 1 °C/min using a programmable Peltier device attached to the thermostated cell holder.

2.4. Binding constant determination

The camphor-binding equilibrium constant was determined by titration of the substrate-free enzyme with camphor in the presence of 100 mM KCl. The change in the enzyme with increasing concentration of the substrate was determined both from the decrease in the absorbance at 417 nm corresponding to decrease in the substrate-free enzyme and also from the increase in the absorbance at 392 nm corresponding to formation of the substrate-bound enzyme. Data were analyzed by the reported method [14] to obtain the equilibrium dissociation constant (K_d) values.

2.5. Stopped-flow kinetics

Kinetics of camphor binding was studied for wild type, T192E and S190D mutants of P450cam by measuring the rate of decrease in absorption at 417 nm corresponding to the low-spin heme of the substrate-free enzyme using a SF61 MX2 stopped-flow spectrometer (HiTech, England). Equal volumes (75 μl) of 0.5 μM substrate-free P450cam and different concentrations of camphor solutions (5–30 μM) were mixed using a pneumatic syringe drive. The reaction solutions were maintained at pH 7.4 in 50 mM Tris–HCl, 100 mM KCl. The decay of the absorption signal at 417 nm was found to fit to single exponential decay mechanism different camphor concentrations at different temperatures ensuring that the reaction followed pseudo-first order kinetics.

3. Results and discussion

Fig. 2 shows the absorption spectra of wild type, S190D and T192E mutant cytochrome P450cam (P450b: camphor-bound form and P450f: camphor-free form). Absorption bands at 392 nm and 510 nm for Soret and β bands respectively and at ~645 nm

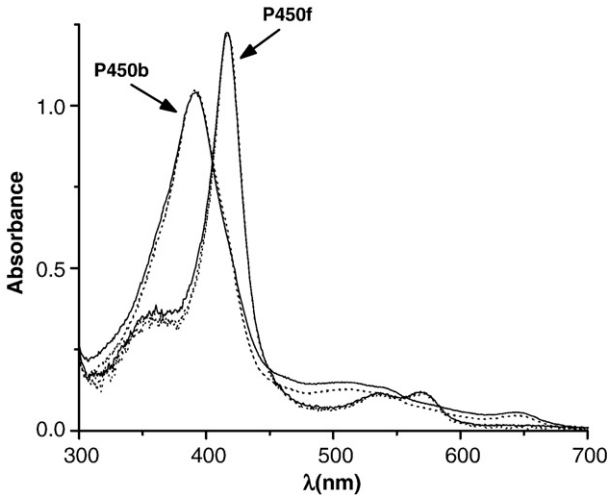

Fig. 2. UV–visible absorption spectra of substrate-bound (10.25 μM, labeled 'P450b') and substrate-free P450cam (11 μM, labeled 'P450f') of wild type enzyme (Solid line), S190D (dashed lines) and T192E (dotted lines) mutant enzymes.

characteristic of the high-spin ferric heme proteins were observed in the wild type as well as the mutants of P450b [1,18], which match with the absorption spectra of the substrate-bound enzyme. The Soret, β and α bands were observed respectively at 417 nm, 536 nm and 569 nm for the wild type as well as the mutant P450f [18,20,21], which match with the absorption spectra of the substrate-free enzyme. Thus the mutation of the enzyme did not cause any change in the absorption spectra of the enzyme in the present case and the visible spectra matched with those reported earlier.

The tryptophan fluorescence emission spectra of the substrate-free P450cam (P450f) were taken at the temperature range 4–65 °C. The intrinsic fluorescence maximum of the protein was observed at 325 nm, which agreed with earlier report [17]. A red shift in the tryptophan fluorescence emission peak position was observed at temperatures above 40 °C, indicating that thermal unfolding of the protein becomes significant at temperatures above 40 °C as reported earlier [20]. A very small decrease in the intrinsic fluorescence intensity at 325 nm with increase in the temperature in the range 4–30 °C was observed due to increase in non-radiative energy loss processes at higher temperature [22], but no bathochromic shift in the tryptophan fluorescence emission spectra was observed in this temperature range. These results indicate that there was no conformational change of the protein in the temperature range of 4–30 °C.

Fig. 3 shows the near-UV and visible CD spectra of camphor-bound wild type and T192E mutant proteins and inset shows the temperature dependence of the ellipticity values at 389 nm for the substrate-bound enzymes. Analogous results were obtained for the S190D mutant of the enzyme. The CD spectra in the near-UV and visible region correspond to the tertiary structure around the aromatic residues and the heme in the protein [23]. The results in Fig. 3 suggest that the tertiary structure of the enzyme was not affected on mutation. Moreover, the tertiary structure of the WT and the mutant proteins remains unchanged over the temperature range 4–30 °C and the unfolding midpoint temperature (Tm) for the protein was unchanged on mutation [18].

