Reflections having intensities greater than 3.0 times their standard deviation were used in the refinements. All calculations were performed on a VAX computer using SDP/VAX or a PDP-11 computer using SDP-PLUS.[31]

Molecular Dynamics. Molecular dynamics calculations were performed with the software Biograf (version 2.0) from Biodesign, Inc., on an Ardent Titan Computer.

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Registry No. **1**, 105810-22-8; **2**, 123641-32-7; **3**, 105810-21-7; **4**, 105810-24-0; **5**, 105821-52-1; **6**, 123641-33-8; **7**, 123641-34-9; **8**, 123641-35-0; **9**, 123641-36-1; **10**, 123674-06-6; **11**, 123641-37-2; **12**, 123674-07-7; **13**, 123641-38-3; **14**, 123641-39-4; **15**, 84191-23-1; H_2L^1, 123641-18-9; H_2L^2, 123641-19-0; H_2L^3, 121788-87-2; H_2L^4, 121788-88-3; H_2L^5, 123641-20-3; H_2L^6, 123674-04-4; H_2L^7, 123641-21-4; H_2L^8, 123641-22-5; H_2L^9, 123641-23-6; H_2L^{10}, 123674-05-5; H_2L^{11}, 123641-24-7; H_2L^{12}, 123641-40-7; H_2L^{13}, 123641-25-8; H_2L^{14}, 123641-26-9; H_2L^{15}, 123641-27-0; $MoO_2(acac)_2$, 17524-05-9; $[(n\text{-}Bu)_4N]_4Mo_8O_{26}$, 59054-50-1; CH_2O, 50-00-0; *N*-methylethylenediamine, 109-81-9; salicylaldehyde, 90-02-8; 3-*tert*-butylsalicylaldehyde, 24623-65-2; *N,N'*-dimethylethylenediamine, 110-70-3; *N,N'*-diethylethylenediamine, 111-74-0; thiosalicylic acid, 147-93-3; benzyl chloride, 100-44-7; *S*-benzylthiosalicylic acid, 1531-80-2; *S*-benzylthiosalicylic acid chloride, 1531-81-3; *N,N'*-bis(2-(benzylthio)benzoyl)-*N,N'*-dimethyl-1,2-diaminoethane, 123641-28-1; *N,N'*-bis(2-(benzylthio)benzyl)-*N,N'*-dimethyl-1,2-diaminoethane, 123641-29-2; *N*-(2-(benzylthio)benzyl)-*N,N'*-dimethyl-1,2-diaminoethane, 123641-30-5; *N*-(2-(benzylthio)-*N'*-(2-hydroxybenzyl)-*N,N'*-dimethyl-1,2-diaminoethane, 123641-31-6; 2-(aminomethyl)pyridine, 3731-51-9; 2-(2-aminoethyl)pyridine, 2706-56-1; 1-(2-aminoethyl)piperidine, 27578-60-5; 2-(ethylthio)ethylamine hydrochloride, 54303-30-9; glyoxal bis(2-hydroxyanil), 1149-16-2; propylene sulfide, 1072-43-1; hydroxylase, 9046-59-7.

Supplementary Material Available: Listings of positional parameters (Tables 3.1, 6.1, 8.1, 9.1, 10.1, 14.1, and 15.1), general temperature factors, *U*'s (Tables 3.2, 6.2, 8.2, 9.2, 10.2, 14.2, and 15.2), and additional bond distances and angles (Tables 3.3, 3.4, 6.3, 6.4, 8.3, 8.4, 9.3, 9.4, 10.3, 10.4, 14.3, 14.4, 15.3, and 15.4) (31 pages); listings of observed structure factors (Tables 3.5, 6.5, 8.5, 9.5, 10.5, 14.5, and 15.5) and unobserved structure factors (Tables 3.6, 6.6, 8.6, 9.6, 10.6, 14.6, and 15.6) (68 pages). Ordering information is given on any current masthead page.

