Articles

Resonance Raman Spectra and the Active Site Structure of Semisynthetic Met80Cys Horse Heart Cytochrome *c*

Giulietta Smulevich,*,† Morten J. Bjerrum,‡,§ Harry B. Gray,§ and Thomas G. Spiro*,‖

Dipartimento di Chimica, Universitá di Firenze, Via G. Capponi 9, 50121 Firenze, Italy, Department of Chemistry, The Royal Veterinary and Agricultural University, Thorvaldsensvej 40, DK-1871 Frederiksberg C, Denmark, Beckman Institute, California Institute of Technology, Pasadena, California 91125, and Department of Chemistry, Princeton University, Princeton, New Jersey 08544

Received April 13, 1994[⊗]

Resonance Raman (RR) spectra are reported for semisynthetic Met80Cys horse heart cytochrome *c*. For the Fe(III) form, the porphyrin high-frequency skeletal modes are at positions close to those of the imidazole adduct of cytochrome P450, confirming earlier spectroscopic indications that the Cys80 side chain is bound to the Fe^{3+} as a thiolate anion. The low-frequency RR spectrum shows changes indicating a relaxation of the protein-induced heme distortion present in native cyt *c*; a change in the protein fold is suggested, due to the shortening of the ligating side chain from methionine to cysteine. When the $Fe^{II}CO$ adduct of the cyt *c*-Cys80 mutant is photolyzed, the product contains low-spin heme, implying religation by an endogenous ligand. The position of the ν_{11} porphyrin back-bonding marker indicates that the endogenous ligand donor strength is greater than that of methionine but less than that of lysine. Cys80 ligation as a neutral thiol is suggested, with proton uptake accompanying the Fe(III)/Fe(II) reduction. Two forms of the $Fe^{II}CO$ adduct, A_0 and A_{-1}, were detected, with RR ν_{FeC} bands at 486 and 491 cm^{-1} and IR ν_{CO} bands at 1966 and 1981 cm^{-1}. These pairs of frequencies are located in the ν_{FeC}/ν_{CO} back-bonding graph near the line characteristic of imidazole (rather than thiolate) ligation. This behavior is consistent with CO displacement of the Cys80 ligand, but His18 substitution cannot be ruled out if Cys80 is bound as thiol, since its donor strength might then be comparable to that of imidazole. The frequencies of form A_0, $\nu_{FeC} = 486$ cm^{-1} and $\nu_{CO} = 1966$ cm^{-1}, are characteristic of CO bound in a hydrophobic pocket, while those of form A_{-1}, $\nu_{FeC} = 491$ cm^{-1} and $\nu_{CO} = 1981$ cm^{-1}, indicate significant inhibition of back-bonding, perhaps via an interaction with a lone pair of the displaced endogenous ligand, either Cys80 or His18.

Introduction

The method of protein semisynthesis has permitted construction of cytochrome *c* (cyt *c*) mutants with designed substitutions of side chains that ligate the heme Fe atom.[1] These constructs are expected to enhance our understanding of the factors that determine the electron-transfer thermodynamics and kinetics in heme proteins. To this end, it is important to characterize the chemical interactions at the active site of the mutants. Resonance Raman (RR) spectroscopy is a useful tool for this purpose, since vibrational modes of the heme group are strongly enhanced by laser excitation in the porphyrin absorption bands.[2] These vibrations have been assigned and analyzed in model compounds and heme proteins, including cyt *c* itself.[3] Marker bands have been identified for heme conformation and ligation state and, in the case of CO ligands, for polar interactions in the heme pocket.[4]

In this study, we report RR spectra for the semisynthetic horse heart cytochrome *c* mutant in which methionine-80 is replaced by cysteine.[1] In cyt *c*, the axial ligands of the heme Fe are the imidazole side chain of His18 and the thioether side chain of Met80. The thiol side chain of cysteine often binds to metal centers as a thiolate anion. For example, cysteine thiolate binding is well established in the cytochrome P450 (cyt P450) class of proteins.[5] The significant issue raised by the construction of the cyt *c*-Cys80 mutant is how the potential for thiolate ligation influences the protein structure and function. From absorption[1] and MCD[6] spectra, thiolate ligation has been inferred for the Fe(III) form but not for the Fe(II) form or for its CO adduct. RR spectroscopy corroborates these inferences and provides significant additional details of structure and bonding.

Experimental Section

Horse heart cytochrome *c* (Sigma Type VI) was used as received. Semisynthetic cyt *c*-Cys80 was prepared as described previously.[1] The imidazole adduct of cytochrome $P450_{cam}$ was supplied by J. H. Dawson (University of South Carolina).

Samples were dissolved in 25 mM phosphate (pH 7) or in 0.1 M CHES (2-(cyclohexylamino)ethanesulfonic acid) buffer (pH 10.8) to give protein concentrations of 0.2–0.4 mM for RR spectroscopy and 1.5–2.5 mM for IR spectroscopy.

* To whom correspondence should be addressed.
† Universitá di Firenze.
‡ The Royal Veterinary and Agricultural University.
§ California Institute of Technology.
‖ Princeton University.
⊗ Abstract published in *Advance ACS Abstracts,* September 1, 1994.

