Endogenous Cysteine Ligation in Ferric and Ferrous Cytochrome P-450

DIRECT EVIDENCE FROM X-RAY ABSORPTION SPECTROSCOPY*

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Extended x-ray absorption fine structure spectroscopy has been applied to the elucidation of the structure of the heme iron site of bacterial cytochrome P-450. The low spin ferric, high spin ferric, ferrous, and ferrous carbonyl states of the enzyme have been examined. Curve-fitting analysis of the data provides direct and compelling evidence for the presence of a sulfur atom in the first coordination sphere of the iron. The iron-nitrogen (porphyrin) distances indicate five coordination in high spin ferric and ferrous P-450 and six coordination in low spin ferric and ferrous carbonyl P-450. The iron-sulfur distances are consistent with thiolate ligation, presumably from cysteinate, in all four states of the enzyme. In each case, the iron-sulfur bond distance is equal to or shorter than the analogous Fe-S bonds in model iron porphyrin thiolate complexes whose crystal structures have been determined. Since known thiol-sulfur:iron-heme bond distances are noticeably longer than the corresponding thiolate bonds, the X-ray absorption fine structure results strongly suggest that, in each P-450 state examined, the sulfur donor is a thiolate. The results reported in this paper concerning the ligand identity, state of protonation, and metal-ligand bond distances are of critical importance to a complete description of the P-450 reaction cycle and its mechanism of oxygen activation.

The mechanism of activation of molecular oxygen for insertion into organic molecules is a problem of fundamental significance. Despite the vital importance of oxidative processes, our ability to direct the incorporation of one or both atoms of dioxygen into a particular substrate remains extremely limited. This lack of expertise is not shared by nature; indeed, the oxidation of organic compounds is a ubiquitous biochemical reaction (2). Numerous enzyme systems have evolved to carry out this process, utilizing a variety of biological oxidants. These enzymes include the mono- and dioxygenases (3, 4) and the peroxidases and catalases (5, 6). The cytochromes P-450 are monooxygenase enzymes that have been isolated from a wide variety of sources (7-9). Mammalian P-450 is a ubiquitous membrane-bound heme iron enzyme that catalyzes the hydroxylation of membrane-trapped nonpolar molecules as well as the activation of environmental carcinogens (10, 11). Despite extensive study, the detailed metal ion coordination structure of the active site of P-450 remains a matter of controversy (12). To provide additional structural evidence, as a basis for describing the mechanism of oxygen insertion and, in particular, to determine what changes occur at the iron center during catalysis, we have undertaken a structural study of the ferric and ferrous states of P-450 utilizing EXAFS spectroscopy.

Cytochrome P-450 enzymes effect the hydroxylation and epoxidation of steroids, drugs, and xenobiotics using molecular oxygen as the source of the incorporated oxygen atom while reducing the remaining oxygen atom to water. As shown in Fig. 1, four isolable states (1-4) have been characterized in the P-450 catalytic cycle. The low spin, substrate-free, six-coordinate ferric form (1) becomes high-spin, five-coordinate (2) upon binding substrate. Addition of one electron produces the high spin, five-coordinate ferrous enzyme (3) which can then bind molecular oxygen to give oxy-P-450 (4). One-electron reduction of this enzyme-oxygen-substrate ternary complex leads to product formation and regeneration of the low spin, ferric resting state (1). No intermediates have been isolated between 4 and 1, although high valent metal-oxo species have been proposed as possible intermediates (4, 7-9, 13). Addition of carbon monoxide to ferrous P-450 gives a stable, low spin ferrous carbonyl adduct (5) for which the Soret absorption maximum has shifted to approximately 450 nm.