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Influence of Thiolate Ligation on the Heme Electronic Structure in Microsomal Cytochrome P-450 and Model Compounds: Resonance Raman Spectroscopic Evidence

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Resonance Raman spectra are reported for rat liver microsomal (RLM3) cytochrome P-450 in oxidized, reduced, and CO-bound forms, and are compared with spectra of model compounds. Contrary to a previous report, thiolate coordination per se is sufficient to shift the ν_4 porphyrin mode frequency of Fe^{II} heme to 1341 cm^{-1}, from the normal 1357-cm^{-1} frequency when bound to imidazole in a five-coordinate complex. A corresponding downshift of ν_3 from 1471 to 1463 cm^{-1} is noted. Other bands can also be assigned on the basis of expected shifts upon increased back-bonding to the porphyrin ring. No additional protein influences are required to explain the RR spectra. The CO-bound and oxidized forms are likewise adequately modeled by protein-free complexes with thiolate ligation. In these cases the shifts relative to complexes with imidazole ligands are small. These differential frequency shifts are discussed in terms of the donor properties of the thiolate ligand, which are transmitted to the porphyrin ring for Fe^{II} heme, but are accommodated by the ferric ion of Fe^{III} heme and by the CO ligand in the Fe^{II}–CO adduct (as seen in the C–O and Fe–CO stretching frequencies). There is a striking difference between the thiolate influence on the porphyrin ground state of the CO adduct (minimal effect, small vibrational frequency shifts) and on its excited state (major perturbation resulting in a split Soret band with a low-energy 450-nm component).

Introduction

The cytochromes P-450 are ubiquitous heme enzymes that catalyze the insertion of one O atom from O_2 into organic substrates, the other O atom being reduced to water.[1] They have attracted widespread attention because of the importance of this reaction for numerous metabolic processes and because of its intrinsic chemical interest. The mechanism of the reaction has been worked out in considerable detail. It involves one-electron reduction of the Fe^{III} substrate-bound enzyme to the Fe^{II} state, which binds O_2. Addition of a second electron induces O—O heterolysis and generates a putative intermediate, analogous to the Fe^{IV}=O cation radical intermediate compound I of horseradish peroxidase,[2] which transfers the O atom to the substrate, regenerating the Fe^{III} enzyme. An important feature of the enzyme is that the proximal ligand of the heme Fe atom is the thiolate side chain of a cysteine residue. A variety of spectroscopic information about this ligand has been assembled,[3] and the coordination of Cys-357 to the heme Fe is evident in the X-ray crystal structure of the camphor-metabolizing enzyme P-450_{cam} in sub-

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strate-free[4] and bound[5] forms. The donor propensity of the thiolate ligand is thought to be an important factor in the O–O cleavage process and in the subsequent O atom transfer.

Resonance Raman spectroscopy has been fruitfully applied to various cytochromes P-450.[6-14] Indeed the definitive evidence for cysteine ligation of the heme came from the identification by Champion et al.[10] of the Fe–S stretching RR band, 351 cm^{-1}, in cytochrome P-450_{cam} isolated from *Pseudomonas putida* grown on $^{34}SO_4^{2-}$. Despite the numerous spectra that have been reported, the influence of the thiolate ligand on the porphyrin vibrational frequencies has remained uncertain. Although most of the porphyrin RR bands are at positions that are more or less expected by analogy with other heme proteins and analogues in corresponding heme spin and oxidation states,[15] it was early noticed[6-7] that the ν_4 band was at an anomalously low frequency, ~1345 cm^{-1}, in reduced cytochrome P-450 vs ~1357 cm^{-1} for high-spin Fe^{II} heme with an imidazole fifth ligand. Electron donation from the thiolate ligand out to the porphyrin ring was suggested to account for the frequency anomaly. Yet, Chottard et al.[16] have reported that the ν_4 frequency of five-coordinate iron(II) "picket-fence" porphyrin (TpivPP) is essentially the same, 1342–1345 cm^{-1}, whether the ligand is $C_6HF_4S^-$, $(CH_3)_3CS^-$, $C_6HF_4O^-$, OH^-, or Cl^-. They suggested that the frequency anomaly for cytochrome P-450 was due to an unspecified protein effect on the heme environment. In this study, we report RR spectra of cytochrome P-450 isolated from rat liver microsomes and compare them with spectra of selected model complexes. We show that thiolate coordination to Fe^{II} heme *does* induce the same ν_4 downshift as seen in the protein when the physiological porphyrin, protoporphyrin IX, is used for the comparison, and we report a corresponding downshift of ν_3. Other modes can also be assigned on the basis of shifts induced by back-donation to the porphyrin ring. On the other hand, there is very little influence of thiolate coordination on the porphyrin frequencies in the case of Fe^{III} heme or the Fe^{II} CO adduct. We discuss this difference in behavior in terms of different mechanisms for the accommodation of the electrons donated by the thiolate ligand. A preliminary report of some of these results has appeared previously.[17]