The time evolution of the formation of the camphor-bound form (P450b) or decrease of camphor-free form (P450f) (P450f + camphor ⇌ P450b) was analyzed to determine the observed rate constants (k_{obs}) for substrate recognition by the WT enzyme and its mutants at each camphor concentration. Typical plot of variation of absorbance of the substrate-free enzyme (P450f) at 417 nm with time is shown in Fig. 4. The observed rate constant k_{obs} increases with increasing concentration of camphor and a linear fit to the plot of k_{obs} vs camphor

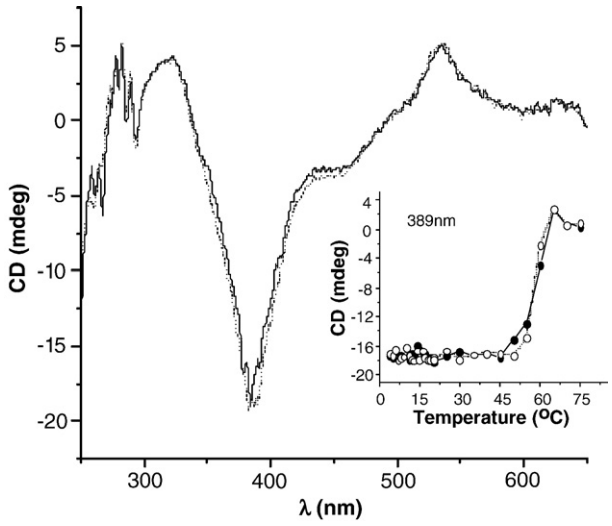

Fig. 3. Near-UV and visible CD spectra of substrate-bound forms of the WT (solid line) and T192E mutant (dotted line) of cytochrome P450cam. The protein concentration was 15 μM. Inset shows temperature dependence of the CD values at 389 nm for substrate-bound form of WT (solid circle) and T192E mutant (hollow circle) of cytochrome P450cam.

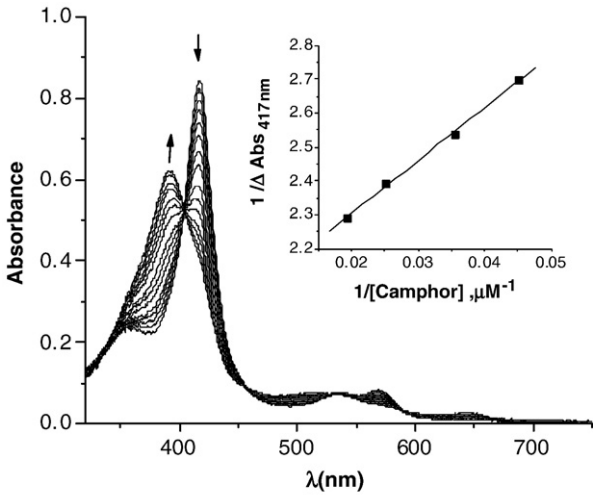

Fig. 5. Variation of absorption spectrum of T192E mutant cytochrome 450cam during titration of camphor to its camphor-free form. The concentration of camphor-free T192E mutant was 7.5 μM and the final concentration of camphor in the solution were varied in the range of 15–50 μM. Inset shows double reciprocal plot of absorbance at 417 nm as a function of camphor concentrations.

concentration (Eq. (1)) was analyzed to determine the rate constants (Inset in Fig. 4) for substrate binding (k_{on}) and substrate release (k_{off}) at each temperature.

$$k_{obs} = k_{off} + k_{on}[\text{camphor}] \quad (1)$$

The apparent substrate dissociation constant (K_d) was determined from the kinetic data as $K_d = k_{off}/k_{on}$ at each temperature. The values of k_{on} and k_{off} of the WT enzyme at different temperature agreed well with those reported earlier [1,24–26]. The values of K_d were also determined by equilibrium titration of the substrate-free enzyme with camphor (Fig. 5). The values of K_d for the WT enzyme agreed well with those reported earlier [14,18,23,24,26]. Furthermore, the values of K_d determined by steady-state method at different temperatures agreed well with those obtained from the kinetic analysis. Both the k_{on} and k_{off} values for the T192E mutant protein were found to be significantly smaller than those for the WT P450cam enzyme. On the other hand, the rate constant values for the S190D mutant were higher than those for the WT enzyme. It was interesting to note that the substrate dissociation constant, K_d was also found to be affected by the mutation though the site of mutation was far away from the substrate binding site of the enzyme. These results indicate that the mutations at S190 as well as at T192 indeed have important effects on the binding or release of the substrate in the P450cam.