A question of considerable importance in understanding the catalytic activity of P-450 is the identity of the nonporphyrin, axial ligand(s) to the heme iron. Of particular interest are the changes in ligand identity or bonding that occur during the...
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reaction cycle. A variety of spectroscopic tools have been applied to elucidate the structures of the different states of P-450 and a great deal is now known about this enzyme, especially about states 1, 2, and the nonphysiological state 5. Spectral comparisons, primarily using UV-visible absorption, magnetic circular dichroism, and EPR, between the ferric states of P-450 and either synthetic model porphyrin systems or ligand adducts of myoglobin, have provided convincing evidence of thiolate ligation in both of the ferric P-450 states (15-16). The low spin, ferric enzyme (1) contains an additional ligand trans to the thiolate. The identity of this ligand is unclear; recent evidence from several laboratories suggests that it is an oxygen donor such as an alcohol-containing amino acid or water (16-18). Our earlier EXAFS study of mammalian liver P-450 demonstrated that a sulfur donor is one of the axial ligands in the low spin, ferric enzyme with an iron-sulfur bond distance of ~2.2 Å (19). The same study showed that the high spin, ferric form of a related heme iron enzyme, chloroperoxidase, also contains a sulfur atom at about 2.3 Å from the iron.

Similarly, by comparison of the spectroscopic properties of the ferrous carbonyl adduct (5) with those of model porphyrin complexes, state 5 has been convincingly shown to contain an axial thiolate ligand trans to carbon monoxide (15, 20, 21). The ligand assignments for ferrous, 3, and oxygenated, 4, P-450 are much less certain. The optical properties of five-coordinate porphyrin-thiolate complexes designed as models for high spin, ferrous P-450 arg that the cysteine ligand remains deprotonated upon reduction (20). Further support for this view comes from the overall similarity of the unpublished magnetic circular dichroism spectrum of a ferrous porphyrin-thiolate complex to that of ferrous P-450 (22). On the other hand, single crystal polarized absorption spectra, together with iterative extended Hückel calculations, point toward an axial thiol (23, 24). Addition of oxygen to ferrous P-450 (3) leads to formation of a semistable oxygen complex (4) (25, 26). Differences between the spectral properties of oxy-P-450 (4) and oxymyoglobin (or oxyhemoglobin) rule out axial histidine ligation trans to dioxygen (27). Thiol (23) or thiolate (28-31) ligation are the most likely candidates for this ligand. EXAFS spectroscopy has proven to be of particular utility in determining the ligand coordination structure of metalloenzymes (32, 33). Analysis of the EXAFS can identify the number, distance, and identity of the ligands surrounding the x-ray absorbing species. Absorber-ligand distances can, in favorable circumstances, be determined to an accuracy of 0.02 Å or better. EXAFS has been used successfully to determine metal environments in azurin (34), nitrogenase (35, 36), hemocyanin (37, 38), rubredoxin (39, 40), ferredoxin (39), xanthine oxidase (41), sulfite oxidase (42), and cytochrome c oxidase (43, 44). We have previously used EXAFS to examine the resting state (1) of mammalian liver P-450 (19) and have reported preliminary results with P-450/CA 18 (10).

We present here EXAFS studies of bacterial P-450-CAM isolated from Pseudomonas putida grown on camphor as the sole carbon source. We have studied the low spin ferrous (1), the high spin ferric (2), the high spin ferrous (3), and the ferrous carbonyl (5) states of P-450. This work directly confirms the presence of a sulfur donor atom in all four states, presumably arising from cysteinyl ligation. In addition, the Fe(Nporphyrin) and Fe-(axial) bond length determinations support the assignment of the iron coordination number and of thiolate ligation, respectively. The details of these results provide valuable quantitative information upon which to base mechanistic interpretations of the P-450 catalytic cycle.

EXPERIMENTAL PROCEDURES

Sample Preparation—The iron porphyrin model compounds (see Table I) were prepared according to literature methods. The samples were ground and diluted with dry boron nitride or LiBF 4 , and then pressed into pellets held inside an aluminum spacer. All sample preparation for the ferrous porphyrins was performed in an inert atmosphere box. The anaerobic samples were sealed inside the spacer with Mylar tape and the sample-spacer-Mylar assembly was sealed in an air-tight Lucite box containing Mylar windows. During irradiation, the anaerobic samples were continuously flushed with helium. After irradiation, air-sensitive samples were examined spectrophotometrically to ensure that oxidation, oxidation, or degradation had not occurred.

Cytochrome P-450-CAM was purified from P. putida grown on d-camphor as reported elsewhere (17). A typical preparation of the camphor-bound form with the ammonium form of a particulate enzyme, chloride, or protein diluted with buffer (anaerobic when appropriate). In no case was there any loss of spectral integrity throughout the 350-700-nm range. Given the sensitivity of P-450 to conversion to P-420 and/or auto-oxidation when reduced, the above results indicate that each protein sample was in the desired form throughout the period of spectral examination.