Experimental Section

Cytochrome P-450, form RLM3, was isolated from the livers of untreated rats according to the procedure of Cheng and Schenkman.[18,19] This method, which involves successive affinity, ion-exchange, and hydroxy apatite chromatography, yields electrophoretically pure preparations, stabilized by glycerol, with concentrations of 17–19 nmol of P-450/mg of protein. Samples for Raman spectroscopy were 33 μM in 50 mM phosphate buffer, pH 7.25, with 25% (v/v) glycerol; they were free of detergent and had low fluorescence. A small excess of dithionite was added to produce reduced samples, and these were converted to the CO adduct by gentle stirring under a stream of CO.

Model complexes were prepared by a modification of the procedure of Berzinis and Traylor.[20] A concentrated solution of (Cl)Fe^{III}P-PIXDME (protoporphyrin IX dimethyl ester) in dimethyl sulfoxide (DMSO) was deoxygenated under N_2 for 30 min and then reduced by the addition of a methanolic solution of sodium dithionite and a crown ether, 18-C-6. A benzenemethanethiolate complex was prepared by addition of a DMSO solution of benzenemethanethiol to which excess solid NaH had been added. The CO adduct was then formed by bubbling CO. The reactions were monitored spectrophotometrically.

Raman spectra were obtained with a Spex 1401 double monochromator with a cooled photomultiplier and photon-counting electronics. Excitation was provided by Spectra Physics 171 Kr^+ (413.1-nm) and 170 Ar^+ (457.9-nm) lasers with 40 mW (20 mW for the CO adducts) of power at the sample. Backscattering geometry was used with spinning NMR tubes cooled with a flow of N_2 through a heat exchanger in liquid N_2.

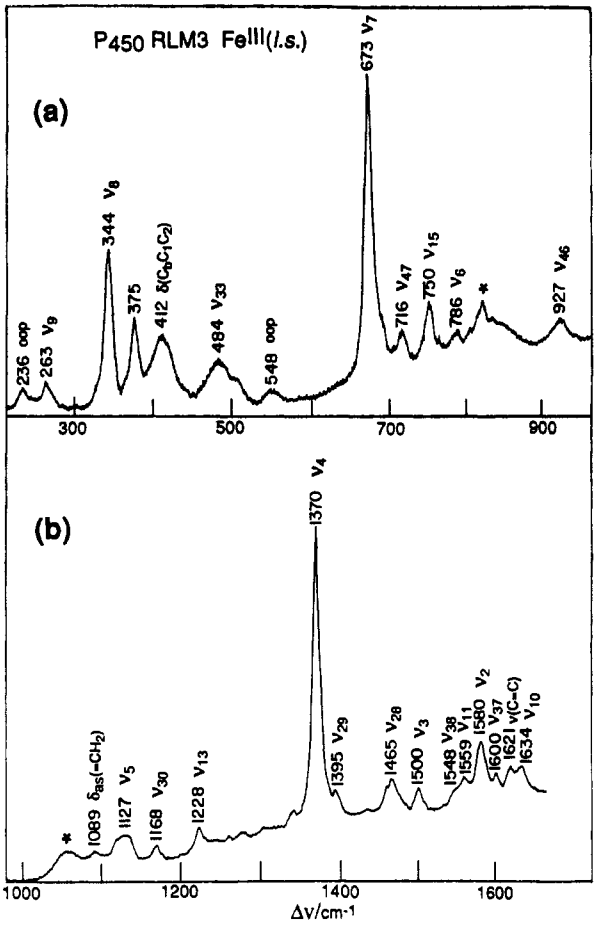

Figure 1. Low- (a) and high-frequency (b) RR spectrum of Fe^{III} P-450 (RLM3) (33 μM in 50 mM phosphate buffer with 25% glycerol, pH = 7.25), obtained with 413.1-nm excitation, 40-mW power at the sample, and a 6-cm^{-1} slit width. The bands are typical of a low-spin Fe^{III} heme; mode assignments are as in ref 21 (oop = out-of-plane mode). Peaks marked with an asterisk are due to the glycerol. Data acquisition: 0.5-cm^{-1} intervals; 3-s accumulation.

