The Active Site of the Thermophilic CYP119 from *Sulfolobus solfataricus* *

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CYP119 from *Sulfolobus solfataricus*, the first thermophilic cytochrome P450, is stable at up to 85 °C. UV-visible and resonance Raman spectroscopy show that the enzyme is in the low spin state and only modestly shifts to the high spin state at higher temperatures. Styrene only causes a small spin state shift, but 

\[ T_2 NMR \] 

studies confirm that styrene is bound in the active site. CYP119 catalyzes the 

\[ \text{H}_2\text{O}_2 \text{-dependent epoxidation of styrene, cis-\beta-methylstyrene, and cis-stilbene with retention of stereochemistry.} \]

This catalytic activity is stable to preincubation at 80 °C for 90 min. Site-specific mutagenesis shows that Thr-213 is catalytically important and Thr-214 helps to control the iron spin state. Topological analysis by reaction with arylazidanes shows that Thr-213 lies above pyrrole rings A and B and is close to the iron atom, whereas Thr-214 is some distance away. CYP119 is very slowly reduced by putidaredoxin and putidaredoxin reductase, but these proteins support catalytic turnover of the Thr-214 mutants. Protein melting curves indicate that the thermal stability of CYP119 does not depend on the iron spin state or the active site architecture defined by the threonine residues. Independence of thermal stability from active site structural factors should facilitate the engineering of novel thermostable catalysts.

The cytochrome P450 superfamily of enzymes catalyzes a wide range of oxidative and reductive reactions that are important in xenobiotic detoxification, carcinogen activation, steroid biosynthesis, fatty acid metabolism, and reactions required for the survival of microorganisms on selected nutrients (1). These reactions include hydrocarbon hydroxylation, olefin epoxidation, and heteroatom oxidation (2). The ability of P450 enzymes to catalyze a diversity of transformations, combined with the ability of their active sites to bind a range of unrelated substrates, makes these enzymes highly versatile catalysts. CYP119, as the first thermophilic member of the P450 superfamily to be identified and cloned (3), is of great interest as a starting point for the construction of useful thermostable P450 catalysts through protein engineering methods.

The CYP119 gene was cloned out of *Sulfolobus solfataricus*, a hyperthermophilic, acidophilic archaeabacteria found in sulfuric acid hot springs (3). *Sulfolobus* are sulfur autotrophs that can be found attached to sulfur crystals at Pisciarelli Springs in Italy and in mud pools at Yellowstone National Park. Optimal growth conditions for *Sulfolobus* occur at temperatures between 78 and 86 °C and pH values between 3 and 4. The growing interest in thermophilic proteins has led to an ongoing effort to sequence the complete *Sulfolobus* genome (5–7).

Several recognizable structural features are evident from the CYP119 primary sequence. First, CYP119 lacks a hydrophobic tail and is therefore expected, as found, to be a soluble protein. Second, CYP119 has a high identity in the heme binding region with mammalian, fungal, and bacterial P450 enzymes and exhibits a particularly high overall identity of 33% with P450cam. Last, CYP119 possesses both the conserved cysteine involved in heme coordination and a highly conserved threonine within the I helix thought to facilitate the coordination of a water ligand to the ferric heme, the binding of oxygen to the ferrous prosthetic group, and cleavage of the bound dioxygen molecule to give the final activated oxidizing species (8, 9). In P450cam, the conserved threonine is Thr-252. This residue is crucial for catalysis, and its mutation to an alanine or valine impairs substrate binding and greatly enhances the uncoupled reduction of oxygen to \( \text{H}_2\text{O}_2 \) and \( \text{H}_2\text{O} \) (8). In addition, mutation of the conserved threonine to a large hydrophobic residue can increase the fraction of the protein in the high spin state due to disruption of the water ligand by the amino acid side chain (8–10).

Conversion of P450 enzymes from the low to the high spin state, which correlates with loss of the distal water ligand, can be indirectly measured by monitoring the shift in the Soret absorbance maximum from 415 to 390 nm. The P450 spin state is temperature-, pH-, and ionic strength-dependent (11–13). It is worth noting that the conserved threonine (Thr-213) in CYP119 is followed by two additional threonine residues (Thr-214 and Thr-215). Such a sequence of three consecutive threonines is not unique within the P450 family, but is unique among the well studied bacterial P450 enzymes (14).

We report here expression and characterization of CYP119, including the first determination of its catalytic properties. We also describe the construction, expression, and characterization of its Thr-213 and Thr-214 mutants. These studies show that the threonine residues in CYP119 play an important role in controlling the active site structure, spin state, and catalytic activity of the enzyme. They are not, however, critical for its thermal stability. An abstract describing some of our work on...
the basic properties of CYP119 has been published (15), as has a communication on the same enzyme by another laboratory (16).

**EXPERIMENTAL PROCEDURES**

**Materials.—**Styrene, cis-stilbene, R- and S-styrene oxide, thiophenol, 4-ethylthiophenol, and other chemicals were purchased from Aldrich. cis-β-Methylstyrene was from K & K Laboratories (Cleveland, OH), and methylphenyldiazene carboxylate azo ester was from Research Organics (Cleveland, OH). Methyl or ethyl arylidene carboxylate azo esters were prepared from the corresponding nitrile, amino, and hydrazino precursors according to the method of Huang and Kosower (17).

**General Procedures —**UV-visible spectra were recorded on a Cary 1E double beam spectrophotometer. Gas chromatography was performed on an HP5890 Series II chromatograph with a flame ionization detector. CD measurements were performed on a Jasco J-710 instrument fitted with a Polarier water bath. 1H spectra were recorded on a Varian Unity Inova AS400 NMR spectrometer.