In order to determine the activation parameters for the binding of the substrate to the enzyme ($E_{a(on)}$ and $E_{a(off)}$), the temperature dependence of the rate constants of camphor association (k_{on}) and dissociation (k_{off}) was studied. The protein conformation was stable over the temperature range from 4–30 °C, hence we studied the kinetics of camphor binding in this temperature range and the results were analyzed using the Arrhenius Equation (Eq. (2)).

$$\begin{aligned} k_x &= A_x \ \exp\ (-E_{a(x)}/\mathrm{RT}) \\ \ln\ [k_x] &= \ln\ A_x - (\mathrm{E}_{a(x)}/\mathrm{RT}) \end{aligned} \quad (2)$$

Where rate constant k_x represents k_{on} or k_{off}, $E_{a(x)}$ is the activation energy ($E_{a(on)}$ or $E_{a(off)}$ are activation energies obtained from k_{on} and k_{off} respectively), A_x (x=on or off) are the pre-exponential factors, R is the universal gas constant, and T is the absolute temperature. The plots of ln [k_x] vs 1/T (Fig. 6A and B) were found to be linear and obeyed the Arrhenius Equation over the temperature range 277–292 K (i.e., 4–19 °C). As the CD and fluorescence results show that structure of the enzyme remains unchanged both for the substrate-bound and substrate-free forms of the enzyme in this temperature range [18,20], the observed temperature dependence in the rate constants could thus be ascribed solely to the activation energy associated with the interaction of the substrate with the enzyme. A distinct deviation from the Arrhenius equation was observed at temperatures above 20 °C. This deviation indicates that though there was no significant change in the protein structure at temperatures in the range 20 °C to 30 °C, the subtle thermal perturbations in the conformation of the enzyme possibly affect the rate constants of substrate association leading to non-Arrhenius behavior. Such non-Arrhenius behavior of the kinetics of substrate association could arise due to increased fluctuations in the protein [12,13,17,27] without causing any apparent change in the equilibrium conformation in the temperature range 20–30 °C as supported by the steady-state fluorescence and CD spectra of the protein discussed in the previous section. The deviations however become more significant at higher

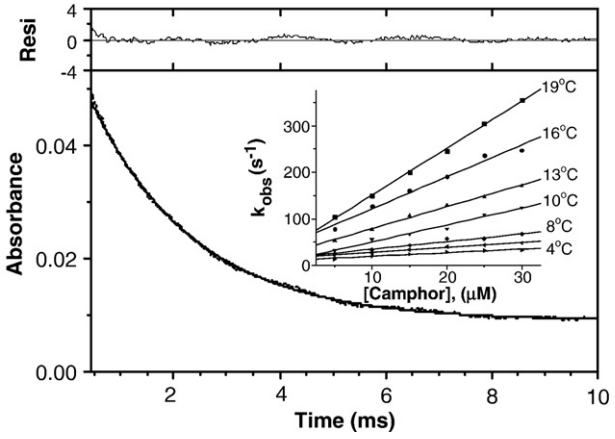

Fig. 4. Typical stopped-flow kinetic trace for the reaction between substrate-free cytochrome P450cam (P450f) and camphor (pH=7.4, 100 mM KCl) at 13 °C for T192E mutant at 417 nm. The protein concentration was 0.5 μM. The solid line through data indicates single exponential fit. The upper panel shows residuals distribution for the single exponential fit. Inset shows plots of observed pseudo-first order rate constant k_{obs} as a function of camphor concentrations at 19, 16, 13, 10, 8, 6 and 4 °C. The solid lines through the data points are fits using Eq. (1).