Results

Fe^{III} P-450. Figure 1 shows a well-resolved RR spectrum of oxidized cytochrome P-450 RLM3 obtained with 413.1-nm excitation. The bands are marked with labels assigning them to various normal modes of the porphyrin ring on the basis of previous assignments of nickel and iron porphyrins.[15] With 413.1-nm excitation, in resonance with the protoporphyrin Soret absorption band, totally symmetric A_{1g} modes are enhanced via Franck–

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Table I. Porphyrin Marker Band Frequencies (cm^{-1}) for P-450 RLM3 and Model Compounds

	band freq						
assignt[a]	Fe^{III} P-450[b]	$(ImH)_2Fe^{III}PP$[c]	$(DMSO)(RS^-)Fe^{III}PP$[d]	Fe^{II} P-450[b]	$(2\text{-MeImH})Fe^{II}PP$[c]	Δ[e]	$\Delta\pi$[f]
ν_{10}	1634	1640	1637	1583	1604	−21	−13
ν_{37}	1600	1602		1600	1583	+17	+12
ν_2	1580	1579	1579	1564	1562	+2	+10
ν_{11}	1559	1562		1521	1547	−26	−20
ν_{38}	1548	1554		1532	1521	+11	+20
ν_3	1500	1502	1500	1463	1471	−8	−1
ν_{28}	1465	1469					0
ν_4	1370	1373	1373	1341	1357	−16	−17

[a] See ref 15. [b] Present work. [c] From ref 21; ImH = imidazole, 2-MeImH = 2-methylimidazole, and PP = protoporphyrin IX. [d] From ref 22; DMSO = dimethyl sulfoxide and RS^- = *p*-nitrobenzenethiolate. [e] Frequency difference Fe^{II} P-450 minus $(2\text{-MeImH})Fe^{II}PP$. [f] Frequency deviations for $(ImH)_2Fe^{II}PP$ from the value expected on the basis of the porphyrin core size, and attributed to π-back-donation effects; from ref 21 but modified according to the subsequent core size parameters in ref 41.

Condon (*A* term) scattering, as are E_u (infrared) modes and the vinyl C=C stretch, which become activated by the conjugation of the asymmetrically disposed vinyl substituents on the porphyrin IX ring. In addition, B_{1g} and B_{2g} modes (ν_{10}, ν_{11}, ν_{28}) can be enhanced by the Jahn–Teller effect. Not seen with measurable intensity are the vibronically active A_{2g} modes, which can be studied in resonance with the α, β absorption bands.[15]

The spectrum is essentially the same as those previously reported[6-14] for substrate-free forms of Fe^{III} cytochromes P-450 from mammalian sources and $P\text{-}450_{CAM}$. These all contain predominantly low-spin six-coordinate Fe^{III} heme. The $P\text{-}450_{CAM}$ crystal structure shows the fifth ligand to be a cysteine S atom and the sixth ligand to be a bound water molecule. Spectroscopic evidence[3] suggests an O atom ligand, presumably H_2O as well, for all the substrate-free P-450's. Table I lists frequencies of marker bands that are known to be sensitive to structural features of the heme group, including core expansion, doming, and π-electron back-donation from the metal. Similar frequencies have been reported for other low-spin 6-coordinate Fe^{III} protohemes, including the bis(imidazole) complex[21] and the dimethyl sulfoxide adduct of the nitrobenzenethiolate complex[22] (Table I). The comparison establishes that the last named complex is indeed low-spin. This is not a self-evident conclusion, since the five-coordinate nitrobenzenethiolate complex[23] is high-spin as is the six-coordinate bis(dimethyl sulfoxide) adduct.[21] When both DMSO and nitrobenzenethiolate are bound, however, the ligand field is strong enough to force a low-spin configuration. Likewise, the combination of cysteinate and a water molecule as ligands in P-450 is sufficient to produce a low-spin heme, but when the water is displaced by the binding of substrate (itself not bound to the Fe), the result is a five-coordinate high-spin complex.[4,5] The similarity of the frequencies for Fe^{III} P-450 and the bis(imidazole) heme complex shows that a thiolate ligand has little influence on the porphyrin vibrational frequencies other than its ligand field effects that produce a low-spin Fe^{III} heme.