Data Collection and Reduction—The protein x-ray absorption data were collected at the Stanford Synchrotron Radiation Laboratory using the focused beam line (beam line II-3) during dedicated running conditions. The radiation was monochromatized using a double crystal monochromator with Si(111) crystals. The energy scale was calibrated using an iron foil absorption spectrum, with the first inflection point of the foil spectrum defined as 7111.2 eV. The data were collected as fluorescence excitation spectra using Fe-Ka fluorescence (46). The sample was placed in the beam at a 45° angle and the fluorescence was measured perpendicular to the beam using an array of five NaI scintillation detectors.

$^1$ J. H. Dawson, T. N. Sorrell, and J. P. Collman, unpublished results.

$^4$ Complete details of bacterial growth and protein purification procedures were reported by L. A. Anderson, Ph.D. thesis, submitted to the University of South Carolina (1982).
The spectra presented here are averages of 12-24 25-min scans. Data were collected on one sample of the low spin ferric enzyme (21 scans), one sample of the high spin ferric enzyme (22 scans), one sample of the ferrous carbonyl enzyme (24 scans), and two samples of the ferrous enzyme (14 and 12 scans).

Every fluorescence channel of each scan was examined visually for glitches before being included in the average. This examination revealed that certain fluorescence detectors were much more sensitive to noise and glitches than were others. Indeed, three of the fluorescence channels were judged too noisy to be used at all. In general, the closer that a fluorescence detector was to being perpendicular to the x-ray beam, the less sensitive it was to noise and glitches. The data presented here represent either the best fluorescence channel or the average of the two best channels, weighted by counting statistics (47).

After averaging, substantial data reduction was necessary to obtain the EXAFS. The EXAFS \( \chi(k) \) is defined as the modulation in the absorption coefficient \( \mu \), given by \( \chi = (\mu - \mu_0)/\mu_0 \) where \( \mu_0 \) is the absorption that would be observed in the absence of interference (EXAFS) effects. Data reduction was performed as previously described in detail (48, 49). In brief, a polynomial was fit to the pre-edge region (approximately 6600-7100 eV) and then subtracted from the data. The resulting data is the absorption, \( \mu_0 \), of the iron corrected for the background absorption of the other atoms in the sample. A two-region cubic polynomial spline was then fit to \( \mu_0 \) above the edge. This spline gave \( \mu_0 \), the smoothly varying part of the absorption which is approximately equal to \( \mu_0 \) above the edge. The spline was subtracted from the observed data and the data were normalized to a per iron basis. The normalization factor, \( \mu_{norm} \), was an approximation of \( \mu_0 \) obtained by a linear interpolation between the measured absorption at the assumed is electron binding energy, \( E_0 \), of 7130 eV and an assumed falloff to 0.75 of this value at 1000 eV above \( E_0 \). Normalization gave \( (\mu - \mu_0)/\mu_0 \), which is an approximation of \( (\mu - \mu_0)/\mu_0 \).

The data were converted to \( k \) space using an \( E_0 \) of 7130 eV. \( E_0 \) represents the threshold energy for liberating a core electron. In principle, \( E_0 \) changes among compounds depending on the oxidation state and the ligation of the absorbing atom. In practice, \( E_0 \) correlates highly with the absorber-scatterer distance, \( R \), hindering attempts to simultaneously determine \( E_0 \) and \( R \). Extensive experience with iron porphyrin EXAFS has shown that the assumption of a constant \( E_0 \) does not introduce a detectable inaccuracy in the determination of \( R \), as long as the same \( E_0 \) value is used for all compounds. The EXAFS data for the four P-450 samples, plotted as a function of \( k \), are shown in Fig. 2.