(1) Raphael, A. L.; Gray, H. B. *J. Am. Chem. Soc.* **1991**, *113*, 1038–1040.
(2) Spiro, T. G.; Li, X.-Y. In *Biological Applications of Raman Spectroscopy*; Spiro, T. G., Ed., 1988; Vol. 3, pp 1–38.
(3) Cartling, B. In *Biological Applications of Raman Spectroscopy*; Spiro, T. G., Ed.; 1988; Vol. 3, pp 217–248.
(4) Li, X.-Y.; Spiro, T. G. *J. Am. Chem. Soc.* **1988**, *110*, 6024–6033.
(5) (a) Champion, P. M. In *Biological Applications of Raman Spectroscopy*; Spiro, T. G., Ed.; 1988; Vol. 3, pp 249–292. (b) Dawson, J. H.; Sono, M. *Chem. Rev.* **1987**, *87*, 1255–1276.
(6) Rux, J.J.; Dawson, J. H. *FEBS Lett.* **1991**, *290*, 49–51.

0020-1669/94/1333-4629$04.50/0

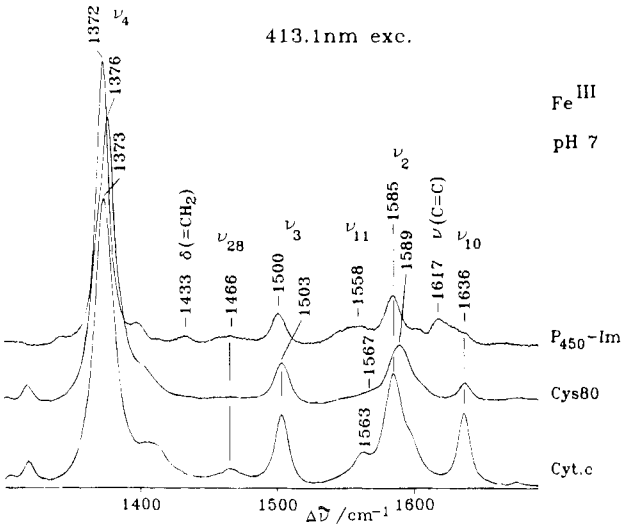

Figure 1. High-frequency-region RR spectra of the Fe(III) forms of cyt *c*, cyt *c*-Cys80, and the imidazole adduct of cyt P450, at pH 7, taken with 413.1-nm excitation. Experimental conditions: 30-mW laser power at the sample; 5-cm^{-1} resolution; 10 min/cm^{-1} (cyt *c* and cyt *c*-Cys80) and 5 s/0.5 cm^{-1} (P450 + Im) collection intervals.

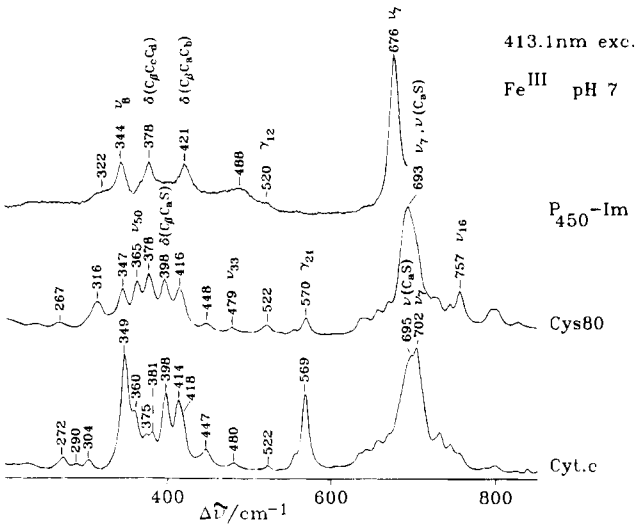

Figure 2. Low-frequency-region RR spectra of the same samples as in Figure 1: experimental conditions as reported in Figure 1, except 10 s/0.5 cm^{-1} collection interval for cyt P450 + Im.

The Fe(II) forms were prepared by adding a minimum volume of dithionite solution to deoxygenated buffered solutions of cyt *c* and by photolyzing the ligand from the CO complex of cyt *c*-Cys80. The CO adducts were obtained by gently flowing CO (1 atm) (Matheson) from a gas cylinder over the surface of the reduced protein for 15 min. The ^{13}CO (99%) adducts were obtained by introducing the gas (Cambridge Isotope Laboratories) from a 0.1-L glass flask to an evacuated NMR tube. For the IR spectra of the CO adducts, the samples were transferred by a gastight syringe flushed with CO to a CaF_2 IR cell (0.1-mm path length) that had been flushed with CO. The CaF_2 windows permitted UV−vis absorption spectra to be recorded immediately before and after IR spectroscopy to check sample integrity.

The RR spectra were obtained with excitation from the 413.1-nm line of a Kr^+ laser (Coherent). The backscattered light from a slowly rotating NMR tube was dispersed with a Spex triplemate monochromator (2400 groove/nm grating) and collected with an optical multichannel analyzer (Princeton Instruments). For the cyt P450 sample, the scattered light was collected and focused into a computer-controlled double monochromator (Yobin-Yvon HG2S) equipped with a cooled photomultiplier (RCA) and photon-counting device. All the spectra were collected at room temperature and calibrated with indene as a standard; the frequencies are accurate to ±1 cm^{-1} for isolated bands.