Fe^{II} P-450. There is something special, however, about the thiolate ligand in a five-coordinate Fe^{II} complex. This is the coordination state of reduced cytochrome P-450, the RR spectrum for which is shown in Figure 2. Again, the spectrum for the RLM3 protein is similar to those of other reduced P-450's.[6-14] Comprehensive band assignments have not previously been made, although the anomalously low frequency, 1341–1347 cm^{-1}, for the prominent band that is obviously ν_4 from its position in the spectrum has frequently been noted. In addition, Champion et al.[6] assigned ν_3 at 1466 cm^{-1} in $P\text{-}450_{CAM}$, and noted its 5-cm^{-1} downshift relative to other high-spin ferrous hemes. The present assignments are made in relation to those of a model five-coordinate high-spin Fe^{II} heme, $(2\text{-MeIm})Fe^{II}PPIX$.[21] The band

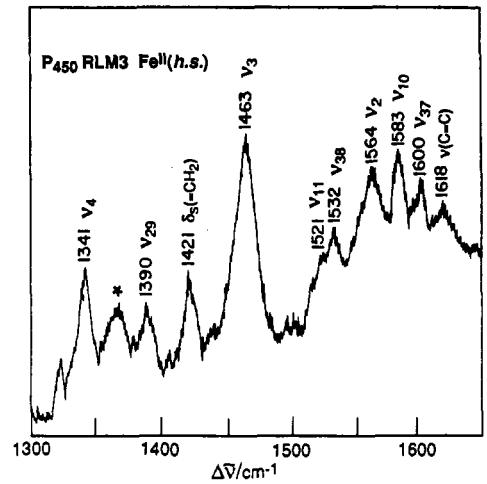

Figure 2. RR spectrum of Fe^{II} P-450 RLM3 under conditions identical with those of Figure 1. Mode assignments are as in ref 21, with the expected π-back-donation shift pattern (see text). Data acquisition: 1-cm^{-1} intervals; 2-s accumulation. Spectra of two separate samples were coadded to improve the signal.

correspondences are shown in Table I, and the large frequency differences (Δ) are also listed. These differences are shown in the last columns to match surprisingly well the deviations ($\Delta\pi$) noted[21] for the low-spin complex $(ImH)_2Fe^{II}PPIX$ from the frequencies expected on the basis of its core size. The latter deviations have been explained[21] in terms of the effects on the porphyrin ring of back-donation of the Fe^{II} d_π electrons, an electronic effect that is pronounced for low-spin Fe^{II}. Back-donation seems to be much less important for high-spin Fe^{II}, presumably because of the longer Fe–pyrrole bonds.[21] But the unusual frequencies observed for Fe^{II} P-450 suggest that thiolate coordination can greatly enhance the extent of back-donation. We note that the Fe^{II} P-450 assignments given in Table I are tentative and need to be secured with polarization measurements. Assignment reversals are possible for the ν_{10}/ν_{37} and ν_{11}/ν_{38} pairs; the result would be smaller Δ values in this case. Since the ν_4 assignment is free of ambiguity, its large Δ value points to a back-donation effect that should also influence the other modes. The match is far from perfect, especially for ν_2 (substantial $\Delta\pi$, small Δ) and ν_3 (substantial Δ, small $\Delta\pi$). Other factors, including the out-of-plane geometry of five-coordinate heme, must play a role. But the overall resemblance of the shift pattern does support the idea that thiolate coordination enhances back-donation to the porphyrin.

In Figure 3, we show the effect on the RR spectrum of adding the benzenemethanethiolate ion to $(Cl)Fe^{III}PPDME$ that has been reduced by sodium dithionite/crown ether. Before the addition, one sees a prominent band at 1365 cm^{-1}, which is assignable to ν_4 of a four-coordinate intermediate-spin Fe^{II} heme,[24,25] this unusual spin state being the result of very weak or absent axial ligation.[26] Intermediate-spin RR frequencies cover a range of

