Resonance Raman enhancement of phenyl ring vibrational modes in phenyl iron complex of myoglobin

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ABSTRACT Resonance Raman spectra are reported for the organometallic phenyl-Fe^{III} complexes of horse heart myoglobin. We observed the resonance enhancement of the ring vibrational modes of the bound phenyl group. They were identified at 642, 996, 1,009, and 1,048 cm^{-1}, which shift to 619, 961, 972, and 1,030 cm^{-1}, respectively, upon phenyl ^{13}C substitution. The lines at 642 and 996 cm^{-1} are assigned, respectively, as in-plane phenyl ring deformation mode (derived from benzene vibration No. 6a at 606 cm^{-1}) and out-of-plane CH deformation (derived from benzene vibration No. 5 at 995 cm^{-1}). The frequencies of the ring "breathing" modes at 1,009 and 1,048 cm^{-1} are higher than the corresponding ones in phenylalanine (at 1,004 and 1,033 cm^{-1}) and benzene (at 992 and 1,010 cm^{-1}), indicating that the ring C—C bonds are strengthened (or shortened) when coordinated to the heme iron. The excitation profiles of these phenyl ring modes and a porphyrin ring vibrational mode at 674 cm^{-1} exhibit peaks near its Soret absorption maximum at 431 nm. This appears to indicate that these phenyl ring modes may be enhanced via resonance with the Soret π-π^* transition. The Fe^{III}—C bond stretching vibration has not been detected with excitation wavelengths in the 406.7–457.9-nm region.

INTRODUCTION

Phenylhydrazine is a suicide substrate for hemoproteins. It inactivates hemoglobin in vivo and forms a protein-stabilized σ-bound phenyl-Fe^{III} complex (1). The phenyl group shifts from the heme iron to one of the pyrrole nitrogens as the protein denatures after adding acidic methanol (2, 3) It was found that hemoglobin-catalyzed phenylhydrazine oxidation results in hemoglobin precipitation as Heinz-body hemolytic anemias (4–7). Myoglobin also reacts with phenylhydrazine, but it does not precipitate from the solution (2). The mechanism of the hemoglobin-catalyzed phenylhydrazine reaction has been intensively studied in recent years. It has been shown that the reaction is terminated by inactivation of the prosthetic groups after each heme moiety catalyzes the consumption of six oxygen molecules and six phenylhydrazines, and the formation of five benzenes. No reaction occurs when phenylhydrazine and methemoglobin are incubated anaerobically. The partial reaction stoichiometry has been determined (2): 6 $C_6H_5NHNH_2$ + 6 O_2 + 1 heme (active) → 5 C_6H_6 + 1 heme (inactive) + N_2. Although alkylhydrazine (e.g., methyl and ethyl) also react with Hb and Mb to form σ alkyl-Fe^{III} bond, only the phenyl-Fe^{III} complex is much more stable than the alkyl-Fe^{III} complexes toward oxygen (8). Thus, the phenyl-Fe^{III} complex was chosen for the present resonance Raman studies.

The distal residues His-E7 and Val-E11 are believed to play an important role in the regulation of ligand binding affinity (9–12). The structure of the phenyl-iron Mb complex has been determined by a high resolution (1.5 Å) x-ray crystallographic method (13). It shows that these two distal residues are pushed away by the axially bound phenyl group (see Fig. 1) to create a channel from the protein surface to the interior of the heme pocket. The iron-carbon (phenyl) bond distance is 1.9 Å, which is in agreement with the bond distances observed for the Fe^{III}-carbon coordination complexes (14). The iron atom is in the plane of the heme. The plane of the phenyl ring is perpendicular to the heme plane and has an orientation angle of 50° relative to the proximal histidine. A rotation about the iron-carbon bond axis is not permissible.

The electronic absorption spectrum of phenyl-Fe^{III} Mb exhibits a Soret band at 431 nm and a band at 540 nm with a poorly resolved shoulder at ~560–570 nm (1). A comparison of the absorption spectra between phenyl-Fe^{III} Mb and Fe^{III} Mb is shown in the inset of Fig. 2.