RESULTS AND DISCUSSION

Data Analysis—Theoretically, the EXAFS \( \chi(k) \), in terms of calculable physical quantities and simplified with a number of approximations, is

\[
\chi(k) = \frac{1}{k} \sum_{N_i} \frac{[f(k, R) \sin(2kR + \alpha(k))]}{R^2} e^{-2kR} e^{i \delta}
\]

where \( N_i \) is the number of scatterers (s) of the same element at the same distance \( R_{ij} \) from the absorber (a), \( [f(k, R) \sin(2kR + \alpha(k))] \) is the scatterer's electron back-scattering amplitude function, \( \alpha(k) \) is the k-dependent total phase shift, and \( \delta \) is the mean square deviation of \( R_{ij} \) (50-54). Due to other physical processes, this expression breaks down at low values of \( k \). It also is incorrect for scatterers outside the first coordination shell because its derivation fails to account for multiple scattering effects, which change the phase shift and amplitude as a function of the intervening first shell atoms (55).

Initial examination of the data (Fig. 2) reveals a complex pattern, with "beats" in the amplitude occurring as a maximum at \( k = 6 \text{ Å}^{-1} \) and a minimum at \( k = 9 \text{ Å}^{-1} \). Beats are the result of the interference between the frequencies from different ligands at different distances from the absorber. We thus recognize immediately that there are several different shells of atoms contributing to the Fe EXAFS. Unfortunately, the beat pattern reveals only this qualitative information about the structure of the iron site.

The photoelectron wave vector \( k \) is defined by \( k = \sqrt{2m(\varepsilon - E_0)/\hbar} \).

More detailed information can be obtained from the Fourier transform of the EXAFS data. It is readily shown that the Fourier transform of the EXAFS yields the phase-shifted radial distribution function around the absorbing atom (50). The Fourier transform of the ferric low spin data is shown in Fig. 3. The peaks represent Fe-ligand distances, offset by an atom-dependent phase shift of approximately 0.4 Å. The most striking feature of this transform is that contributions are observed from atoms as far as 4.2 Å (peak at 3.8 Å) away from the iron. This is quite different from the EXAFS observed for simple inorganic complexes, where damping effects frequently "wash out" the EXAFS from atoms beyond the first coordination sphere. The observation of such extended structure for P-450 is a reflection of the relative rigidity and high degree of structural order of the porphyrin group. The general appearance of this transform is typical of metalloporphyrin EXAFS.

The transform also reveals the low resolution of the data. The three major transform peaks are due to the pyrrole nitrogens and the axial ligands (1.7 Å ± 0.4 Å = 2.1 Å), the pyrrole a-carbons and the porphyrin mesocarbons (2.8 ± 0.4 ± 3.2 Å), and the pyrrole β-carbons (3.8 ± 0.4 ± 4.2 Å). The minor peak at approximately 3.3 Å is due to the overlap of the mesocarbon and β-carbon peaks. The transform thus shows that the a- and mesocarbons, known to be separated by approximately 0.4 Å, are not resolved in these data. Resolution is limited by the range of the data in \( k \) space, which, due to the glitches in our data, was 12.5-13 Å. Clearly, the transforms alone do not allow determination of the nature of the axial ligands since the contributions from the axial ligands and from the porphyrin nitrogens are not resolved.

The most sensitive technique for analyzing EXAFS data is curve fitting. In this procedure, the overall EXAFS is modeled as a sum of sine waves, where each sine wave represents the contribution from a single absorber-scatterer pair. In practice, all atoms of the same type located at approximately the same distance from the absorber are grouped together in one "shell." The contribution from a given shell can either be calculated from first principles (56) or can be determined empirically. The \textit{ab initio} method uses four adjustable parameters per shell and gives correspondingly good fits to the data.

See Ref. 19 for a detailed discussion of Fe-porphyrin Fourier transforms.
which are combined and then summed over each wave to give a description of the total EXAFS

\[ \chi(k) = \sum_{i} N_i(c_i)e^{-\alpha_i k^2} \sin(\alpha_i + (\alpha_i + 2R_{\alpha}k) + \alpha_i) \]

The parameters \( c_i, c_1, c_2, a_0, a_1, \) and \( a_2 \) were all allowed to vary for the model compounds. These parameters were then fixed for all other refinements while \( N \) and \( R_{\alpha} \) were allowed to vary. Alternately, when fitting data in which \( N \) was known, \( N \) could be fixed at the correct value while \( c_1 \), which resembles a Debye-Waller type factor, was varied. The change in \( c_1 \) corresponds to the difference in Debye-Waller factor between the original model compound and the compound under study.