**Expression and Purification of CYP119 —**The CYP119 cDNA was a kind gift from Peter Konnolly (Virginia Tech). For overexpression, CYP119 was subcloned into pCWori using the NdeI and XhoI sites. An overnight culture (3 ml) of a clone picked from a single colony in 2YT medium with ampicillin was used to inoculate 1.5 liters of 2YT medium. Protein expression in *Escherichia coli* DH5α cells was induced using 1 mM isopropyl-1-thio-β-D-galactosidase after the cell density had reached an OD of 0.8. The incubation temperature was then lowered to 30 °C, and the cells were grown for 40 h. Cells were harvested and lysed using lysozyme in 50 mM Tris buffer, pH 7.0, at 4 °C. After sonication and centrifugation, the supernatant was heated to 60 °C for 1 h. Precipitated proteins were pelleted (20 min at 15,000 rpm), and the supernatant was loaded onto a Q Sepharose column; washed with 50 mM Tris, pH 7.0, buffer; and eluted using a gradient of 0–250 mM NaCl. All of the reddish-brown fractions were combined and dialyzed overnight against 50 mM bis-Tris, pH 7.0. The protein was then loaded onto a PBE94 column equilibrated in 50 mM bis-Tris, pH 7.0, buffer and eluted using Polybuffer 74 at pH 6.6. CYP119 eluted at pH 6.6 and was judged to be pure based on the method of Huang and Kosower (17).

**Methylphenyldiazene carboxylate azo ester** was from Research Organics (Cleveland, OH), and 5′-ATT TTA CTT CTC ATA GC was from the University of California, San Francisco, as a 0.5 M solution in acetonitrile to give a final acetonitrile concentration of 1.5%. Control incubations were done without CYP119 or without H₂O₂. Each reaction was allowed to proceed for 30 min at 30 °C. Samples were then extracted with CHCl₃ (2 × 150 μl). The combined CHCl₃ layers were concentrated under a stream of argon and then analyzed by isothermal gas chromatography at 80 °C on a fused silica DB-1 column (30 m × 0.25 mm inner diameter). Under these conditions, the retention times for styrene and styrene oxide were 4.9 and 11.6 min, respectively. Authentic standards of the other epoxides were obtained by adding meta-chloroperbenzoic acid to the olefins in CHCl₃, allowing the mixture to react for 30 min at room temperature, and then injecting the products directly onto the gas chromatographic column. The retention times for cis-β-methylstyrene and cis-β-methylstyrene oxide were 4.6 and 11.9 min, respectively. The gas chromatographic conditions were modified to 130 °C for 2 min, followed by 7 °C/min rise to 180 °C, and finally 180 °C for 5 min, for the separation of cis-stilbene and cis-stilbene oxide (retention times of 10.5 and 12.2 min, respectively).

**Styrene epoxide formation** was quantitated using relative peak areas with excess styrene as the internal standard. The initial CYP119 concentration was used for all calculations. The enantiomeric selectivity of styrene epoxidation was analyzed by isothermal gas chromatography at 100 °C on a Chiraldex G-TA column (30 m × 0.25 mm inner diameter). Pd- and PdR-dependent styrene epoxidation assays were also carried out using wild-type, T213S, T214A, T214V, and T213A/T214A CYP119. Reaction mixtures (total volume 200 μl) contained CYP119 (12.5 μM), Pd (125 μM), PDr (25 μM), styrene (saturated, 5 μM), and 5′-ATT TTA CTT CTC ATA GC (3 μM). CYP119 was allowed to proceed for 5 h at 30 °C. Products were analyzed and quantitated as described above. Reactions with wild-type CYP119 were used as the control incubation.

**Characterization of CYP119 —**For thermal stability studies, 75 μM CYP119 in 50 mM bis-Tris buffer, pH 6.0, was incubated in a water bath at temperatures ranging from 20 to 90 °C for 1.5 h. An aliquot of each sample was taken and immediately reduced and analyzed at room temperature for CO complex formation. Each CYP119 sample was then used in the styrene epoxidation assay carried out at room temperature. For comparison, P450_0 was incubated in 50 mM Tris buffer, pH 7.0, at 40 and 60 °C and assessed for reduced-CO complex formation after 1.5 h.

**Styrene binding studies** were performed in 50 mM bis-Tris buffer at pH 6.0 saturated with styrene (5 μl undiluted in 5 μl of buffer) using 1.3 or 2.5 μM CYP119. Under these conditions, the styrene concentration exceeded that of the enzyme by at least 1000-fold. CYP119 was added to the styrene-saturated buffer in a capped cuvette at the temperature of interest, and the mixture was incubated for 3 min before the UV-visible spectrum was recorded. The percentage conversion of the high spin state was calculated using an absorption coefficient of 6.4 mM⁻¹ cm⁻¹ at λmax = 646 nm. The binding of thiophenol and 4-ethylthiophenol to 4 μM CYP119 in 50 mM bis-Tris buffer, pH 6.0, saturated with each of the two compounds (3 μl undiluted in 1 μl of buffer) was monitored by UV-visible spectroscopy.

The initial rates of CYP119 bleeding to H₂O₂ were calculated for the reaction from 2 to 6.5 min. A 12.5 μM solution of CYP119 in 50 mM bis-Tris buffer, pH 6.0, was incubated for 3 min at 30 °C before the H₂O₂ (final concentration 10 mM) was added, and the increase in the Soret absorbance was recorded.

**Resonance Raman Spectroscopy —**RR spectra were obtained using a custom McPherson 2061/207 spectrograph (0.67 m) with a Princeton Instruments (LN-1100PB) liquid N2-cooled CCD detector. Rayleigh scattered light was attenuated with Kaiser Optical notch filters. Excitation sources consisted of a Coherent Innova 302 krypton laser (413 nm) and an Innova 90-6 argon laser (514.5 nm). Spectra were collected in a 90° scattering geometry with collection time of a few min. Frequencies were calibrated relative to indene and CCl₄ standards and are accurate to 1 cm⁻¹. CCl₄ was also used to check the polarization conditions. The samples contained in glass capillaries were either inserted in a copper finger immersed in a water bath or directly exposed to a thermostatted air flow that maintained the sample at ~22 and 70 °C (18). Typical enzyme concentrations were ~90 μM in 50 mM bis-Tris (pH 6.0) and ~1 mM in styrene when present. The integrity of the Raman samples, before and after laser illumination, was confirmed by direct monitoring of their UV-visible spectra in the Raman capillaries with a Perkin-Elmer Lambda 9 spectrometer.