To gain insight into the nature of the phenyl-iron interactions, we have carried out resonance Raman excitation profile studies of the phenyl-iron complex of horse heart myoglobin near Soret region. The profiles of the phenyl ring modes at 642, 996, 1,009, and 1,048 cm^{-1} (identified via ^{13}C isotope shifts) exhibit a maximum at ~440 nm, indistinguishable from that of a porphyrin ring

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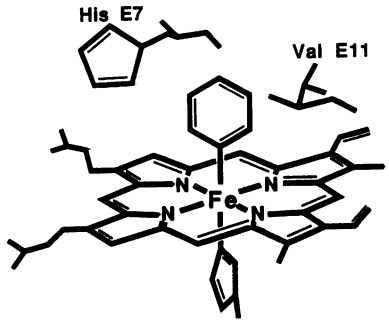

FIGURE 1 Structure of phenyl-Fe^{III} myoglobin around the heme binding site.

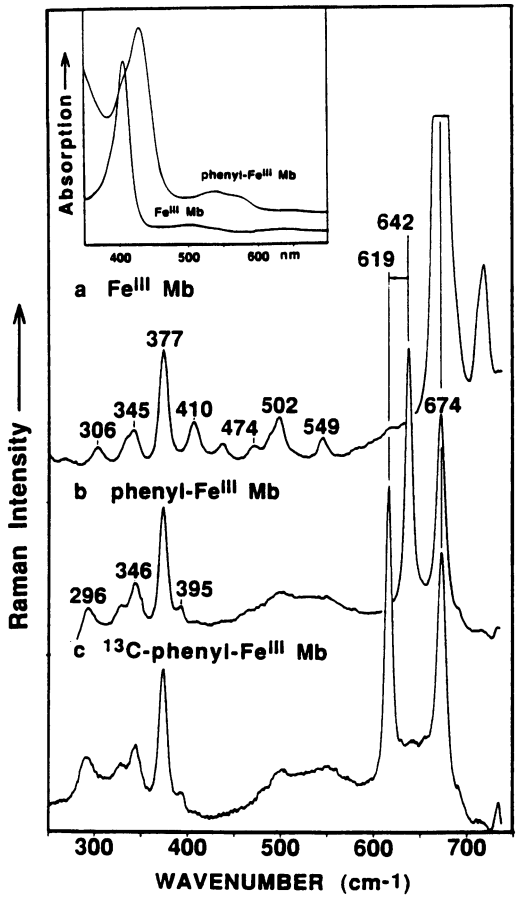

FIGURE 2 Isotope effects on resonance Raman spectrum (250–750 cm^{-1}) of phenyl-Fe^{III} Mb at pH 6.8, (*a*) Fe^{III} Mb at pH 6.8 excitation wavelength (λ_{exc}) = 406.7 nm; (*b*) phenyl-Fe^{III} Mb at pH 6.8, λ_{exc} = 441.6 nm; (*c*) ^{13}C-phenyl-Fe^{III} Mb at pH 6.8, λ_{exc} = 441.6 nm.

mode (at 674 cm^{-1}). This suggests that the phenyl ring modes may be in resonance with the Soret π–π^* transition.

MATERIALS AND METHODS

Preparation of phenyl-iron complex

The samples of horse heart myoglobin (Sigma Chemical Co., St. Louis, MO) were purified following the method described by Romero-Herrea et al. (15). The phenyl-iron Mb complex was prepared according to the procedure of Oritz de Montellano et al. (16) with some modifications. The met Mb sample (0.15 mM) in 0.2 ml of 0.1 M phosphate buffer at pH 6.8 (with 0.2 mM EDTA) was mixed with 0.2 ml phenylhydrazine (Sigma Chemical Co., St. Louis, MO) (2 mM in 0.1 M phosphate buffer at pH 6.8). The reaction was terminated when the Soret band shifted completely from 408 to 431 nm. The mixture was then centrifuged and the supernatant was passed through the MSI (Micron Separation Inc.) cameo II 25 mm disposable HPLC syringe filter (Nylon 66 membranes, 0.22-μm pore size, Thinline, 25 mm diameter, Fisher Scientific Co., Pittsburgh, PA, Cat No. DDDN-02T25-50), and was then transferred into a cylindrical quartz Raman cell (with a rubber septum) filled with N_2 gas.