Parameters were derived in this way, using Fe(III)(TPP)(imidazole)Cl as a model for Fe-N parameters, Fe(II)(TPP) as a model for Fe-C(a) parameters and Fe-C(meso) parameters, Fe(diethylidithiocarbamate) as a model for Fe-S parameters, Fe(acetylacetonate) as a model for Fe-O parameters, and HFe(CO)₄(bis(triphenylphosphine)iminium cation) as a model for Fe-C(CO) and Fe-O(CO) parameters. We have performed numerous tests of the reliability of these parameters (19). Recently, we have recollected the data for some of the Fe porphyrin models that were studied previously (19). By comparing the best fits obtained for several different data sets representing the same compound, we found that the reproducibility of the data among data sets is approximately 0.02 Å and 25% for the coordination number determination. The best fits to a number of model compounds are shown in Table I and in Table I of Ref. 19. The data in Ref. 19 give a measure of the accuracy of the EXAFS results. Table I includes the EXAFS results for different data sets of the same compound and thus gives a measure of the precision of the EXAFS measurements. These tests also revealed that attempts to vary the Debye-Waller factor with a fixed \( N \) reduced the reliability of the distance determination. This correlation occurs because the shape of the amplitude envelope changes as \( c_1 \) is changed.

All curve-fitting was based on a least-squares minimization (49) using \( k^2 \)-weighted data. Fourier filtering was used to isolate the first shell (pyrrole nitrogens and axial ligand(s)) or the second shell (porphyrin \( \alpha \)- and mesocarbons) or the first two shells. The typical filter limits are indicated by bars on the transform for low spin ferric P-450 (Fig. 3). Fits were also performed on the unfiltered P-450 data. The curve-fitting results for low spin ferric P-450 are reported in Fig. 4; the results for all states of P-450 examined are collected in Table II.

The first goal of the curve fitting for the P-450 data was to determine whether a sulfur atom was in the Fe coordination sphere. This was accomplished by comparing the goodness of fit, \( F \), obtained by fitting the data with either nitrogen alone, nitrogen and a non-sulfur ligand, and nitrogen and sulfur. The curve-fitting results for Fourier-filtered first shell data fit with

\[ F = \sqrt{k^2 (\text{data-fit})^2 / \text{number of points.}} \]
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TABLE I

Best fits for model porphyrin EXAFS

The reported EXAFS results are the best fit to filtered first shell data. Similar results were obtained by fitting the unfiltered data (see text). Multiple entries for \((\mu\)-oxo Fe(II)TPP and Fe(II)TPP represent the best fits to different data sets.

| Compound          | \(R_s^a\) | \(n_s^a\) | \(R_c^e\) | \(n_c^e\) |
|-------------------|-----------|-----------|-----------|-----------|
| \(\mu\)-oxo       | 2.06      | 3.7       | 1.74      | 1.3       |
| Fe(II)TPP         | 2.06      | 4.0       | 1.73      | 1.6       |
| Fe(II)TPP         | 2.08      | 3.4       | 1.76      | 1.3       |
| Fe(II)TPP         | 1.96\(^f\) | 4.4\(^f\) | 1.76\(^f\) | 0.9\(^f\) |
| Fe(III)TPP(C1)    | 2.04      | 3.3       |           |           |
| Fe(III)(TpivPP)\(\cdot\)(2MeIm)\(^f\) | 2.09      | 4.4       | 2.2       | 1.0       |

\(^a\)The Fe-N(porphyrin) distance.
\(^b\)The number of nitrogens (N) determined to be present at the distance indicated.
\(^c\)The Fe-X(axial) distance.
\(^d\)The number of atoms (X) determined to be present at the distance indicated.

compared with these numbers, providing direct evidence that P-450 tend to be longer than those interpreted in the P-450 bond distances and numbers are discussed below.

**Comparison with Model Structures**—Relatively few compounds that directly model the P-450 active site have been structurally characterized. However, the structural properties of iron porphyrins in general are very well known (57, 58). Typically, low spin, six-coordinate, ferric porphyrins have Fe-N(porphyrin) distances of about 1.99 \(\text{Å}\) while high spin, five-coordinate, ferric porphyrins have Fe-N(porphyrin) distances of approximately 2.065 \(\text{Å}\). The corresponding Fe-N(porphyrin) distances for ferrous porphyrins are approximately 0.02 \(\text{Å}\) longer. The EXAFS Fe-N(porphyrin) distances are consistent with these numbers, providing direct evidence that P-450 states 1 and 5 contain six-coordinate iron while 2 and 3 have five-coordinate iron. The conversion of the P-450 heme iron from six to five coordination upon substrate addition is important in order to provide a vacant coordination site for ligation of dioxygen and in order to increase the reduction potential (9).