Infrared spectra were recorded at 25 °C with a Bruker 1F120HR FTIR spectrophotometer.

Results

Fe(III) Form. Figure 1 compares Soret-band RR spectra, in the region of the high-frequency porphyrin skeletal modes, for cyt *c* and its Cys80 mutant and also for the imidazole adduct of cytochrome $P450_{cam}$.[6] We note that the RR spectrum of cyt *c*-Cys80 is unaltered at pH 10.8 (data not shown), although the spectrum of cyt *c* is altered at this pH, via replacement of the Met80 by another ligand.[3] Many workers in the field have long suspected that the high-pH ligand is the amine side chain of an adjacent residue, Lys79, and ligation by this side chain was recently established for alkaline yeast-iso-1-ferricytochrome *c*, via NMR and mutational analysis.[7] These experiments also showed the alkaline form to consist of two species, involving ligation by Lys79 in one and by another lysine in the other.

The labeling of the bands in Figure 1 corresponds to the standard mode numbering of octaethylporphyrin and protoheme.[2] Cyt P450 also shows modes of the vinyl substituents, ν(C=C) and δ(=CH_2), but these are missing from both cyt *c*'s, since the vinyl double bonds are saturated through the formation of covalent links to cysteine side chains of the protein. The skeletal modes ν_{10}, ν_2, ν_{11}, and ν_3 are well-characterized markers of the porphyrin core size and therefore of the spin state; they are at expected low-spin positions for all three proteins.[2] ν_2 is coupled to the vinyl ν(C=C) mode, in protohemes, and saturation of the vinyl groups raises its frequency by about 10 cm^{-1}.[8a] This effect is seen in Fe^{II}(cyt *c*),[3] for which ν_2 is at 1592 cm^{-1}, but the frequency is anomalously low, 1585 cm^{-1}, for Fe^{III}(cyt *c*) and happens to coincide with the cyt P450-Im frequency. All the core-size marker bands of native cyt *c* are depressed by 2−4 cm^{-1}, relative to the alkaline form of Fe^{III}-(cyt *c*),[3] as is ν_4, which is a marker of oxidation state but is also mildly dependent on core size.[2] This effect is attributed to steric interactions of the thioether ligand with the porphyrin, which expand the core slightly and are relieved by the replacement with lysine. A similar core size expansion is apparent for cyt *c*-Cys80, since ν_2, ν_{11}, and ν_4 are all 4 cm^{-1} higher than in native cyt *c*. We note that ν_{11} is a weak shoulder for excitation at 413.1 nm, but upon excitation at 363.8 nm, it becomes a prominent band at 1567 cm^{-1} (data not shown).

Figure 2 compares low-frequency spectra for these three proteins. The cyt *c* spectrum is very rich in this region, due to the activation of substituent and porphyrin out-of-plane modes. These modes have been assigned via enzymatic reconstitution of cyt *c* with isotropically labeled heme.[8b] Their activation is attributed to heme distortions imposed by the protein, since the spectrum simplifies substantially when cyt *c* is unfolded, or converted to the A ("molten globule") state.[9a] The high-resolution crystal structure of cyt *c* reveals a pronounced saddling of the entire porphyrin ring.[9b] Of particular interest are the prominence of the RR band at 569 cm^{-1}, assigned to the pyrrole-swiveling mode γ_{21},[8b] and the frequency elevation of the in-plane pyrrole deformation mode ν_7, from 676 cm^{-1} in

(7) Ferrer, J. C.; Guillemette, G.; Bogumil, R.; Inglis, S. C.; Smith, M.; Mauk, A. G. *J. Am. Chem. Soc.* **1993**, *115*, 7507−7508.

(8) (a) Choi, S.; Spiro, T. G.; Langry, K. C.; Smith, K. M. *J. Am. Chem. Soc.* **1982**, *104*, 4337. (b) Hu, S.; Morris, I. K.; Singh, J. P.; Smith, K. M.; Spiro, T. G. *J. Am. Chem. Soc.* **1993**, *115*, 12446−12458.

(9) (a) Jordan, T.; Austin, J.; Spiro, T. G. Submitted for publication. (b) Louie, G. V.; Brayer, G. D. *J. Mol. Biol.* **1990**, *214*, 527−555.

(10) (a) Smulevich, G.; Miller, M. A.; Gosztola, D.; Spiro, T. G. *Biochemistry* **1989**, *28*, 9905−9908. (b) Kerr, E. A.; Yu, N.-T. In *Biological Applications of Raman Spectroscopy*; Spiro, T. G., Ed.; 1988; Vol. 3, pp 39−96.