Synthesis of ^{13}C-labeled phenylhydrazine

The ^{13}C-labeled aniline sample (0.1 g) (Cambridge Isotope Laboratories, Woburn, MA) was dissolved in 10 ml of 40% (vol/vol) HCl/H_2O solution. The mixture was stirred and cooled in an ice bath. The 1.2 ml of 1 M $NaNO_2$ solution was added to the mixture to allow the reaction to take place in ~2 h. The 5 ml of $SnCl_2$ reducing agent which was made up by dissolving 1 g of $SnCl_2$ in 5 ml of concentrated HCl, was slowly added into the mixture. After an additional 2 h, the mixture was analyzed by thin-layer chromatographic method (silica gel, 1:1 ether/hexane, R_f = 0.4 for amine, R_f = 0.1 for hydrazine). The product was extracted with ether. The ether solution of phenylhydrazine was dried by adding Na_2SO_4, and was concentrated to ~10 ml in a water bath. Then the oxalic acid solution (100 mg in 5 ml ether) was added and the precipitated oxalate salt was recrystallized from methanol/ether.

Resonance Raman spectroscopy

All the spectra except those used for constructing the excitation profiles were obtained with a dry ice–cooled vidicon multichannel laser Raman system (17). The laser power at the sample was <30 mW and the cell was spun throughout each measurement to avoid local heating. The resonance Raman spectra for the excitation profile studies were recorded with a conventional scanning Raman system equipped with a photomultiplier tube (model C-313034; RCA, Lancaster, PA). The following laser lines were used for excitation: 406.7 and 415.3 nm (krypton-ion laser; model 171-01 from Spectra-Physics Inc., Mountain View, CA), 441.6 nm (He-Cd laser; model 4240NB from Liconix, Sunnyvale, CA), 457.9 and 488.0 nm (argon-ion laser; CR-6 from Coherent Inc., Palo Alto, CA). All the spectra were obtained with the samples at room temperature.

RESULTS AND DISCUSSION

Resonance Raman spectra of met Mb (λ_{exc} = 406.7 nm), phenyl-Fe^{III}Mb (λ_{exc} = 441.6 nm) and ^{13}C-phenyl-Fe^{III}Mb (λ_{exc} = 441.6 nm) at pH 6.8 are compared in Fig. 2 (250–750 cm^{-1}), Fig. 3 (800–1,200 cm^{-1}), and Fig. 4 (1,300–1,650 cm^{-1}). The ^{13}C-isotope–sensitive lines were identified at 642, 996, 1,009, and 1,048 cm^{-1}, which shift to 619, 961, 972, and 1,030 cm^{-1}, respectively. These lines are therefore attributed to the ring vibrational modes of the bound phenyl group.

In Fig. 4 the so-called "oxidation state marker" of the porphyrin (ν_4) (18, 19) appears at 1,371 cm^{-1}, indicative of the Fe^{III} oxidation state for both met Mb and phenyl-Fe Mb. The Fe-C bond distances in the Fe^{III}-CN and phenyl-Fe^{III} complexes are 1.908 Å (20) and 1.9 Å (13), respectively. Because the iron in both complexes is in the same low spin state, the force constants for the two Fe^{III}-C bonds may be similar. Previously, the Fe^{III}-CN stretching frequencies were observed around 445–451 cm^{-1} (21, 22); the Fe^{III}-C(phenyl) stretching frequency should be around 250 cm^{-1} if the phenyl group is treated as a unit mass. However, we were not able to detect this line, presumably due to its weak intensity.

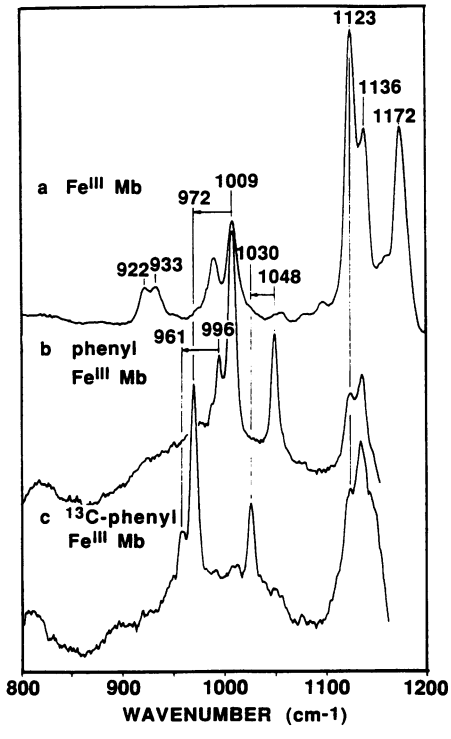

FIGURE 3 Isotope effects on resonance Raman spectrum (800–1,200 cm^{-1}) of phenyl-Fe^{III} Mb at pH 6.8. (*a*) Fe^{III} Mb at pH 6.8, λ_{exc} = 406.7 nm; (*b*) phenyl-Fe^{III} Mb at pH 6.8, λ_{exc} = 441.6 nm; (*c*) ^{13}C-phenyl-Fe^{III} Mb at pH 6.8, λ_{exc} = 441.6 nm.