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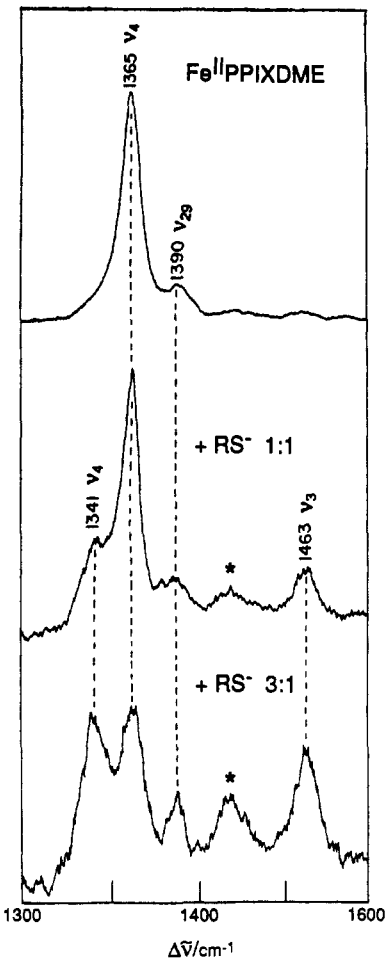

Figure 3. 413.1-nm excited RR spectrum of Fe^{II}PPIXDME in DMSO (top) to which the indicated molar ratios of benzenemethanethiolate ion (prepared by NaH reaction with RSH) were added (middle and bottom). Growth of ν_3 and ν_4 bands at the same frequencies seen for Fe^{II} P-450 reflect thiolate adduct formation. A DMSO band (asterisk) also grows in, due to decreased solution absorbance (shifted electronic spectrum). Data acquisition: 0.5-cm^{-1} intervals; 3-s accumulation.

frequencies, depending on the extent of interaction with solvent molecules, but they are always higher than the high-spin frequencies due to the emptying of the antibonding Fe $d_{x^2-y^2}$ orbital, and the consequent contraction of the porphyrin core.[15] As the benzenemethanethiolate ion is added, new bands grow in at 1341 and 1463 cm^{-1}. These are the same ν_4 and ν_3 frequencies observed for reduced cytochrome P-450, and clearly establish that the protein spectrum is adequately modeled with a five-coordinate thiolate–Fe^{II} complex. This finding is in marked contrast to that of Chottard et al.[16] who found the same ν_4 frequency 1342–1344 cm^{-1} for five-coordinate complexes of Fe^{II}TpivPP whether the ligand is thiolate ($C_6HF_4S^-$, $(CH_3)_3CS^-$) or Cl^-, OH^-, $C_6HF_4O^-$, or *N*-MeIm. We have no explanation for the ν_4 invariance observed by Chottard et al. beyond noting that ν_4 may be different in character for meso-substituted than for β-substituted porphyrins. The ligation and spin-state sensitivities of iron–TPP Raman spectra are quite different from those of iron protoporphyrins.[27] When protoporphyrin itself is used, then thiolate coordination to Fe^{II} clearly induces the same dramatic frequency lowering of ν_2 and ν_4 as it does in Fe^{II} P-450. No special protein influence is required to explain the effect.

An additional unusual aspect of the Fe^{II}–thiolate species is the strength of the ν_3 RR band in relation to ν_4. Usually ν_4 is the strongest band in heme RR spectra, and often it completely dominates the spectrum as it does in unligated Fe^{II}PPDME (Figure 3). In the thiolate adduct, however, ν_3 is about as strong as ν_4. Thus, in addition to ground-state frequency shifts, the electron donation from the thiolate ligand also produces a change in the excited-state geometry involving a larger displacement along the ν_3 coordinate. For Fe^{II} P-450 (Figure 2), the 1463-cm^{-1} band is the strongest one in the spectrum. Although the glycerol in the solution may contribute to this band (see e.g. the starred glycerol peak in Figure 4), the titration data of Figure 3 leaves no doubt that a strong ν_3 band is an intrinsic aspect of the thiolate–Fe^{II} heme RR spectrum.

Fe^{II}CO P-450. Figure 4 shows that ν_4 is at 1369 cm^{-1} for the CO adduct of Fe^{II} P-450, as it is for the CO adduct of the benzenemethanethiolate complex of protoheme. This is nearly the same frequency as seen for the CO adducts of myoglobin and hemoglobin, 1370–1372 cm^{-1}.[28] The reason that ν_4 of heme–CO adducts is at nearly as high a frequency as in Fe^{III} hemes is that the π-acid CO ligand competes effectively with the porphyrin for the Fe d_π electrons, thereby diminishing back-donation to the ring and raising ν_4. Other features of the RR spectra of cytochrome P-450 (data not shown) and myoglobin CO adducts are also quite similar. The strong thiolate influence on the heme RR frequencies is eliminated upon CO binding.