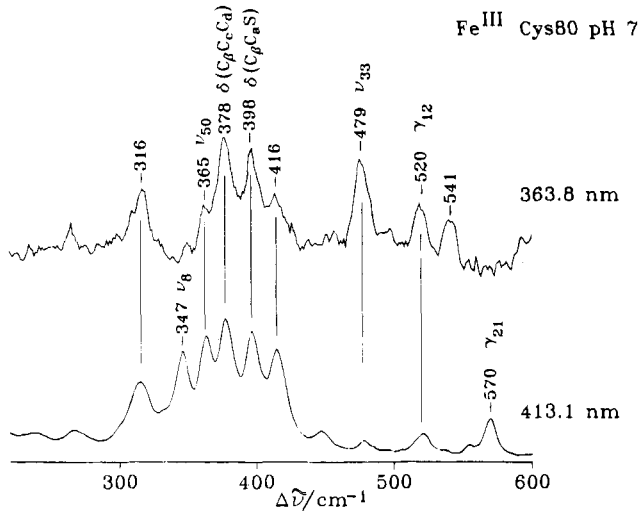

Figure 3. Low-frequency-region RR spectra comparing 413.1- and 363.8-nm excitations for cyt *c*-Cys80. The 363.8-nm-excited spectrum of native cyt *c* is also shown.

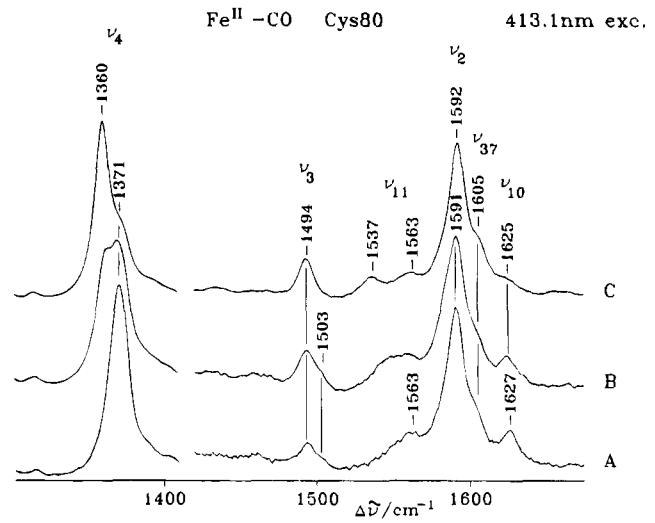

Figure 4. High-frequency-region RR spectra of the $Fe^{II}CO$ adduct of cyt *c*-Cys80, with 413.1-nm excitation: (A) pH 7, using 3-mV laser power at the sample with a defocused beam, 5-cm^{-1} resolution, 20-min collection; (B) pH 7, using 12-mW laser power at the sample, 5-cm^{-1} resolution, 5-min collection; (C) pH 10.8, using 2-mW laser power at the sample, 5-cm^{-1} resolution, 5-min collection.

P450 (and many other heme proteins) to 702 cm^{-1} in cyt *c*. The γ_{21} band attenuates significantly in the cyt *c*-Cys80 spectra, and the ν_7 band shifts down, under the 693-cm^{-1} band, which is assigned[8b] to a CS stretching vibration of the thioether linkage between the heme and the protein. Since both these effects are also seen in the cyt *c* A state,[9a] we infer that the heme distortion is partially relaxed by the Met80 → Cys substitution.

There are other subtle differences between the spectra of cyt *c*-Cys80 and of native cyt *c*. Most notable is the appearance of a band at 316 cm^{-1}, which is missing in the spectrum of cyt *c*. The cyt P450 imidazole adduct, however, shows a weak band at 322 cm^{-1}. For the adduct-free high-spin form of cyt P450, a band at 351 cm^{-1} has been assigned, via ^{34}S and ^{54}Fe substitution, to the FeS(cysteine) stretching vibration.[5a] It is possible that the 322-cm^{-1} band of the cyt P450 imidazole adduct and the 316-cm^{-1} band of cyt *c*-Cys80 are FeS(cysteine) stretches as well; the frequency lowering from the high-spin position is expected from the kinematic effect of the trans imidazole ligand. The FeS band of high-spin cyt P450 is specifically enhanced at 363.8 nm, on the blue side of the Soret band, via resonance with an S → Fe charge-transfer transition.[5a] In the 363.8-nm-excited spectrum of cyt *c*-Cys80 (Figure 3), the 316-cm^{-1} band is enhanced to a much greater extent than, e.g., the 347-cm^{-1} ν_8 band. Other bands are prominent as well, however, including the 378-cm^{-1} propionate bend, the 398-cm^{-1} thioether bend, the 479- and 522-cm^{-1} ν_{33} and γ_{12} bands, and an extra peak at 540 cm^{-1}, which may correspond to a feature of Fe^{II}(cyt *c*) at 536 cm^{-1} that has been assigned to the skeletal mode ν_{49}.[8b] This change in the heme mode enhancement pattern obscures the significance of the 316-cm^{-1} band enhancement with 363.8-nm excitation. In the absence of isotopic data, the FeS assignment remains speculative at this stage.

Fe(II) Form. Reduction of Fe^{III}(cyt *c*-Cys80) is sluggish (reflecting the low reduction potential, −390 mV vs NHE[1]), and we were unable to prepare satisfactory RR samples by dithionite reduction. Addition of CO to the dithionite-containing solution nevertheless produces the $Fe^{II}CO$ adduct.[1] This adduct is photolabile, and we were able to obtain RR spectra of the adduct and its photolysis product by adjusting the laser power, as shown in Figure 4. The ν_4 and ν_3 bands are particularly sensitive markers of photolysis,[11] appearing at 1360 and 1494 cm^{-1} in the spectra of photolyzed samples and at 1371 and 1503 cm^{-1} in the spectra of unphotolyzed protein. (ν_3, however, is much stronger for the photolyzed than for the unphotolyzed form, as has also been observed for cytochrome *c* peroxidase,[10a] while the ν_4 intensities are comparable for both forms.) At pH 7, excitation with 12 mW of laser power at the sample is sufficient to photolyze about half of the molecules (spectrum B), while lowering the power to 3 mW and defocusing the laser lower the photolyzed fraction substantially (spectrum A). At pH 10.8, photolysis is more efficient, and even 2 mW of laser power is sufficient to produce mainly the photolyzed product (spectrum C).