**NMR Relaxation Rate Data Acquisition —**Relaxation rates in three independent titrations of styrene with CYP119 were measured using the standard inversion recovery method at 400 MHz with a spectral width of 4500 Hz and 0.1-Hz digital resolution at 298 K using a 5 mm probe. The 700 μM D₂O samples contained a saturated amount of styrene (2.4 mM) (19), 0.1 M potassium phosphate, 100 mM NaCl, and 0.5–10 μM CYP119 at a pH (uncorrected for isotope effects) of 5.5. All solutions were prepared in D₂O (99.9%, Cambridge Isotope Laboratories, Inc.) and were saturated with each of the two compounds (3 μl undiluted in 1 μl of buffer) before acquisition.
were pretreated with Chelex to remove free metal ions. The typical pulse sequence contained a long delay ($5-7 \times T_1$) between scans, 30 s of low power irradiation on the residual water peak, a 180° pulse, a variable time delay, a 90° read pulse, and acquisition. When enzyme concentrations exceeded 1 $\mu$M, the integrity of the protein was verified by formation of the ferrous carbon monoxide complex. All $T_1$ data sets contained 15 time delay points over a 10–15-fold range centered around the null point.

**Relaxation Rate Data Analysis** —The amplitudes of the peak heights were fit to standard relaxation equations using a nonlinear method (20–22). Under the limits of fast exchange, the observed rate ($R_{obs}$) is a weighted average of the rate in free solution ($R_{1,p}$) and the rate bound to the paramagnetic enzyme ($R_{1,p}$) (23, 24).

$$R_{obs} = pR_{1,f} + (1-p)R_{1,p}$$

(Eq. 1)

When $S_i > K_i$, the fraction of enzyme bound at 1.5 mM styrene may be approximated as unity. With $p = 1$, Equation 1 may be expressed in terms of total enzyme concentration, total substrate concentration, and the dissociation constant (25).

$$R_{obs} - R_{f} = \frac{E_0}{K_d + S} (R_{1,p} - R_{1,f})$$

(Eq. 2)

The relaxation enhancement of a ligand in the presence of a paramagnetic protein contains paramagnetic and diamagnetic components. The paramagnetic contribution may be dissected from the total enhancement by duplicating the relaxation measurement with the heme protein in the diamagnetic ferrous carbon monoxide complex (25).

$R_{1,p}$ and $K_d$ may be calculated from a two parameter curve fit when Equation 2 is varied as a function of enzyme and/or substrate concentration. The $K_d$ value may also be approached by measuring the spectroscopic binding constant and evaluating $R_{1,p}$ as the sole unknown. The Solomon–Bloembergen equation relates the paramagnetic effect to a function of the square of the interaction energy, the frequency of the nuclear and electronic transitions, and a correlation time for the motion that modulates the interaction (26, 27).

The assumptions and restrictions of this equation have been discussed in detail elsewhere (28, 29).

$$R_{1,p} - R_{1,f} = \frac{E_0}{K_d + S} (R_{1,p} - R_{1,f})$$

(Eq. 3)

The parameter $r$ is the distance from the heme iron to the ligand proton, $s$ is the spin state of the heme iron, $\omega_1$ and $\omega_2$ are the nuclear and electronic Larmor frequencies, and $\tau_d$ is the correlation time for the motion that modulates the interaction (28, 29). Due to the uncertainty in the precision of high to low spin protein in the solution, limiting values were calculated by assuming that the protein was all high spin or all low spin.

The electronic relaxation rate for heme proteins is usually $10^{10}$ to $10^{13}$ s$^{-1}$ (29). Chemical exchange rates rarely exceed $10^5$ s$^{-1}$, and rotational rates rarely exceed $10^6$ s$^{-1}$ based on the Stokes-Einstein equation. $\tau_d$ is therefore usually dominated by $\tau_d$ and has been estimated by the frequency dependence of $R_{1,p}$ for several proteins. In most cases, $\tau_d$ lies between $10^{-12}$ and $10^{-11}$ (25, 30, 31). In accord with these previous results, we have assumed that $\tau_d$ is equal to $5 \times 10^{-11}$. $\tau_d$ is the correlation time for the motion that modulates the interaction (28, 29). Due to the uncertainty in the precision of high to low spin protein in the solution, limiting values were calculated by assuming that the protein was all high spin or all low spin.

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Styrene Epoxidation by CYP119—Since the endogenous electron transfer partners for CYP119 remain unknown, the ability of alternate proteins to transfer electrons to CYP119 was investigated. Incubation of CYP119 with Pd/PdR, spinach ferredoxin/ferredoxin reductase, and human cytochrome P450 reductase under a CO atmosphere, conditions that should lead to formation of the ferrous-CO complex with a λ\text{max} at 450 nm, showed that none of these proteins functioned effectively as an electron donor partner for CYP119. This conclusion was confirmed by unsuccessful efforts to use these electron donor proteins to support the CYP119-catalyzed oxidation of styrene (see below). However, the catalytic activity of CYP119 could be assayed in the absence of electron donor proteins using H\textsubscript{2}O\textsubscript{2} as the source of oxidizing equivalents.

No information is available on the endogenous substrates for CYP119, although the growth of \textit{S. solfataricus} in sulfur-rich environments makes sulfur compounds good candidates for this role. We have used styrene and substituted styrenes as test substrates because they are readily available, they give products that are readily analyzed, and there are extensive data on their oxidation by other P450 enzymes (34–37). H\textsubscript{2}O\textsubscript{2} was used for the assays to minimize the likelihood of homolytic oxygen radical formation, although preliminary experiments suggest that tert-butylhydroperoxide is a more efficient donor of oxidizing equivalents. The sole detectable product detected by gas chromatography in a 60-min incubation of CYP119 with styrene and 10 mM H\textsubscript{2}O\textsubscript{2} was styrene oxide (Fig. 3A). The styrene oxide was identified by direct chromatographic comparison with an authentic standard. Although a longer incubation time increased the yield of epoxide, the reaction time was held at 30 min to minimize the concomitant, H\textsubscript{2}O\textsubscript{2}-dependent bleaching of the CYP119 porphyrin chromophore. Kinetic studies show that with 10 mM H\textsubscript{2}O\textsubscript{2} at 30 °C CYP119 converts styrene to styrene oxide with a V\textsubscript{max} of 0.6 nmol min\textsuperscript{-1} nmol\textsuperscript{-1} protein. Control experiments establish that the reaction requires both H\textsubscript{2}O\textsubscript{2} and CYP119. This is the first CYP119 catalytic activity to be identified.