There are only three ferric porphyrin thiolate complexes that have been structurally characterized in detail. Fe(III)(TPP)(SC\(\text{CH}_2\)H\(_2\))\(_2\) anion has an Fe-S distance of 2.336 \(\text{Å}\) (59). This is longer than the EXAFS Fe-S distance we found for 1, 2.22 \(\text{Å}\). However, Fe-S bonds \(\text{trans}\) to a thiolate tend to be longer than those \(\text{trans}\) to other ligands. For example, low spin ferric tris(diethylidithiocarbamate), with \(\text{trans}\) thiolates, has an Fe-S bond of 2.31 \(\text{Å}\) (60) while Fe(III)(SC\(\text{CH}_3\)CHN\(\text{CH}_2\)NH\(_2\))\(_2\), in which the thiolate is \(\text{trans}\) to an amine, has an Fe-S distance of 2.21 \(\text{Å}\) (61). The complex Fe(III)(TPP)(HSC\(\text{CH}_3\))\(_2\)(SC\(\text{CH}_3\)) exists as a distorted structure containing a mixture of a six-coordinate thiolate-thiol adducts and five-coordinate thiolate adducts (62). The thiolate-thiol adduct, a possible model for 1, has an Fe-S(thiolate) bond length of 2.27 \(\text{Å}\). The five-coordinate thiolate adduct has an Fe-S bond length of 2.32 \(\text{Å}\), identical with the Fe-S bond length found in Fe(III)(PPIXDMB)(SC\(\text{H}_2\)N\(_2\))\(_2\)(14), the third structurally characterized ferric porphyrin thiolate.

If the thiolate-thiol adduct is assumed to be a good model for 1 and the five-coordinate para-nitrobenzenethiolate complex is assumed to be a good model for 2, then the EXAFS Fe-S distances for P-450 states 1 and 2 are 0.05–0.1 \(\text{Å}\) shorter than expected. A possible explanation for this apparent discrepancy is that the model \(\text{aryl}\) thiolate porphyrins are not representative of the Fe-S ligation (presumably \(\text{alkyl}\) thiolate).
### Table II

| Structural details for P-450-CAM and relevant porphyrin model compounds |
|---|---|---|---|---|
| | Fe-N(porphyrin) | Fe-S(axial) | Fe-C(CO) | Fe-O(CO) | Reference |
| | R | n° | R | n° | R | n° | R | n° |
| Low spin Fe(III) | | | | | | | | |
| 1 | 2.00 | 5.0 | 2.22 | 0.6 | This work |
| (mammalian P-450) | 2.00 | 4.8 | 2.19 | 0.8 | 19 |
| Fe(TPP)(SC_6H_5)_2b | 2.008 | 4 | 2.336 | 1 | 59 |
| Fe(TPP)(HSC_6H_5)- (SC_6H_5)_2b | Not reported | 2.27° | 1 | 62 |
| High spin Fe(III) | | | | | | | | |
| 2 | 2.06 | 5.2 | 2.23 | 0.8 | This work |
| Chloroperoxidase | 2.05 | 4.2 | 2.30 | 0.9 | 19 |
| Fe(PPIXDME)- (SC_6H_4NO_2)_b | 2.064 | 4 | 2.324 | 1 | 14 |
| High spin Fe(II) | | | | | | | | |
| 3 | 2.06 | 3.0 | 2.34-2.38 | 0.6 | This work |
| Fe(TPP)(SC_6H_5)_2b | 2.064 | 4 | 2.360 | 1 | 63 |
| Low spin Fe(II) | | | | | | | | |
| 4 | 1.98 | 3.3 | 2.32 | 1.0 | 1.72 | 1.4 | 2.90 | 0.5 | 63 |
| Fe(TPP)(SC_6H_5)- (CO)_b | 1.993 | 4 | 2.362 | 1 | 1.78 | 1 | 2.95 | 1 | 63 |

* The number (n) of ligands at the distance indicated.

b Data from crystal structure determination.