The frequency shifts in ν_4 and ν_3 are similar to those observed between Fe(III) and Fe(II) forms of cyt *c*[3] and are due to the electron-withdrawing effects of the CO ligand, which diminishes back-bonding from Fe(II) to the porphyrin π^* orbitals.[2] A small shift is seen as well for ν_{10} (1627 vs 1625 cm^{-1}), and a much larger shift is seen for ν_{11} (1563 vs 1537 cm^{-1}). The ν_2 frequency is insensitive to back-bonding changes.

Loss of the CO ligand by photolysis should produce a five-coordinate high-spin Fe(II) heme, but the band positions of the photolysis product (ν_4 = 1360 cm^{-1}, ν_3 = 1494 cm^{-1}, ν_2 = 1592 cm^{-1}) are characteristic of *low-spin* Fe(II),[2] implying the presence of a strong-field sixth ligand. This observation indicates that CO photolysis is accompanied by rapid binding of a protein ligand. Rebinding of CO competes with this process, so that a photostationary state is formed in which the ratio of CO adduct to the adduct with the endogenous ligand depends on the laser power. At pH 10.8, CO rebinding is apparently less rapid than that at pH 7, suggesting a change in conformation of the binding pocket. This change does not, however, affect the nature of the endogenous ligand, since the spectrum of the photolysis product is unaltered.

While the frequencies of the photolysis product are similar to those of Fe^{II}(cyt *c*), ν_{11} is appreciably lower, 1537 vs 1544 cm^{-1}.[3] (This relatively weak band shows up most clearly in the pH 10.8 spectrum, which is subject to the least CO recombination.) An even lower frequency, 1533 cm^{-1}, has been reported for Fe^{II}(cyt *c*) modified by carboxymethylation of the Met80 ligand;[3] in this derivative, the sixth ligand is believed to be Lys79. The same low frequency is seen for the transient intermediate generated by rapid reduction of alkaline Fe^{III}(cyt

(11) Spiro, T. G. *Adv. Protein Chem.* **1985**, *37*, 110.

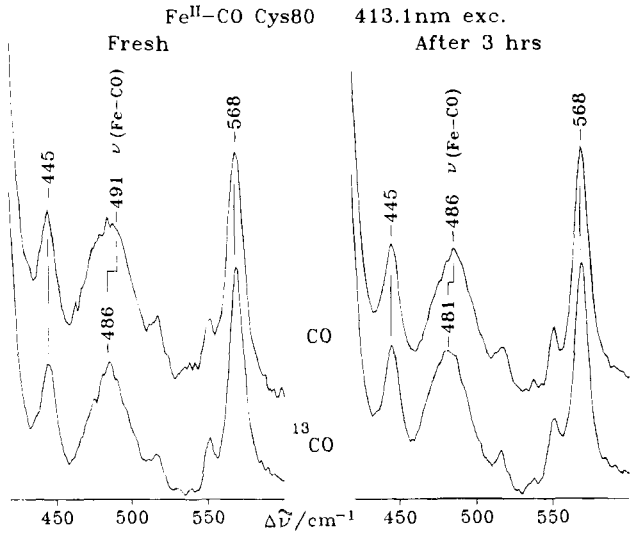

Figure 5. Low-frequency-region RR spectra of the Fe^{II}CO adduct of cys *c*-Cys80 with ^{12}CO and ^{13}CO, at pH 7. Experimental conditions: 413.1-nm excitation; 2-mW laser power at the sample with defocused beam; 5-cm^{-1} resolution; 20-min collection. The left panel shows the spectra of fresh samples, while the right panel gives the spectra of the same samples 3 h after preparation.

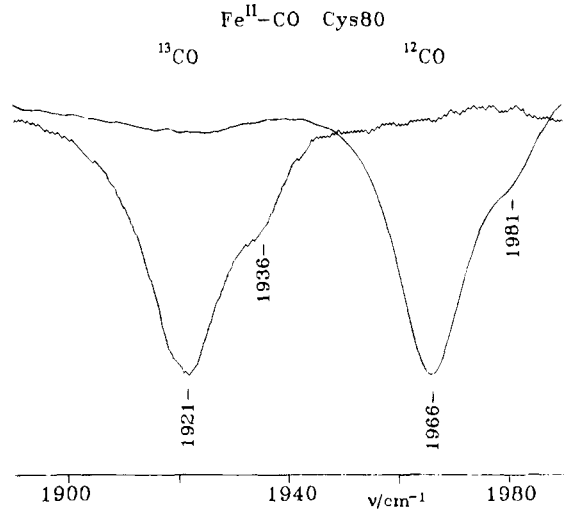

Figure 6. IR spectra in the CO stretching region for the Fe^{II}CO adduct to cys *c*-Cys80 with ^{12}CO and ^{13}CO, at pH 7. A total of 1200 transients were accumulated, (4-cm^{-1} resolution).

c),[3] in which Met80 is again replaced as a ligand by lysines.[7] The 11-cm^{-1} lowering of ν_{11}, relative to native Fe^{II}(cyt *c*), has been attributed to stronger σ donation to the Fe(II) from lysine than from methionine, resulting in greater back-donation to the porphyrin.[3] In this model, the intermediate frequency seen for the (cyt *c*-Cys80)Fe^{II}CO photoproduct implies a donor strength for the endogenous ligand that is intermediate between those of methionine and lysine.