Incubation of CYP119 with cis-β-methylstyrene and cis-stilbene shows that the epoxidation reaction takes place with complete retention of the olefin stereochemistry. The only products identified in these reactions by gas chromatography were cis-β-methylstyrene oxide (Fig. 3B) and cis-stilbene oxide (data not shown), respectively. As observed with other P450 enzymes (34), the epoxidation of styrene by CYP119 is enanti-
oselective, yielding a 75:25 mixture of the S- and R-styrene oxide enantiomers.

Thermal Stability—The effect of temperature on both the CYP119 heme chromophore and styrene epoxidation activity was investigated. After preincubation of CYP119 at temperatures between 20 and 80 °C for 90 min, the absorbance maximum of the ferrous CO complex of the enzyme determined at 25 °C remained entirely at 450 nm. Abrupt precipitation occurred when the protein was preincubated between 80 and 90 °C (Fig. 4). This thermal stability contrasts with that of P450cam, which at 60 °C partially precipitated and gave mixtures of species absorbing at 420 and 450 nm (data not shown). Preincubation of CYP119 for 90 min at temperatures between 20 and 80 °C did not measurably alter the styrene epoxidation activity of the enzyme when subsequently measured at 25 °C (Fig. 4).

Substrate Binding Studies by UV-visible Spectroscopy—As shown by changes at 646 nm in the difference absorption spectrum of substrate-bound versus substrate-free CYP119, styrene only converts a small fraction of the enzyme to the high spin state at temperatures between 20 and 80 °C (Fig. 5). Similar results were obtained using cis-β-methylstyrene, cis-stilbene, and 4-methoxystyrene (data not shown). The fraction of the high spin species increased slightly with increasing temperature, but it has not been possible to determine whether a temperature-dependent correlation exists between the spin state and the catalytic activity. Preliminary experiments suggest that styrene oxidation increases as the incubation temperature is raised, but substrate and product recovery were not sufficiently quantitative at the higher temperatures to make possible accurate rate comparisons.

Resonance Raman Characterization of Styrene Binding—The minor spin state changes that accompany the addition of styrene to CYP119 suggest that this substrate either very poorly displaces the distal water ligand from the iron or does not actually bind in the active site and only allosterically perturbs the spin state. We have therefore also examined the binding of styrene to CYP119 by resonance Raman and NMR methods. Fig. 6 shows the effect of temperature and excess substrate on the high frequency region of the CYP119 RR spectrum obtained with Soret excitation. The data obtained at room temperature are very similar in the absence and presence of substrate (Fig. 6, A versus B). The heme core marker bands \( \nu_{3p}, \nu_{4p}, \nu_{10p} \), and \( \nu_{10p} \) at 1370, 1500, 1581, and 1635 cm\(^{-1}\), respectively, are characteristic of a hexacoordinate low spin (Fig. 6, 6cLS) heme and are very similar to the literature values for substrate-free P450 enzymes (38). When the temperature is raised to 70 °C, a significant amount of the pentacoordinate high spin species (Fig. 6, 5cHS) is observed. This low to high
spins shift appears as a \( v_3 \) component at 1485 cm\(^{-1}\) and additional intensity in the \( v_2 \) region at 1570 cm\(^{-1}\), while hexacoordinate low spin contributions are diminished (Fig. 6).

Although at 70 °C, the addition of excess styrene increases the conversion of LS (Fig. 6D) to HS (Fig. 6C), a large proportion of LS heme is still present. The percentage of LS to HS conversion deduced from the RR data is consistent with the UV-visible absorption data (Table I).

Resonance Raman using excitation wavelengths away from the Soret absorption can result in resonance enhancement of iron-ligand stretching vibrations. In heme proteins with cysteinate-proximal ligands, the Fe-S stretching mode is observed only in pentacoordinate high spin species ferric hemes using excitation wavelengths around 540 or 365 nm (39–41). The low frequency RR spectra of substrate-bound CYP119 at 22 and 70 °C was obtained with 514.5-nm excitation (Fig. 7). A new signal at 352 cm\(^{-1}\) can be isolated from the 70 °C data by subtraction of the room temperature spectrum (Fig. 7C). The 352 cm\(^{-1}\) band is assigned to the \( v(\text{Fe-S}) \) of the pentacoordinate high spin species substrate-bound CYP119, since it is absent in the purely LS sample, and it is not observed with 413-nm excitation. The relatively small amount of HS species formed in CYP119 even with excess styrene at 70 °C is consistent with the low intensity of the 352-cm\(^{-1}\) band as compared with modes insensitive to spin states such as \( v_{16} \) at 753 cm\(^{-1}\). Whereas the intense \( v_{16} \) vibration at 753 cm\(^{-1}\) is subtracted out in the difference spectra, the positive signal at 352 cm\(^{-1}\) is accompanied by a negative band of comparable intensity at 390 cm\(^{-1}\).

The signal at 352 cm\(^{-1}\) is comparable with the Fe-S vibration observed at 351 cm\(^{-1}\) in P450cam (41). The similarity in \( v(\text{Fe-S}) \) frequencies between the two P450s indicates that the interaction within the proximal side of the heme pocket of pentacoordinate high spin species CYP119 does not differ from that of other P450 enzymes.