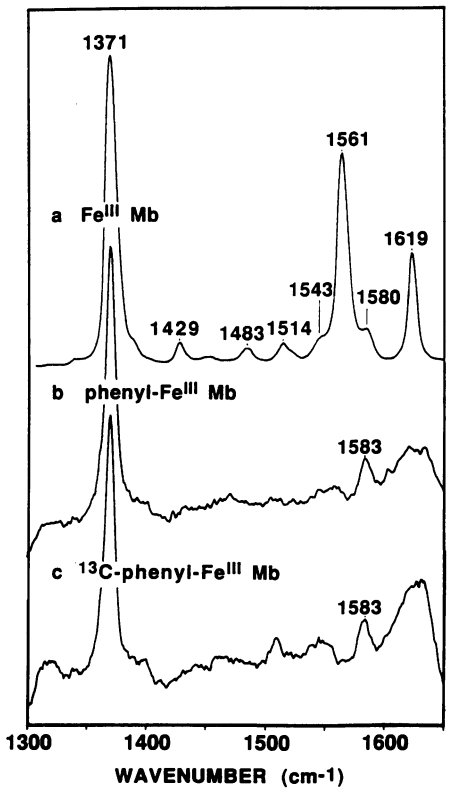

FIGURE 4 Isotope effects on resonance Raman spectrum (1,300–1,650 cm^{-1}) of phenyl-Fe^{III} Mb at pH 6.8. (*a*) Fe^{III} Mb at pH 6.8, λ_{exc} = 406.7 nm; (*b*) phenyl-Fe^{III} Mb at pH 6.8, λ_{exc} = 441.6 nm; (*c*) ^{13}C-phenyl-Fe^{III} Mb at pH 6.8, λ_{exc} = 441.6 nm.

The characteristic frequencies of the substituted benzenes in the IR and Raman spectra are well known. The normal vibrations of the complete series of monosubstituted benzene have been studied by numerous investigators (23–31). The numbering of the vibrations of substituted benzene is generally made by analogy with those proposed by Wilson (23) and extended by Langseth and Lord (24). Upon monosubstitution of the benzene molecule the symmetry is lowered from D_{6h} to C_{2v}. The symmetry species of the fundamental vibrations of the monosubstituted benzene molecule are:

$$11\,a_1(IR, R) + 10\,b_2(IR, R) + 3\,a_2(R) + 6\,b_1(IR, R)$$

in which 24 vibrations of the phenyl ring are essentially independent of the substituent attached to the ring and the other six are "x-sensitive" vibrations, i.e., in these modes the substituent moves with appreciable amplitude (32). In the spectra of the phenyl-Fe^{III} Mb (250–1,650 cm^{-1}), there are four lines identified as phenyl ring vibrations via isotope shifts. The study of the depolarization ratios showed that the lines at 642, 1,009, and 1,048 cm^{-1} are polarized ($\rho \leq 0.75$), and the line at 996 cm^{-1} is

depolarized ($\rho \sim 0.75$). The 642 cm^{-1} line is an in-plane phenyl ring deformation mode (a_1) derived from benzene vibration No. 6a (606 cm^{-1}); the one at 996 cm^{-1} is an out-of-plane CH deformation of the phenyl ring (b_1) derived from benzene vibration No. 5; the one at 1,048 cm^{-1} is a trigonal ring "breathing" mode (a_1) derived from benzene No. 12 (1,010 cm^{-1}); and the one at 1,009 cm^{-1} is a ring "breathing" vibration (a_1) derived from benzene vibration No. 1 (992 cm^{-1}). These assignments are further supported by results from resonance Raman studies of $(py)_2Fe^{II}$ porphyrin complex (33), phenylalanine (34, 35), and the monosubstituted phenyl rings (36, 37). The frequencies of the phenyl ring "breathing" modes in phenyl-Fe^{III} Mb (at 1,009 and 1,048 cm^{-1}) are higher than the corresponding ones in phenylalanine (at 1,004 and 1,033 cm^{-1}) and benzene (at 992 and 1,010 cm^{-1}), indicating that the C—C bonds of the bound phenyl ring are somewhat shorter than those in phenylalanine and benzene. Comparison of frequencies with $(py)_2Fe^{II}$(MP), phenylalanine, benzene and pyridine is given in Table 1.