A thiolate influence is seen, however, on the Fe–CO stretching frequency, as has previously been noted.[12,13] Figure 5 shows the relevant region of the RR spectrum; the band at 474 cm^{-1} shifts to 469 cm^{-1} upon ^{13}CO substitution, as expected for the Fe–CO stretch.[29] This band is in the 460–480-cm^{-1} region for CO adducts of different P-450's, but is close to 500 cm^{-1} for heme proteins with imidazole ligands.[12,13,30,31]

Discussion

In heme complexes, there can be electronic communication between axial ligands and the porphyrin ring via the central iron atom.[31,33] The iron d_π orbitals are of proper symmetry to overlap with the porphyrin e_g^* orbitals. The d_π orbitals are filled in low-spin Fe^{II} complexes, and substantial back-donation to the e_g^* orbitals is evident in large deviations of porphyrin RR band frequencies, when, e.g., the bis(imidazole)–Fe^{II} heme frequencies are compared with those expected on the basis of its porphyrin core size[21] (see Table I). Both up- and downshifts are found depending on the mode symmetries in a manner than can be related to the symmetry properties of the e_g^* orbitals. These deviations are, however, diminished when the axial ligands have empty π^* orbitals that can compete with the porphyrin e_g^* orbitals for the Fe d_π electrons.[24] In the case of CO or O_2 binding, the competition strongly favors the axial ligand, and the porphyrin frequencies approach those seen for low-spin Fe^{III} complexes, in which one d_π electron is entirely removed.[24,32,33] For these complexes, the frequencies are as expected for the core size, and there is no evidence of back-donation.[21]

These considerations provide a basis for understanding the spectroscopic effects of thiolate ligation. The present study shows that thiolate binding in a high-spin five-coordinate Fe^{II} complex is sufficient to induce the large ν_4 downshift that has previously been noted for reduced cytochrome P-450. This downshift is the same as the π-back-donation effect on ν_4 observed in $(ImH)_2Fe^{II}PP$. Shifts of the remaining Fe^{II} P-450 marker bands relative to (2-MeImH)Fe^{II}PP (or deoxymyoglobin) likewise reflect a clear back-bonding pattern (Table I). We infer that the strong donor properties of the thiolate ligand induce back-donation of the Fe^{II} d_π electrons to the porphyrin e_g^* orbitals despite the

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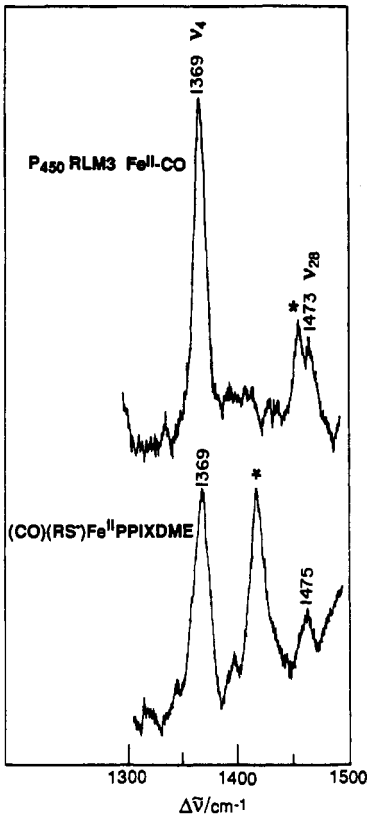

Figure 4. Comparison of the RR spectrum of the reduced Fe^{II} P-450–CO complex (33 μM, upper trace) with that of the model compound [(CO)Fe^{II}PPIXDME(benzenemethanethiolate)] in DMSO. Conditions: 457.9-nm excitation; 30-mW power (lower trace, 15 mW) at the sample; 6-cm^{-1} slit width. Peaks marked with asterisks are due to glycerol (upper trace) or DMSO (lower trace). Data acquisition: 0.5-cm^{-1} intervals; 1-s accumulation.

high-spin character of the complex.