\( \Delta A_{450} \) absorbance value \( e = 8.4 \text{ M}^{-1} \text{ cm}^{-1} \). Values are reported as the mean of triplicate experiments ± S.D.

| Protein   | High spin state |
|-----------|-----------------|
|           | 40 °C  | 70 °C  | 40 °C  | 70 °C  |
| CYP119    | 0      | 5      | 6      | 5      | 19 ± 5 |
| T213A     | 2      | 8      | 15     | 22     |
| T213V     | 3      | 11     | 4      | 18     |
| T213S     | 6      | 18     | 11     | 30     |
| T213F     | 9      | 14     | 25     | 34     |
| T213W     | 17     | 38     | 31     | 51     |
| T214A     | 15     | 18     | 30     | 32     |
| T214V     | 9      | 15     | 30     | 40     |
| T213A/T214A | 4  | 9      | 19     | 24     |

**Table I**

**Spin states of CYP119 and its mutants as a function of the temperature and the binding of styrene**

![FIG. 6. High frequency region of the RR spectra of ferric wild-type CYP119 obtained with 413-nm excitation (5 milliwatts) at 22 °C in the presence of excess styrene (A) or without substrate (B) and at 70 °C in the presence of excess styrene (C) or without any substrate (D).](http://www.jbc.org/)

![FIG. 7. Low frequency region of the RR spectra of ferric wild-type CYP119 obtained with 514.5-nm excitation (20 milliwatts) in the presence of excess styrene at 70 °C (solid trace A) and 22 °C (dotted trace B) and the difference spectrum A minus B (trace C). The inset is an enlargement of the same spectra in the 300–500-cm\(^{-1}\) region.](http://www.jbc.org/)

\[ ^2 \text{A } v(\text{FeII-OH}_2) \text{ assignment for the 390-cm}^{-1} \text{ band is ruled out by the absence of an isotopic shift upon } ^{16} \text{H}^{18} \text{O}/^{18} \text{O substitution.} \]
spin state could account for the failure of the surrogate electron residues. Furthermore, the inability to significantly shift the bonding interactions between the water ligand and protein high spin state, could be due to unusually strong hydrogen-iron, as indicated by the very small shift from the low to the similarity of the distances.

Tumbling rather than a fixed orientation is responsible for the distances for all of the protons suggests either that (a) styrene binds in a single position from which the distance of each of the styrene protons to the iron of approximately 6.4 Å. (Table II). If the assumption is made that the protein was completely in the high spin state, yields distances from the high spin state of the enzyme. Furthermore, the equivalence of the high spin species almost completely resolved chromatographically. The basis for this separation of the proteins into the first band contained predominantly high spin protein (Fig. 10). Both species were predominantly low spin protein, and the second band contained bands during chromatofocusing. The first band contained protein with ferricyanide (43). The distribution of the four possible states of the Thr-213 mutants appear to be due mostly to the increase in temperature. CYP119 has an unusual sequence of three threonine residues beginning with the conserved Thr-213 (Fig. 9). To determine whether Thr-214 is also involved in stabilization of the CYP119 water ligand, it was mutated to an alanine and valine. These mutants were found to have 10–15% high spin character at room temperature in the substrate-free form (Table I). Interestingly, the T214V mutant separated into two distinct bands during chromatofocusing. The first band contained predominantly low spin protein, and the second band contained predominately high spin protein (Fig. 10). Both species were shown by their ferrous CO spectra, which exhibited maxima at 450 nm, to be entirely in the undenatured P450 state (data not shown). Similar phenomena were observed with the other T213 and T214 mutants, but only in the case of T214V were the low and high spin species almost completely resolved chromatographically. The basis for this separation of the proteins into two fractions differing in spin state is not known at this time. The T213A/T214A double mutant was constructed to see if the spin state effects of the two mutations were additive. Surprisingly, the changes in Thr-214 appear to have a much greater impact on the spin state than the changes in the putative conserved Thr-213 residue (Table I).

**Active Site Topology of Threonine Mutants**—As indicated above, sequence alignments suggest that Thr-213 and Thr-214 are probably situated within the CYP119 active site. To examine their proximity to the heme moiety and their role in determining the active site topology, the Thr-213 and Thr-214 mutants were allowed to react with arylidiazene probes. Arylidiazenes react with P450 enzymes to give aryl-iron complexes that can be induced to undergo a migration of the aryl group from the iron to the porphyrin nitrogens by incubation with ferricyanide (43). The distribution of the four possible shift in P450cam is associated with a change in the redox potential that facilitates reduction of the iron by Pd/PdR (42).

| Styrene proton | Distance (Å) |
|----------------|-------------|
| Para           | 6.2 ± 0.1   |
| Meta           | 6.5 ± 0.1   |
| Ortho          | 6.3 ± 0.1   |
| α              | 6.6 ± 0.2   |
| β              | 6.4 ± 0.4   |

**TABLE II**

Maximum distances of the styrene protons from the heme iron atom determined from 1H NMR T2 relaxation data assuming a completely high spin protein.
N-aryl-PPIX isomers isolated from the reaction can then be related to the active site topology and can be used, in particular, to explore changes in the topology caused by mutations. We have found that CYP119 is unusual in that it forms the aryl-iron complex, but the complex is unstable in the presence of oxygen and directly undergoes a shift of the aryl group to the porphyrin nitrogens (44). The CYP119 studies thus do not require the use of ferricyanide but simply require anaerobic incubation of the enzyme with the aryldiazene to generate the aryl-iron complex followed by aerobic incubation to induce migration of the aryl group. The process is thus described by the following equation, where \( A \) and \( B \) are the enzyme and the aryldiazene, respectively, \( C \) is the aryl-iron complex, and \( D \) is the N-aryl heme.

\[
A + B \rightarrow C \rightarrow D \quad \text{(Eq. 6)}
\]

![Fig. 9. Sequence alignment of the I helix region of selected bacterial P450 enzymes. Beginning with the conserved residue Thr-213 (boldface type), CYP119 has three threonine residues in a row.](http://www.jbc.org/)