We have obtained resonance Raman spectra of phenyl-Fe^{III} Mb excited at various wavelengths. The 982 cm^{-1} line of SO_4^{2-} (in the form of 2% (wt/wt) $(NH_4)_2SO_4$) serves as an internal intensity standard. The intensities of the four lines (at 642, 996, 1,009, and 1,048 cm^{-1}) and that of the internal standard were obtained after baseline subtraction and curve deconvolution. The excitation profiles (relative intensities vs. excitation wavelengths) for these four phenyl modes (Fig. 5) exhibit maxima near 440 nm. Included in Fig. 5 is the excitation profile of a porphyrin ring mode at 674 cm^{-1}, which also displays a maximum near 440 nm. Based on these data alone, one would conclude that for four phenyl ring modes observed in the spectra of phenyl-Fe^{III} Mb are enhanced via the Soret π–π^* transition. However, the enhancement of axial ligand internal ring modes via the porphyrin π–π^* transition is unusual. Equally unusual is the enhancement of an out-of-plane CH deformation mode at 996 cm^{-1}. Previously, Spiro and Burke (33) observed the enhancement of axial pyridine internal ring modes via a $Fe^{II} \rightarrow py \cdot (d_{\pi \rightarrow \pi^*})$ charge-transfer transition (at ~475 nm).

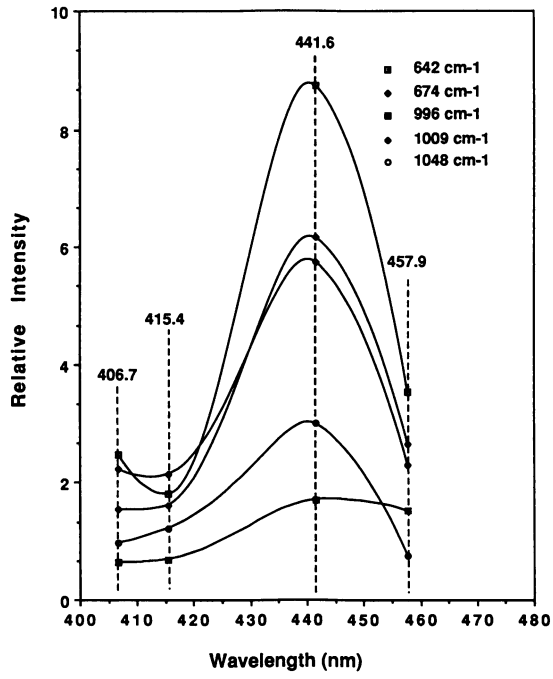

FIGURE 5 Excitation profiles of five Raman lines (642, 674, 996, 1,009, 1,048 cm^{-1}) of phenyl-Fe^{III} Mb at pH 6.8.

Thus, there is a possibility that the four phenyl ring modes reported here might also be enhanced via a charge-transfer transition near the Soret maximum. The coupling between the charge-transfer transition and the Soret transition might bring out the out-of-plane CH deformation mode at 996 cm^{-1}.

In conclusion, we show that the reaction of horse heart myoglobin with phenylhydrazine does form a stable organometallic Fe^{III}—C bond. The internal phenyl ring vibrations (at 642, 996, 1,009, and 1,048 cm^{-1}), identified via ^{13}C isotopic shifts, exhibit excitation profile maxima near 440 nm. Because the excitation profile of a porphyrin ring mode at 674 cm^{-1} also exhibits a similar maximum at 440 nm, we suggest that the Soret π–π^* transition may be responsible for the observed enhancement of the phenyl ring modes. The present unambiguous identification of

TABLE 1 Comparison of phenyl and pyridyl ring vibrational modes

	Trigonal ring "breathing"	Ring "breathing"	Out-of-plane CH deformation	In-plane ring deformation
Phe-Fe^{III} Mb	1048	1009	996	642
$(py)_2Fe^{II}$(MP)	1046	1010	—	634
Phenylalanine	1033	1006	—	622
Benzene	1010	992	995	606
Pyridine	1030	992	—	605

MP, mesoporphyrin.

these bound phenyl modes is important because these Raman signals are useful for quantitative monitoring of the reaction of hemoglobin/myoglobin with phenylhydrazine in dilute aqueous solution.

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