Fe^{II}CO Adduct. The low-frequency RR spectrum of the CO adduct (Figure 5) contains a broad band at 486 cm^{-1} that can be assigned to the FeC stretching vibration[12] because of its 5-cm^{-1} downshift upon ^{13}CO substitution. The frequency is 491 cm^{-1} in the spectrum of a freshly prepared solution (486 cm^{-1} for the ^{13}CO isotopomer), but over the course of 3 h, the band shifts to 486 cm^{-1} (481 cm^{-1} for ^{13}CO). Thus, the structure of the CO adduct appears to change with time. Figure 6 shows the infrared spectrum in the region of the CO stretching frequency. The main band is at 1966 cm^{-1}, but there is a shoulder at 1981 cm^{-1}. Upon ^{13}CO substitution, both the main band and the shoulder shift down 45 cm^{-1}, which is the expected isotope shift for CO stretching.[10b] Thus, there again appear to be two distinct forms of the CO adduct. In the IR experiment, a clear time evolution of the two components was not observed; because reduction is slow, freshly prepared samples gave a high background attributable to the Fe(III) protein. We were also able to detect the CO stretching band weakly in the RR spectrum, however, and found its main component to be at 1966 cm^{-1} for the 3-h-old sample. We therefore associate the 1966-cm^{-1} CO stretch with the 486-cm^{-1} FeC stretch and the 1981-cm^{-1} CO stretch with the 491-cm^{-1} FeC stretch. These two pairs of frequencies define a pair of interconverting substates, which we label A_0 and A_{-1}, respectively (see below).

Discussion

Replacement of the Met80 residue with Cys yields an Fe(III) protein whose absorption[1] and MCD[6] spectra closely resemble those of the imidazole adduct of Fe^{III}(cyt P450). This similarity extends to the RR spectrum, when due allowance is made for the alterations expected by the conversion of the heme vinyl groups to thioether substituents in cyt *c*. Thus, all the spectroscopic indicators point to imidazole and thiolate ligation in cyt *c*-Cys80, as in cyt P450, implying that the side chain of the Cys80 residue is ligated to Fe^{3+} and is deprotonated.

Moreover, the RR spectrum is quite clean, showing no sign of heterogeneity. Had there been any high-spin heme present, we would have seen extra bands at shifted positions. In particular, a ν_3 band would have been apparent at 1491 cm^{-1} (five-coordinate) or 1478 cm^{-1} (six-coordinate).[11] These positions are at the baseline of the Fe^{III}(cyt *c*-Cys80) spectrum (Figure 1). We emphasize this point because of a recent report, by Wallace and Clark-Lewis,[12] that semisynthetic cyt *c*-Cys80 was produced in two forms, one like that reported by Raphael and Gray[1] and another form, which has a much higher reduction potential, ~65 mV vs NHE, a 404-nm Soret band, and an acid transition to a high-spin form with a pK_a of 6.6. We have not observed this second form in repeated preparations using the Raphael–Gray procedures,[1] and it is not present in the samples used in this study. According to Wallace and Clark-Lewis, the second form is obtained when peptide fragment religation is carried out under highly reducing conditions and also when reduced cyt *c*-Cys80, obtained from either form, is reoxidized. It is possible that, in the second form, the Cys80 S atom is converted to sulfone or sulfoxide by the action of O_2 in the presence of reducing agents.

The ligation by Cys80 relaxes the protein-induced heme distortion seen in native cyt *c* to a significant extent, as evidenced by intensity loss of the 569-cm^{-1} γ_{21} pyrrole-swiveling mode and by the downshift of the ν_7 pyrrole deformation mode; similar changes are seen when cyt *c* is converted to the A state, in which tertiary constrains are significantly relaxed.[9] This relaxation no doubt reflects a change in the steric requirements for Cys80 ligation. The sulfur atom is connected to the backbone C_α atom by two methylene groups in methionine but only one in cysteine, so the protein fold of native cyt *c* must be altered somewhat to permit Cys80 ligation.

The photolysis product of the Fe^{II}CO adduct of Cys80 is a low-spin heme, implying rapid ligation by an endogenous ligand, following CO dissociation. The likeliest candidate is the Cys80 side chain. Lysine side chains are less likely alternatives because the position of the ν_{11} porphyrin back-bonding marker band is higher than that of carboxymethylated Fe^{II}(cyt *c*), or the alkaline reduction intermediate of native cyt *c*. The

(12) Wallace, C. J. A.; Clark-Lewis, I. *J. Biol. Chem.* **1992**, *267*, 3852–3861.