This effect is *not* seen in thiolate–Fe^{III} hemes, however. We infer that, owing to its higher effective nuclear charge, Fe^{III} can absorb the electron donation from the thiolate ligand without significant back-donation to the porphyrin. Consequently, the porphyrin frequencies for oxidized cytochrome P-450 do not differ significantly from those of other Fe^{III} hemes at the same ligation number and spin state.

In the case of the Fe^{II}–CO adducts, it is the CO ligand that absorbs the electron donation from the thiolate. The C–O stretching frequency for cytochrome $P\text{-}450_{CAM}$ and for thiolate–heme–CO adducts is quite low, ~1940 cm^{-1},[34] reflecting enhanced back-donation to CO and weakening of the C–O bond. The frequency is ~1970 cm^{-1} (depending on solvent) for imidazole–heme–CO adducts.[30,31,36] In MbCO the frequency is lower, 1947 cm^{-1}, but this reflects the influence of FeCO tilting[31] due to the nonbonded contact with the distal histidine residue;[37] the frequency rises to 1967 cm^{-1} at low pH,[38] probably because the protonated histidine side chain swings out of the heme pocket.[39] Distal residues do not crowd the ligand binding site in cytochrome P-450,[4] and the low CO stretching frequency reflects the electronic influence of the thiolate ligand.

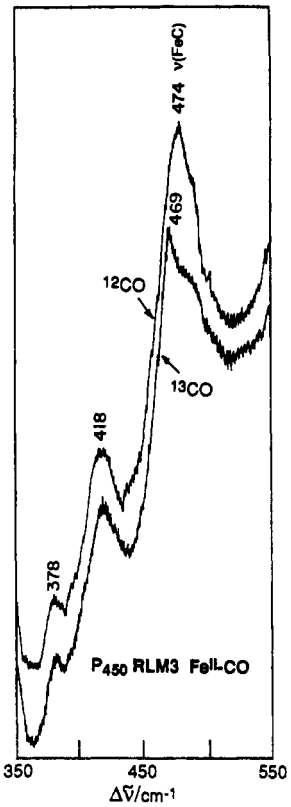

Figure 5. Isotopic shift of the RR band at 474 cm^{-1} ($^{12}C^{16}O$, upper trace; $^{13}C^{16}O$, lower trace) of Fe^{II} P-450–CO (33 μM). Conditions: 457.9-nm excitation; 20-mW power at the sample; 6-cm^{-1} slit width. Data acquisition: 0.5-cm^{-1} intervals; 9-s accumulation.

A σ- as well as π-donor effect of the thiolate is displayed by the CO ligand, the former leading to a lowering of the Fe–CO stretch, seen at 460–480 cm^{-1} in P-450–[11-13,17] and thiolate–heme–CO adducts,[16] but near 500 cm^{-1} for CO adducts with imidazole ligands.[27-31] π-Back-donation leads to a correlated increase in the Fe–CO stretching frequency with decreasing CO frequency, and a plot of these two frequencies gives a negative linear correlation for CO adducts of imidazole complexes and heme proteins with proximal imidazole ligands.[11,12,30,31] The P-450 data fall below this line, reflecting the σ-donating propensity of the thiolate ligand, which leads to decreased FeCO frequencies despite the greater back-bonding.

The capacity of the bound CO to absorb the electron donation from the thiolate ligand eliminates additional effects on the porphyrin RR frequencies. These are essentially the same for CO adducts of cytochrome P-450 as for MbCO. The absence of porphyrin vibrational frequency perturbations may seem paradoxical in view of the strong perturbation of the electronic absorption spectrum of the P-450–CO adduct. The Soret band is split, with a low-energy component at 450 nm (the feature that gives rise to the name of the enzyme).[3] This splitting has been attributed to a mixing of the porphyrin $\pi-\pi^*$ transition with a thiolate → porphyrin charge-transfer transition.[37] The lack of a corresponding perturbation of the porphyrin vibrational frequencies simply reflects the fact that the properties of the ground state (vibrational frequencies) need not be connected with those of the excited states (electronic absorption spectrum). The RR enhancements do reflect the nature of the excited states, however, and excitation profile measurements are expected to give further insight into the nature of the electronic interactions in the various states of P-450.[14b]

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