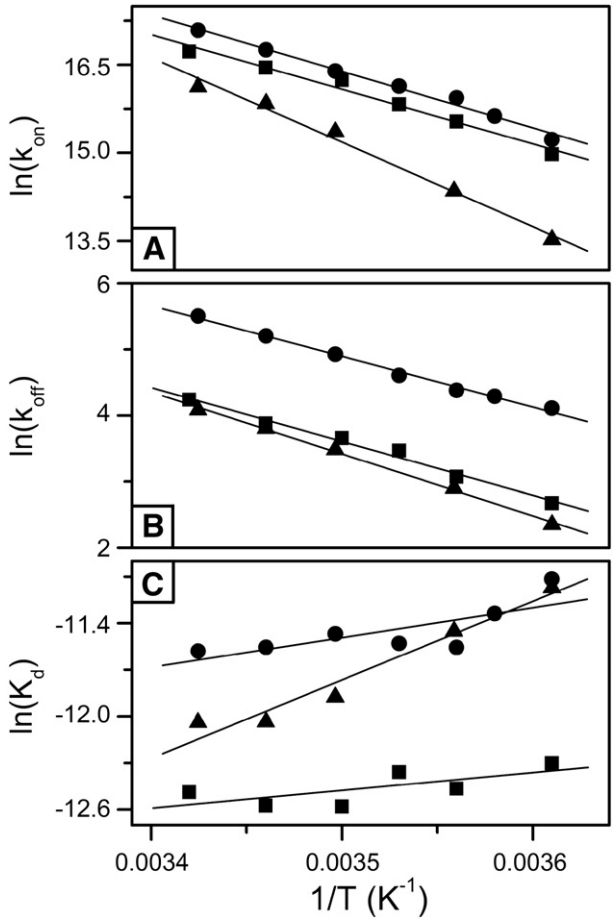

Fig. 6. Temperature dependence of the experimental rate constant for (A) camphor association (k_{on}); (B) camphor dissociation (k_{off}) and (C) the dissociation constant (K_d) from wild type (■), T192E (▲), S190D (●) mutant CytP450cam. Temperature was varied between 4 °C and 19 °C.

temperatures (>40 °C) owing to thermal inactivation possibly due to partial unfolding of the enzyme [18,20]. Earlier studies [24] had reported a bi-phasic behavior of k_{on} with temperature, which possibly arises because of the increased contributions of temperature induced conformation change to the substrate association kinetics of the enzyme. The activation energy $E_{a(on)}$ for camphor association to the wild type and to the mutant enzymes was determined from the slopes of the linear fit to $\ln[k_{on}]$ vs $1/T$ plots (Fig. 6A) in the temperature range 277–292 K (4 °C–19 °C). Variations of $\ln[k_{off}]$ vs $1/T$ (Fig. 6B) for the WT as well as the mutant enzymes gave the activation energies for dissociation of the substrate from the enzyme. The results of the analyses of the temperature dependence of the rate constants are given in Table 1 and shown schematically in Fig. 7.

The values of the dissociation constants (K_d) calculated from the forward (k_{on}) and the reverse (k_{off}) rate constants ($K_d = k_{off}/k_{on}$) at different temperatures agreed with the K_d values determined by steady-state UV–visible titration of the enzyme with the substrate. Fig. 6C shows the temperature dependence of K_d, which was analyzed by the Van 't Hoff equation:

$$\ln[K_d] = \frac{\Delta S^0}{R} - \frac{\Delta H^0}{RT} \quad (3)$$

Where ΔH^0 and ΔS^0 are the standard enthalpy and entropy changes associated with the dissociation of camphor from the substrate-bound enzyme. The values of the thermodynamic parameters obtained for the WT enzyme agreed well with those reported earlier [24]. The results of analyses of the equilibrium dissociation constant are given in Table 1.

Table 1
Comparison of the activation energies for camphor association ($E_{a(on)}$), dissociation ($E_{a(off)}$ and the enthalpy changes (ΔH^0) for dissociation of the substrate-bound wild type and mutant CytP450cam

	ΔH^0 (kJ/mol) [ΔS^0 (J/mol/K)]	$E_{a(on)}$ (kJ/mol) [ln A_{on}]	$E_{a(off)}$ (kJ/mol) [ln A_{off}]
WT	−9.6±1.5 [−137]	77.4±2.8 [404]	67.8±3.9 [266]
T192E	−42.1±1.5 [−245]	119.9±2.1 [546]	77.8±2.2 [299]
S190D	−15.9±1.5 [−151]	79.9±3.2 [416]	64±4.1 [266]

The values of frequency parameters (ln A_x in Eq. (2)) and standard entropy change (ΔS^0) of dissociation are shown in brackets.