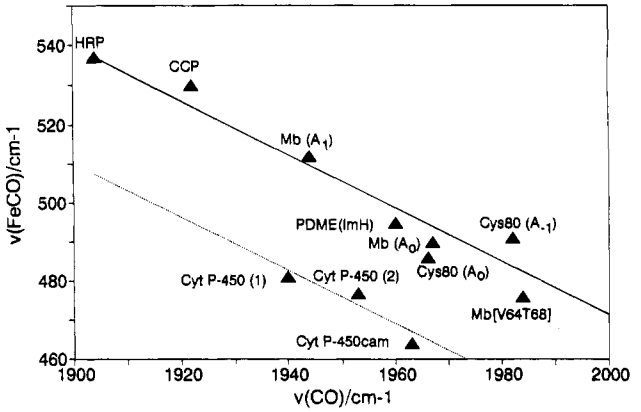

Figure 7. ν_{FeCO} vs ν_{CO} back-bonding correlations. The upper and lower lines are for proteins in which the FeCO is ligated by histidine or cysteinate side chains, respectively. Frequencies are listed in Table 1. The point for the protein-free (ImH)Fe(CO) adduct of protoporphyrin dimethyl ester is also shown.

absorption spectrum of reduced cyt *c*-Cys80 is very similar to that of native Fe^{II}(cyt *c*), and this has been interpreted to imply the absence of a thiolate−iron bond.[1] It is possible, however, that the cysteine is *protonated* when bound to Fe(II) and therefore gives rise to an absorption spectrum similar to that with methionine ligation. Protonation of the cysteinate would maintain charge neutrality in the heme pocket.

The $Fe^{II}CO$ adduct exists in two forms, reminiscent of the multiple substates observed for the CO adduct of myoglobin (Mb).[13] The pairs of ν_{FeC} and ν_{CO} frequencies (491/1981 cm^{-1} and 486/966 cm^{-1}) are associated via the time dependence of the RR spectrum. A normal CO frequency is 1966 cm^{-1} for CO bound to heme proteins having a hydrophobic pocket.[4,14] An example is the A_0 substrate of MbCO, in which the distal histidine is expelled from the heme pocket, leaving the CO in a hydrophobic environment.[15−17] By analogy, we designate this form of the (cyt *c*-Cys80)$Fe^{II}CO$ adduct as A_0. The value 1981 cm^{-1} is unusually high for ν_{CO};[4,12,14] thus we designate this substrate A_{-1}, since the MbCO substates were assigned the symbols A_0, A_1, ..., by Frauenfelder and co-workers,[13] in order of decreasing ν_{CO}.

When ν_{FeC} is plotted against ν_{CO} for a range of heme−CO adducts, an inverse correlation is found, as expected from the back-bonding model, since increased back-bonding strengthens the FeC bond while weakening the CO bond.[4,14] The points fall on lines that are displaced from one another depending on the nature of the axial ligand trans to the bound CO; ligands that are strong σ donors weaken the FeC bond without a proportionate strengthening of the CO bond. Figure 7 shows the back-bonding lines that have been determined[4,14] for many proteins and analogs having imidazole (upper line) or thiolate (lower line) ligands, along with a few examples, to give a sense

Table 1. Vibrational Frequencies (cm^{-1}) of the FeCO Linkage in Carbonyl Complexes of Metalloporphyrins and Heme Proteins

molecule	ν_{FeCO}	ν_{CO}	ref
HRP pH 7	537	1904	19
CCP pH 7	530	1922	20
sperm whale Mb, pH 7 (A_1)	512	1944	13b
PPDME (ImH)	495	1960	19
Cys80 (A_0)	486	1966	this work
Cys80 (A_{-1})	491	1982	this work
sperm whale Mb, pH 4 (A_0)	490	1967	15
Cyt P450 from *P. putida* (1)	481	1940	18a
Cyt P450 from bovine adrenocortical mithocondria (2)	477	1953	18b
Mb[V64T68]	476	1984	22, 23
Cyt P450 from *P. putida* plus camphor substrate	464	1963	16a

of the scatter of the points. The two substates of cyt *c*-Cys80 (labeled Cys80 ($A_{0,-1}$) on the graph) are both far from the thiolate line but close to the imidazole line. Thus, the vibrational data fully support an earlier conclusion (reached on the basis of the absorption band positions[1]) that the trans ligand is not cysteine thiolate and is likely to be the His18 imidazole. We cannot, however, rule out the possibility that the trans ligand is the Cys80 side chain if the S atom is protonated. We do not know where the back-bonding line should be for a trans thiol (as distinct from thiolate) ligand. The donor strength of thiol is certainly less than that of thiolate and may be comparable to that of imidazole.