The EXAFS results were obtained from curve fitting either the unfiltered data or the appropriately filtered (first or second shell) data. Only for reduced P-450 did the filtered and unfiltered fits give different results (see text). Fits to the unfiltered data and to the second shell data also included a C(α, meso) wave (see text). Porphyrin ruffling and doming result in this metal to porphyrin-carbon distance being only weakly dependent on the metal geometry, and uninformative of P-450 structure.

### Table III

| Curve-fitting results for P-450-CAM using various first shell ligands |
|---|---|---|---|---|
| | Fe-N | Fe-S | Fe-O | Fe-C(CO) |
| | R | n° | R | n° | R | n° |
| Low spin ferric | | | | | | | | |
| (1) | 2.01 | 5.9 | 2.00 | 6.0 | 2.00 | 4.9 | 2.22 | 0.6 | 0.634 |
| High spin ferric | | | | | | | | |
| (2) | 2.06 | 5.2 | 2.07 | 5.4 | 2.05 | 5.7 | 2.24 | 0.7 | 0.707 |
| Ferrous | | | | | | | | |
| (3) | 2.10 | 3.3 | 2.08 | 3.0 | 2.08 | 3.3 | 2.34 | 0.6 | 0.690 |
| Ferrous carbonyl | | | | | | | | |
| (4) | 2.01 | 2.5 | 1.99 | 2.1 | 2.00 | 3.2 | 2.34 | 1.0 | 1.68 | 1.1 | 1.10 |

* The number (n) of ligands at the distance indicated.

b Number of N atoms fixed at 3.0 (as found for N + S fit). If the number of N atoms and the number of O atoms were both variable, the fit refines to the chemically unreasonable value of three nitrogen atoms at 2.17 Å and four oxygen atoms at 1.98 Å.

in P-450. For example, Holm et al. (14) have observed that the Fe-S bond length in Fe(II)(PPIXDME)(SC_6H_4NO_2)_b is longer than the distances found in nonporphyrin high spin ferric thiolate complexes, and is 0.07 Å longer than would be predicted from the difference in radii and the Fe-Cl distance in Fe(III)(TPP)Cl. It is possible that the crystallographically characterized Fe(III)-SR models (all having R = aryl) have longer Fe-S bond lengths than would be found for alkyl thiolates. The lack of additional models for ferric thiolate porphyrins (with R = alkyl) precludes any further interpretation of the P-450 Fe-S bond length. The EXAFS results do, however, provide unambiguous proof of thiolate ligation in P-450 states 1 and 2 since a coordinated thiol would undoubtedly have a bond length longer than 2.32 Å. Fe(III)(TPP)(HSC_6H_5)(SC_6H_5)_2 has an Fe-S(thiol) distance of 2.43 Å (62).

Recent spectroscopic studies suggest that the sixth ligand in 1 is an oxygen atom, possibly from an alcohol or from water (16-18). Since O and N are indistinguishable via EXAFS, the sixth ligand is included with the porphyrin nitrogens, resulting in an overall N coordination number of five for 1. This coordination number should decrease to four nitrogens in 2. The fact that it does not indicates a decreased Debye-Waller factor in 2 relative to 1. Ideally, the Debye-Waller factor...
would also be a variable parameter in the curve-fitting, but the large number of correlated parameters and the short range of the data preclude this option. This inflexibility contributes to the 25% estimated error in coordination number determination.

The Fe-N bond lengths found in ferrous P-450 (3), relative to those found in the high spin ferric state (2), are consistent with the bond lengthening expected upon one-electron reduction of a ferric porphyrin. As shown in Table II, the EXAFS results for 3 agree well with the structure of the model Fe(II)(TPP)(SC6H5) anion (63). The lower quality of the EXAFS data and the resulting uncertainty in the Fe-S bond length of 3 (as discussed above) hinder the interpretation of the nature of the sulfur ligation (e.g. thiol versus thiolate).