These results show that the activation energy for substrate association ($E_{a(on)}$) is much higher in case of the T192E mutant compared to that in the S190D mutant and in the WT enzyme. The enthalpy of the substrate-free form of the enzyme and the surface mutants (P450f) were considered to be the same and a relative energy-level diagram was drawn as shown in Fig. 7. It is important to note that the binding of camphor to P450cam is an entropy driven process [16]. Thus, though the enthalpy change on substrate association is positive (substrate dissociation enthalpy is negative, Table 1) [16], the overall free energy for substrate binding to the enzyme is negative. The value of the overall free-energy change on substrate binding to the wild type enzyme determined by this method agreed with that reported earlier [24,28].

The results in Table 1 and Fig. 7 show that mutation of the Threonine 192 residue drastically increases the activation barrier of substrate association to the enzyme. This residue is located on the F–G loop near the proposed substrate entry channel (Fig. 1A). The F–G loop has earlier been proposed [7,10,16,27] to play important role in substrate selectivity of the enzyme. The active site of the enzyme is located deep inside the protein pocket [21]. The putative substrate access channel in the P450cam was proposed to involve the F–G loop and B' helix and specific interactions of several residues in the substrate access channel that are likely to be important for transport of the substrate to the active site of the enzyme [13]. The crystal structures of the enzyme (PDB Code: 1DZ4 and 1PHC) [6,10] show that the T192 resides on top of the putative substrate access channel [13] thus may form the first site of interaction with the substrate camphor and directs the substrate towards the access channel. The OH– group in Threonine may form hydrogen bonds with the carbonyl (C=O) at C2 position of camphor. Mutation of T192 by Glutamic acid in T192E will

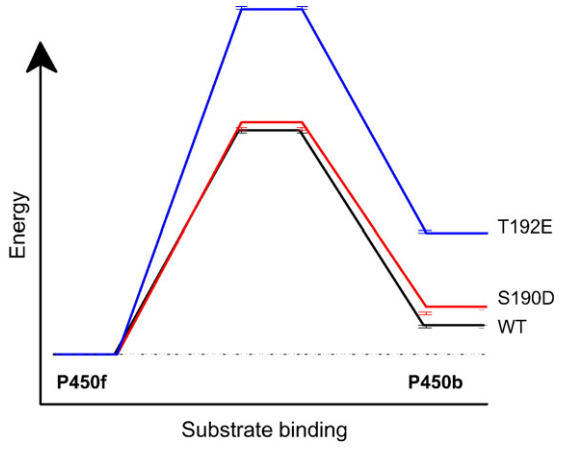

Fig. 7. Schematic representation of the activation energy required for association and dissociation of camphor to/from the Wild type, T192E, S190D mutants of the enzyme.

not allow such H-bond formation with the camphor (Fig. 1B) leading to an increase in the activation barrier for substrate association to the enzyme. These results thus enable us to propose that the T192 residue plays a role in recognition of the substrate at the enzyme surface and helps the substrate to enter into the access channel to go to the active site. Fig. 7 shows that mutation of Serine 190 by Aspartic acid (S190D mutant) in P450cam has almost no effect on the activation energy of substrate binding to the enzyme. The results however indicate that analogous to the T192E mutant, the enthalpy of the substrate-bound form increases on mutation of the S190 residue and the substrate dissociation constant (K_d) increases on the mutation (Table 1). The crystal structure of the enzyme [6,10] shows that though the S190 residue is located on the F–G loop, it is directed away from the putative substrate access channel formed by the intersection of the F–G loop and the B' helix of the enzyme [13]. Hence mutation of S190 by Aspartic acid does not significantly alter the recognition and transport of the substrate by the access channel to the active site. These studies thus indicate that while the T192 residue might serve as a recognition site for the substrate at the enzyme surface, the S190 residue being oriented in the opposite direction does not participate in the substrate recognition by the enzyme to move the substrate to the access channel.

4. Conclusion

The substrate dissociation constant of cytochrome P450cam was found to increase on mutation of the Threonine 192 or Serine 190 residue, though these residues are located at the surface at about 20 Å away from the substrate binding site. The kinetics of substrate association to the enzyme was found to follow the Arrhenius equation in the temperature range 4–19 °C. The substrate binding process was found to have different activation energies for the WT and mutant enzymes. The activation energy for substrate binding was found to be higher in the T192E mutant of the enzyme compared to the WT and S190D mutant cytochrome P450cam. These results thus show that the substrate binding to the P450cam involves a specific recognition site at the enzyme surface that forms the entry site of the substrate to the access channel. The Threonine 192 residue was shown to play an important role in the substrate recognition by the enzyme. This residue possibly acts as the first binding site of the substrate at the surface of the enzyme which enables the substrate to enter into the access channel, while the Serine 190 though located on the same F–G loop, does not have any major role in recognition of the substrate.

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