The spread of points along the lines in Figure 7 is due to variations in the extent of Fe→CO back-bonding, under the influence of different polar interactions. The main substrate of MbCO, A_1,[13] for example, is higher on the correlation than is A_0, because the distal imidazole in the heme pocket provides a positive polar interaction that increases back-bonding.[14] The CO adducts of cytochrome *c* peroxidase (CCP) and horseradish peroxidase (HRP) are even higher on the line, due to the presence of a positively charged arginine side chain in the pocket.[19−21] Form A_{-1} of the (cyt *c*-Cys80)$Fe^{II}CO$ adduct falls *lower* on the line than A_0, implying inhibition of back-bonding, relative to a nonpolar environment. One other protein that falls as low as the line (labeled Mb[V64T68]) is the recently reported double mutant of Mb in which the distal His64 is replaced by Val and the Val68, adjacent to the bound CO, is replaced by Thr.[22,23] The crystal structure of the Thr68 single mutant has been shown[22] to present a lone pair of the threonine OH group to the O atom of the bound CO, because the H atom is tied up in an H-bond to a backbone carbonyl. This lone pair provides a negative polar interaction, which is expected to inhibit back-bonding. A similar effect has been seen in the model porphyrin C_2-Cap, in which a close interaction of the bound CO with a covalently attached benzene cap drives the CO frequency up to 2002 cm^{-1}.[14] We infer that form A_{-1} of the (cyt *c*-Cys80)Fe^{II}-CO adduct likewise experiences a negative polar interaction, possibly with a lone pair from the displaced endogenous ligand, Cys80 or His18. (Lysines are less likely candidates, since their lone pairs are protonated at pH 7.)

Substrates A_0 and A_{-1} might be species in which there are alternative conformations of the displaced endogenous ligand,

(13) (a) Ansari, A.; Berendzen; J.; Braunstein, D.; Cowen, B. R.; Frauenfelder, H.; Hong, M. K.; Iben, I. E. T.; Johnson, J. B.; Ormos, P.; Sauke, T. B.; Scholl, R.; Schulte, A.; Steinbach, P. J.; Vittitow, J.; Young, R. D. *Biophys. Chem.* **1987**, *26*, 337−355. (b) Tsubaki, M.; Srivastava, R. B.; Yu, N.-T. *Biochemistry* **1982**, *21*, 1132.
(14) Ray, G. B.; Li, X.-Y.; Ibers, J. A.; Sessler, J. L.; Spiro, T. G. *J. Am. Chem. Soc.* **1994**, *116*, 162−176.
(15) Ramsden, J.; Spiro, T. G. *Biochemistry* **1989**, *28*, 3125−3128.
(16) Morikis, D.; Champion, P. M.; Springer, B. A.; Sligar, S. G. *Biochemistry* **1989**, *28*, 4791−4800.
(17) Quillin, M. L.; Arduini, R. M.; Olson, J. S.; Phillips, G. N., Jr. *J. Mol. Biol.*, in press.
(18) (a) Uno, T.; Nishimura, Y.; Makino, R.; Iizuka, T.; Ishimura, Y.; Tsuboi, M. *J. Biol. Chem.* **1985**, *260*, 2023−2026. (b) Makino, R.; Iizuka, T.; Ishikava, Y. *Proc. Int. Conf. Raman Spectrosc., 9th* **1984**, 492.
(19) Evangelista-Kirkup, R.; Smulevich, G.; Spiro, T. G. *Biochemistry* **1986**, *25*, 4420−4425.
(20) Smulevich, G.; Evangelista-Kirkup, R.; Spiro, T. G. *Biochemistry* **1986**, *25*, 4426.
(21) Edwards, S. L.; Poulos, T. L. *J. Biol. Chem.* **1990**, *265*, 2588−2595.
(22) Cameron, A. D.; Smerdon, S. J.; Wilkinson, A. J.; Habash, J.; Helliwell, J. R.; Li, T.; Olson, J. S. *Biochemistry*, in press.
(23) Biram, D.; Garratt, C. J.; Hester, R. E. In *Spectroscopy Biological Molecules*; Hester, R. E., Girling, R. B., Eds.; Royal Society of Chemistry: Cambridge, U.K., 1991; pp 433−434.

one of which presents a lone pair to the CO; the time dependence seen in the RR spectrum could then be interpreted in terms of a slow conformational change. Another possibility is that there are two adducts, the first in which Cys80 is replaced and the second in which His18 is dislodged. The lone pair interaction of form A_{-1} would then arise from only one of the displaced ligands; the time dependence is difficult to explain with this model, however.

Conclusions

RR spectroscopy supports other spectoscopic evidence that the Cys80 side chain binds to the Fe as cysteinate in the Fe(III) form of cyt *c*-Cys80. The protein-induced heme distortion is partially relaxed by the mutation, presumably because the Cys80 side chain is shorter than the Met80 side chain and requires a different protein fold for binding. In the Fe(II) form, it is likely that Cys80 still binds, but as neutral cysteine. When CO binds, it may displace Cys80, or alternatively His18, provided the Cys80 is bound as thiol. There are two forms of the CO adduct, and in one of them there is inhibition of back-bonding, possibly reflecting a lone pair interaction with the displaced ligand.

Acknowledgment. We thank Professor John Dawson and Mr. Eric D. Coulter for a generous gift of a sample of cyt P450-Im, Dr. Adrienne Raphael for experimental assistance in the early stages of the work, and Drs. Deborah Wutke and Songzhou Hu for helpful discussions. This work was supported by grants from the National Institutes of Health (GM 33576 to T.G.S.), the National Science Foundation (CHE-9214569 to H.B.G.), and the Italian Consiglio Nazionale delle Ricerche and MURST (to G.S.).