![Fig. 10. Spectra of the two nearly completely resolved protein fractions obtained upon purification of the CYP119 T214V mutant. The chromatographic details are given under "Experimental Procedures." The spectra of the two fractions are characteristic of predominantly high spin (solid line) and low spin (dashed line) proteins.](http://www.jbc.org/)

| CYP119 protein | 4-Trifluoromethylphenyldiazene | 4-Bromophenyl diazene | 3,5-Difluoro-4-nitrophenyldiazene |
|----------------|-------------------------------|------------------------|----------------------------------|
|                | \( k_i \) | \( N_{\text{aryl-PPIX}} \) | \( N_{\text{aryl-PPIX}} \) | \( N_{\text{aryl-PPIX}} \) | \( N_{\text{aryl-PPIX}} \) | \( N_{\text{aryl-PPIX}} \) |
| Wild type      | 656 | 05:03:53:39 | 05:03:55:36 | ND |
| T213A          | 1730 | 23:43:17:16 | 44:56:00:00 | |
| T213V          | 389 | 03:01:47:49 | 43:57:00:00 | 35:40:10:15 |
| T213S          | 775 | 35:45:11:09 | 43:57:00:00 | ND |
| T213F          | 286 | 06:05:70:18 | 43:57:00:00 | ND |
| T213W          | 275 | 10:07:52:31 | 43:57:00:00 | ND |
| T214A          | 825 | 05:02:59:34 | 43:57:00:00 | ND |
| T214V          | 861 | 04:02:50:44 | 43:57:00:00 | ND |
| T213A/T214A    | 2140 | 26:43:16:15 | 40:45:02:03 | |

* Shift induced by addition of ferricyanide.
* \( N_{\text{aryl-PPIX}} \) the N-aryl-PPIX adducts in which the aryl group is attached, in order of elution from the HPLC column, to the nitrogens of pyrrole rings B, A, C, and D, respectively.
* ND, not detected.
mutants with 4-trifluoromethylphenyldiazene. The obtained from the reaction of CYP119 and its T213S and T213F Thr-214 mutants are slightly more resistant to H2O2 bleaching paralleled a lower rate of styrene epoxidation. However, the studies (Table IV). In general, a lower rate of enzyme bleaching with the relative active site access implied by the aryl-iron shift oxide. The rates for the Thr-213 mutants roughly correlate lytic activity, but they produced varying amounts of styrene epoxide ratio. The conditions are given under “Experimental Procedures.”

**Effect of Mutations on CYP119 Catalysis**—All but one of the active site threonine mutants retained H2O2-dependent catalytic activity, but they produced varying amounts of styrene epoxide. The rates for the Thr-213 mutants roughly correlate with the relative active site access implied by the aryl-iron shift studies (Table IV). In general, a lower rate of enzyme bleaching paralleled a lower rate of styrene epoxidation. However, the Thr-214 mutants are slightly more resistant to H2O2 bleaching but are able to epoxidize styrene at higher rates than wild-type CYP119 while still giving the same 75:25 S:R epoxide ratio. Surprisingly, the double alanine mutant had properties similar to the T213A mutant. Given that the T214A mutant undergoes a larger spin state conversion and has a higher activity than the wild-type protein, one might have expected the double mutant to exhibit additive effects due to the two single mutations. This result suggests that Thr-213 plays a catalytic role and/or is involved in substrate binding. Furthermore, the data agree with the findings from the aryldiazene reactions that Thr-214 is further away from the heme than Thr-213 and only indirectly influences the CYP119 spin state.

**Effect of Mutations on CYP119 Thermostability**—To investigate whether CYP119 thermostability was influenced by alteration of the active site cavity, the melting points of wild-type CYP119 and its Thr-213 and Thr-214 mutants were determined using circular dichroism. These melting point studies reveal that the Thr-213 mutations have little or no influence on CYP119 thermostability (Table IV). In all cases, the melting temperature varied, on average, by only 2 °C, and the CD spectra obtained at all temperatures were similar to those containing 100 mM KCl. The following temperatures were employed for the experiment: 40 °C (solid line), 60 °C (dashed line), 80 °C (dashed and dotted line), and 100 °C (dotted line). The conditions are given under “Experimental Procedures.”

The ability of the CYP119 mutants to accept electrons from electron transfer proteins was tested by measuring the ability of Pd/PdR to support styrene epoxidation. The reactions were carried out with wild-type CYP119 and its T213S, T214A, T214V, and T213A/T214A mutants. Styrene epoxidation by the T214A and T214V mutants is supported by Pd and PdR, whereas catalysis by the wild-type protein and the Thr-213

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**Graph 11**

**Graph 12**

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**Table IV**

| Protein   | CYP119 heme loss Pnmol min⁻¹ nmol | Styrene epoxidation Pnmol min⁻¹ nmol | Protein melting temperature °C |
|-----------|----------------------------------|-------------------------------------|-------------------------------|
| CYP119    | 55 ± 6                           | 590 ± 40                            | 90.5                          |
| T213A     | 28 ± 6                           | 490 ± 70                            | 88.9                          |
| T213V     | 6 ± 2                            | 4 ± 4                               | 89.4                          |
| T213S     | 19 ± 2                           | 113 ± 5                             | 88.5                          |
| T213F     | 10 ± 1                           | 0 ± 2                               | 88.1                          |
| T213W     | 6 ± 1                            | 31 ± 3                              | 88.1                          |
| T214A     | 41 ± 2                           | 1580 ± 150                         | 88.9                          |
| T214V     | 36 ± 1                           | 1740 ± 120                         | 92.8                          |
| T213A/T214A| 29 ± 1                         | 840 ± 150                         | 87.0                          |
reactions of the Thr-213 mutants with larger side chains. By both UV-visible (Table I) and RR spectroscopy (Fig. 6), the Thr-213 mutants with large amino acid replacements are at either 40 or 70 °C, with all of the Thr-214 mutants and T213S, T213F, and T213W mutant proteins exhibit a higher proportion of HS species than the wild-type enzyme (Table I). In contrast, the T213A and T213V mutants have spin state data is mutated. Introduction of larger side chains at this position, as in the T213F, T213W, and T213V mutants, causes only small changes in the regioisomer distribution, suggesting a minimal alteration in the active site steric environment. However, the T213A and T213S mutations, in which the size of the side chain is reduced, clearly alter the regioisomer ratio from one that favors pyrrole rings C and D to one that favors pyrrole rings A and B. This is most clearly seen with the 4-bromophenyl probe, which shifts only to the A and B rings in the latter mutants but almost exclusively to pyrrole rings C and D in the wild-type protein (Table III). The aryl shift data suggesting that the distal water ligand is more tightly bound to the iron atom than usual. The basis for the unusual stability of the distal water ligand is not known, but it could involve the formation of an unusually strong set of hydrogen bonds with active site residues or water molecules hydrogen-bonded to those residues or electrostatic effects on the distal side of the active site (45).