Moreover, there are no adequate models of high spin ferrous porphyrin thiol ligation. However, based on the observed 0.094 lengthening of the Fe(III)-S distance on changing a thiolate ligand (59) to a thiol (62) and on the close similarity of the Fe-S distance determined for 3 and reported for the Fe(II)(TPP)(SC6H5) anion, the EXAFS data for ferrous P-450 (4) are quite consistent with thiolate ligation. The 0.04 Å Fe-S bond lengthening between P-450 states 5 and 3 (Table II) is expected as a result of the occupation of the dπ orbital on going from the low spin 5 to the high spin 3 (58). However, in the absence of appropriate thiol models, these data must remain suggestive, rather than conclusive, evidence for thiolate ligation in ferrous P-450.

Ferrous carbonyl P-450 (5) should be well modeled by the Fe(II)(TPP)(SC6H5)(CO) anion synthesized and structurally characterized by Caron et al. (63). Indeed, our EXAFS measurements for 5 are in excellent agreement with the bond lengths found in this complex (see Table II). The Fe-C(CO) bond length, 1.72 Å, is slightly (~0.06 Å) shorter than expected, but is still within the range of bond lengths found in similar ferrous carbonyl adducts (1.706–1.779 Å) (64–67). This somewhat short Fe-C(CO) bond may indicate stronger than usual carbonyl bonding. As in the oxidized enzyme, the presence of a sulfur donor atom is confirmed for 5. Although there are no structurally characterized low spin ferrous porphyrin thiol complexes, such an Fe-thiol bond should be at least as long as the 2.43 Å Fe-thiol bond in low spin Fe(III)(TPP)(HSC6H5)(SC6H5) (62). Hence, the EXAFS bond length of 2.32 Å strongly supports the spectroscopic assignment of thiolate ligation in P-450 state 5. The observed coordination numbers for 5 agree with the proposed structure within experimental error. The Fe-C(CO) coordination number is low, possibly as a result of slight nonlinearity of the Fe-C-O linkage, and a subsequent reduction in the “focusing effect” (55). However, such an interpretation is at best speculative due to the large number of shells being fit and the limited range of the data.

The presence of thiolate, presumably cysteinate, ligation to the heme iron of P-450, which is directly demonstrated in this work, has significance for the enzyme’s catalytic activity. It has previously been suggested (13) that such thiolate ligation in P-450 results in an unusually electron-rich ferric and ferrous iron, as compared to the iron in the histidine-ligated, dioxygen transport proteins myoglobin and hemoglobin. This unusual electron density at the heme iron of P-450 has recently been quantitatively verified in comparative studies with myoglobin (68, 69). Since P-450 must not only bind dioxygen, but also activate it for insertion into substrate molecules, the electronic structure of the active site iron is a critical feature of the catalytic process. High valent metal-oxo species have frequently been suggested to be the ultimate catalytically active agent formed after addition of the second electron and loss of water (4, 7–9, 13). An electron-rich thiolate ligand may help stabilize the normally unfavorable oxidation state of iron in these species.

CONCLUSIONS

Based on our fits of the EXAFS model compounds and the fits reported in Ref. 19, we conclude that our curve-fitting techniques using empirical parameters derived from the analysis of structurally characterized compounds are able to determine the structure of Fe porphyrins to an accuracy of approximately 0.02 Å and ± 255 in coordination number. Using these techniques, we have conclusively shown the presence of a sulfur atom in the first coordination sphere of the iron in the low spin (1) and high spin (2) ferric and in the high spin (4) and carbonyl-bound (5) ferrous states of P-450. This constitutes the first direct observation of sulfur ligation in P-450-CAM and the first measurement of the Fe-S bond length in states 2, 3, and 5. The Fe-N(porphyrin) bond distances determined for the low spin ferric and ferrous forms (1 and 5) and for the high spin ferric and ferrous forms (2 and 3) provide further evidence for six and five coordination, respectively, of the heme iron. Analysis of the Fe-S bond lengths conclusively demonstrates thiolate ligation for 1, 2, and 5 and strongly suggests it for 3. In the absence of ferrous porphyrin thiol models, thiol ligation in ferrous P-450 (3) cannot be completely ruled out. We are currently undertaking an EXAFS investigation of ferrous thiol porphyrins in hopes of resolving this uncertainty. The structural results presented herein should prove extremely useful for future model and mechanistic studies of P-450.

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