If hydrogen bonding is important for stabilization of the water ligand, two possible candidates for the hydrogen-bonding residues are Thr-213, the residue that aligns with the conserved threonine in the sequences of other P450 enzymes, and the adjacent residue Thr-214 (Fig. 9). Indeed, mutation of these threonine residues strongly perturbs the spin state of the enzyme, without affecting either the folding or thermostability of the protein (Table IV). At all temperatures, the T214A, T214V, T213S, T213F, and T213W mutant proteins exhibit a higher proportion of HS species than the wild-type enzyme (Table I). In contrast, the T213A and T213V mutants have spin state equilibria very similar to those of the parent enzyme. The same pattern is observed when the proteins are incubated with styrene at either 40 or 70 °C, with all of the Thr-214 mutants and the Thr-213 mutants with large amino acid replacements showing a considerably increased high spin component. The high spin state of CYP119 is thus favored by groups larger than a threonine at position 213 and by mutation of Thr-214 to nonpolar (non-hydrogen-bonding) residues.

If Thr-213 is hydrogen-bonded to the distal water ligand, removal of this interaction is not sufficient to destabilize the iron–water bond. Either this hydrogen bond does not contribute significantly to stabilization of the water ligand, or the hydrogen bond to Thr-213 is replaced when this residue is mutated by a hydrogen bond to a new water molecule in the active site. However, when the hydrogen bond is eliminated and the new residue at position 213 is large enough to block the positioning of a new water molecule near the distal water ligand, dissociation of the water ligand becomes more favorable, and an increase in the fraction of the protein in the high spin state is observed. In contrast, simple removal of the hydrogen-bonding group from position 214 is sufficient to perturb the stability of the distal water ligand, either because the water and Thr-214 are directly hydrogen-bonded or because Thr-214 participates in a hydrogen-bonding network that helps to stabilize the distal water ligand.

To examine the active site alterations accompanying the threonine mutations, we investigated the reactions of CYP119 and the CYP119 mutants with aryldiazene probes. Analysis of the reaction rates indicates that mutation of Thr-213 to a smaller residue increases, whereas mutation to a larger residue decreases, the rate of formation of the aryl-iron intermediate. In contrast, the Thr-214 mutations have a relatively minor effect on the rate of complex formation. These results clearly suggest that Thr-213 is located close to the iron atom in a position that sterically interferes with formation of the aryl-iron complex, whereas Thr-214 is so placed that changes in the volume of the side chain at that position have little effect on the rate of the reaction despite their major effect on the spin state of the protein.

As already reported (44), the N-aryl-PPIX regioisomer patterns obtained in the reactions of wild-type CYP119 with the 4-trifluoromethylphenyldiazenes and 4-bromophenyldiazenes indicate that the wild-type active site is primarily open above pyrrole rings C and D (Table III). In both cases, 92% of the resulting N-aryl-PPIX adducts bear the aryl moiety on the nitrogens of these two pyrrole rings. The central role of Thr-213 in the CYP119 active site is beautifully confirmed here by the changes in the N-aryl-PPIX regioisomer ratio when this residue is mutated. Introduction of larger side chains at this position, as in the T213F, T213W, and T213V mutants, causes only small changes in the regioisomer distribution, suggesting a minimal alteration in the active site steric environment. However, the T213A and T213S mutations, in which the size of the side chain is reduced, clearly alter the regioisomer ratio from one that favors pyrrole rings C and D to one that favors pyrrole rings A and B. This is most clearly seen with the 4-bromophenyl probe, which shifts only to the A and B rings in the latter mutants but almost exclusively to pyrrole rings C and D in the wild-type protein (Table III). The aryl shift data suggesting that reducing the size of the residue at position 213 decreases steric encumbrance over pyrrole rings A and B are clearly consistent with the finding that the same mutations increase the rates of formation of the aryl-iron complexes (Table III). In contrast, mutation of Thr-214 to an alanine or valine has little effect on the N-aryl PPIX regioisomer ratios, in accord with the observation that the rates of aryl-iron complex formation are also not altered. Thr-214 is therefore in a sterically insensitive region of the active site despite the fact that mutation of this residue strongly alters the spin state equilibrium. This finding supports the inference that the effects of Thr-214 on the distal

| Protein       | styrene epoxidation |
|---------------|---------------------|
| **CYP119**    | ND                  |
| **T213S**     | ND                  |
| **T214A**     | 4 ± 1               |
| **T214V**     | 7 ± 1               |
| **T213A/T214V**| ND                  |

* ND, not detected under assay conditions.

| TABLE V |
|---------|
| Rates of Pd/PdR-dependent styrene epoxidation by CYP119 and selected CYP119 mutants |
| Protein | styrene epoxidation |
|---------|---------------------|
| CYP119  | ND                  |
| T213S   | ND                  |
| T213A   | 4 ± 1               |
| T213V   | 7 ± 1               |
| T213A/T214V | ND               |

* ND, not detected under assay conditions.
water ligand are mediated by polar effects or through a hydrogen-bonding network rather than by a direct hydrogen-bonding interaction.

The unusually high stability of the low spin state of CYP119 and the architecture of the active site defined by Thr-213 and Thr-214 are not linked to the thermostability of the enzyme. Thus, despite the range of spin state compositions and the degree of active site steric crowding represented by the mutants, the melting temperatures of the proteins varied by no more than 2–3 °C (Table IV). Although the stability of the hexacoordinate, low spin state is apparently not structurally important for the thermostability of the protein, it could still be important in preventing oxidative degradation of the protein under the high temperature conditions in which it is normally expressed. The low spin state, as shown below, disfavors electron transfer to the iron and the subsequent activation of molecular oxygen, reactions that are deleterious in the absence of a bound substrate. It is possible that the low to high spin shift is normally facilitated by specific interactions with either the native electron donor or the endogenous substrate, so that catalysis only occurs under optimal conditions.

In the absence of the endogenous redox partners for CYP119, we have examined the catalytic activity of the enzyme with styrene as the substrate and H2O2 as a surrogate donor of oxidizing equivalents. Under these conditions, CYP119 catalyzes the enantioselective oxidation of styrene to styrene oxide (Fig. 3A), giving a 25:75 ratio of the R- and S-enantiomers of the epoxide. Furthermore, the enzyme oxidizes cis-β-methylstyrene exclusively to cis-β-methylstyrene oxide (Fig. 3B) and cis-stilbene to cis-stilbene oxide (not shown). No trace was found of the trans-isomers expected from a non-P450-like epoxidation mechanism. The fact that the oxidation is enantioselective and proceeds with complete retention of the olefin stereochemistry confirms that it occurs within the active site of the enzyme. This epoxidation of styrene and styrene analogues is the first catalytic activity identified for CYP119, although styrene is unlikely to be the natural substrate for the enzyme.

The catalytic oxidation of styrenes by CYP119 would appear to conflict with the observation that these substrates cause only a small shift in the spin state of the enzyme, particularly at the lower temperatures used for the catalytic studies (Table I). However, RR studies confirm that the binding of styrene causes a small shift in the spin state (Fig. 6). Furthermore, 1H NMR relaxation studies demonstrate that styrene is bound in the active site of the enzyme with the styrene protons at a distance of approximately 6.4 Å from the heme iron atom (Table II). Although the binding of the substrate may alter as the enzyme traverses the intermediates required to generate the reactive oxygen species, it is clear that styrene binds in the active site although it does not greatly alter the spin state.

Altering the CYP119 spin state equilibrium and the active site environment by mutating Thr-213 and Thr-214 alters both the stability of the heme toward degradation by H2O2 and the styrene epoxidation activity of the enzyme. In all of the mutants, the heme chromophore is bleached more slowly by H2O2 than it is in the wild-type enzyme. The mutants with large side chains at position 213 are most resistant toward bleaching, but they are also catalytically the least active. Thus, mutation of Thr-213 to a large, non-hydrogen-bonding residue (i.e. valine, tryptophan, or phenylalanine) greatly diminishes the H2O2-dependent epoxidation activity of the enzyme. Even replacement of Thr-213 with a serine, a mutation that increases the high spin state and decreases the active site steric encumbrance, decreases the rate of styrene epoxidation (Table IV). In contrast, a higher epoxidation activity is observed when Thr-213 is replaced by a non-hydrogen-bonding alanine residue in both the single and double mutants. However, one cannot conclude from this that Thr-213 is not important for activation of the ferric peroxide complex, because the role of Thr-213 could be fulfilled by a water molecule in the mutants with smaller side chains. The increased rates of reaction with the aryl diazene probes and the changes in the resulting aryl shift patterns indicate that the smaller side chains decrease steric encumbrance in the vicinity of the iron atom. This decrease in steric interference provides space for additional water molecules and could also help to compensate for a decreased rate of peroxide activation by increasing access of both the peroxide and the substrate to the active site iron. Accessibility of the active site to H2O2, rather than the spin state of the enzyme, appears to be the crucial determinant of the H2O2-dependent activity of the enzyme.

A clearer picture of the catalytic roles of Thr-213 and Thr-214 emerges from studies of the Pd-, PdR-, and NADH-dependent epoxidation of styrene by CYP119 mutants. The native electron donor partner for CYP119 is not known, and efforts to identify alternative electron donor partners among the proteins that support other P450 systems have not been successful. However, even if wild-type CYP119 does not detectably oxidize styrene with Pd and PdR as electron donors, the same is not true of some of the mutants. The T213S mutant, which has an increased high spin component (Table I) and a higher H2O2-dependent activity (Table IV), also does not detectably catalyze styrene epoxidation using Pd and PdR. However, both the T214A and T214V mutants are able to epoxidize styrene using Pd and PdR as electron transfer partners (Table V). The ability of these CYP119 mutants to accept electrons from Pd and PdR is consistent with the fact that in the native P450cam system the change in redox potential that accompanies the low to high spin shift is necessary for efficient electron transfer (46). The T213A/T214A double mutant, in contrast, does not retain the ability to utilize Pd and PdR to epoxidize styrene, confirming that the conserved Thr-213 residue is important for formation of the oxidizing species. The Thr-213 results are in keeping with previous results on P450cam (47). The results also establish that Thr-214 has a major role in controlling the spin state of the enzyme but is not catalytically essential. These results and the evidence obtained from the aryl diazene reactions strongly suggest that Thr-214 influences the spin state and the catalytic activity indirectly, probably as the result of a hydrogen-bonding network that extends some distance away from the iron atom.

In summary, CYP119 is a highly thermostable P450 enzyme. Thr-213, which aligns with the conserved threonine in other P450 enzymes, is close to the heme iron atom as shown by the changes in the spin state, the catalysis of styrene epoxidation, the rates of reaction with aryl diazenes, and the regioisomer patterns obtained from those reactions when Thr-213 is mutated. Thr-214 strongly influences the CYP119 spin state but is at some distance from the iron. As a result, Thr-214 mutants are able to support styrene epoxidation by Pd and PdR, an activity that is lost when Thr-213 is also mutated. These results suggest that Thr-213 interacts with the distal water ligand and functions as a catalytic residue, whereas Thr-214 indirectly influences the protein spin state through a hydrogen-bonding network. Significantly, the data indicate that the thermostability of CYP119 does not depend critically on the spin state or the structural features of the active site represented by Thr-213 and Thr-214. This is important because it implies that site-directed mutagenesis may be employed to change the substrate specificity of CYP119 without altering its thermostability, a critical requirement for the development of novel CYP119-based catalysts through protein engineering.
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