Resonance Raman investigation of the effects of copper binding to iron-mesoporphyrin·histidine-rich glycoprotein complexes

Randy W. Larsen,* David J. Nunez,* William T. Morgan,‡ Barry B. Muhoberac,§ and Mark R. Ondrias*
*Department of Chemistry, University of New Mexico, Albuquerque, New Mexico 87131; ‡Division of Molecular Biology and Biochemistry, University of Missouri at Kansas City, Kansas City, Missouri 64110; and §Department of Chemistry, Purdue University School of Science, Indiana University-Purdue University at Indianapolis, Indianapolis, Indiana 46205 USA

ABSTRACT Histidine-rich glycoprotein (HRG) binds both hemes and metal ions simultaneously with evidence for interaction between the two. This study uses resonance Raman and optical absorption spectroscopies to examine the heme environment of the 1:1 iron-mesoporphyrin·HRG complex in its oxidized, reduced and CO-bound forms in the absence and presence of copper. Significant perturbation of Fe^{3+}-mesoporphyrin·HRG is induced by Cu^{2+} binding to the protein. Specifically, high frequency heme resonance Raman bands indicative of low-spin, six-coordinate iron before Cu^{2+} binding exhibit monotonic intensity shifts to bands representing high-spin, five-coordinate iron. The latter coordination is in contrast to that found in hemoglobin and myoglobin, and explains the Cu^{2+}-induced decrease and broadening of the Fe^{3+}-mesoporphyrin·HRG Soret band concomitant with the increase in the high-spin marker band at 620 nm. After dithionite reduction, the Fe^{2+}-mesoporphyrin·HRG complex displays high frequency resonance Raman bands characteristic of low-spin heme and no iron-histidine stretch, which together suggest six-coordinate iron. Furthermore, the local heme environment of the complex is not altered by the binding of Cu^{1+}. CO-bound Fe^{2+}-mesoporphyrin·HRG exhibits bands in the high and low frequency regions similar to those of other CO-bound heme proteins except that the iron-CO stretch at 505 cm^{-1} is unusually broad with $\Delta\upsilon$ approximately 30 cm^{-1}. The dynamics of CO photolysis and rebinding to Fe^{2+}-mesoporphyrin·HRG are also distinctive. The net quantum yield for photolysis at 10 ns is low relative to most heme proteins, which may be attributed to very rapid geminate recombination. A similar low net quantum yield and broad iron-CO stretch have so far only been observed in a dimeric cytochrome c′ from *Chromatium vinosum*. Furthermore, the photolytic transient of Fe^{2+}-mesoporphyrin·HRG lacks bands corresponding to high-spin, five-coordinate iron as is found in hemoglobin and myoglobin under similar experimental conditions, suggesting iron hexacoordination before CO recombination. These data are consistent with a closely packed distal heme pocket that hinders ligand diffusion into the surrounding solvent.

INTRODUCTION

Elucidation of the interactions governing heme and metal binding to proteins is often crucial in understanding ligand transport and catalysis as well as the structural stability of certain proteins. Heme-protein interactions are also important in the binding, transport and degradation of heme itself, e.g., catalysis by heme oxygenase (Yoshinaga et al., 1982; Kutty and Maines, 1982). Furthermore, a number of important metalloproteins contain heteronuclear substrate binding and/or redox sites that are linked through protein-mediated interactions; Rousseau and Friedman, 1988; Trumpower, 1990). A potentially useful system for studying these many interactions is the histidine-rich glycoprotein (HRG) from rabbit serum. Although the purified protein is devoid of metal centers, its abundance of histidine residues facilitates the binding of a wide variety but specific number of heme groups and metals (Morgan, 1981; Muhoberac et al., 1988). Two thermodynamically preferred heme binding sites can be differentiated from the others on HRG by fluorescence quenching and optical absorption spectroscopy (Morgan, 1981; Burch and Morgan, 1985). Furthermore, titration of the complex formed between ferric heme and HRG with divalent metals produces reproducible, progressive perturbations of the local heme environment (Burch et al., 1988). These characteristics make HRG an attractive candidate for investigating heme-protein interactions and for the systematic examination of interactions between heterogeneous metal binding and/or redox sites in a controlled protein environment.

Rabbit serum HRG is a 94-kDa protein consisting of a 30-kDa histidine-rich domain and a 45-kDa histidine-normal domain containing aromatic residues (Morgan, 1985). HRG can bind up to 20 (±2) equivalents of Cu^{2+} and 25–30 equivalents of heme, and the 45-kDa domain contains two spectroscopically equivalent, thermodynamically preferred (low K_d) heme binding sites (Burch and Morgan, 1985). One equivalent of Fe^{3+}-mesoporphyrin bound to a preferred site on HRG (ferric meso-heme·HRG) produces a primarily low-spin complex whose optical absorption and EPR spectra indicate bis-histidine heme coordination (Muhoberac et al., 1988).

Address correspondence to Dr. Muhoberac or Dr. Ondrias.
Dr. Larsen's present address is Department of Chemistry, University of Hawaii, Honolulu, Hawaii 96822.

The binding of increasing amounts of Cu^{2+} causes specific, sequential spectral changes generally consistent with a low- to high-spin heme transition (Burch et al., 1988), but the situation is somewhat more complicated. While the 620 nm optical absorption band increases with Cu^{2+} addition, the Soret changes differ from those found in the low- to high-spin transition in ferric hemoglobin, myoglobin and many other heme proteins (Smith and Williams, 1970). Furthermore, the EPR spectrum of the 1:1 Fe^{3+}-mesoporphyrin·HRG complex exhibits a decrease in both the high-spin, $g = 6$ and low-spin, $g = 3$ resonances when > 14 equivalents of Cu^{2+} are added, as well as a series of EPR signals that are somewhat unusual and distinct from those found in hemoglobin and myoglobin (Burch et al., 1988). Similar unusual EPR signals are found with heme proteins that are less well-understood than these two globins (Aasa et al., 1976; Brudvig et al., 1981; Armstrong et al., 1983; Maltempo et al., 1979), and often such signals are not related back to the specifics of heme spin state and axial coordination. In addition, almost no information is available concerning the ferrous or ligand-bound forms of mesoheme·HRG.

We have employed resonance Raman spectroscopy for a site-specific investigation of the only partially characterized 1:1 mesoheme·HRG complex with varying amounts of bound copper. Resonance Raman spectroscopy is a powerful probe of the structural characteristics and ligand binding dynamics of a wide variety of heme complexes and proteins (Rousseau and Friedman, 1988; Spiro and Li, 1988; Spiro, 1983), and is especially well suited for this study. Indeed it can complement EPR spectroscopy in helping define axial coordination associated with the ferric high-spin and unusual EPR signals, and it provides information on ferrous and CO-bound mesoheme·HRG not attainable by EPR. More specifically, resonance Raman spectra of the 1:1 Fe^{3+}-mesoporphyrin·HRG complex indicate that the local heme environment is significantly perturbed by Cu^{2+} binding to HRG, which produces five-coordinate iron instead of six-coordinate as found in ferric hemoglobin and myoglobin. In contrast, spectra of the 1:1 Fe^{2+}-mesoporphyrin·HRG complex display little sensitivity to Cu^{1+} binding. Furthermore, the distinctive CO binding dynamics of the ferrous complex suggest a local environment that is closely packed supporting the uniqueness of the thermodynamically preferred mesoheme binding sites.

MATERIALS AND METHODS

Rabbit HRG was prepared by the method of Morgan and stored lyophilized at 5°C (Morgan, 1981). All HRG samples were prepared fresh in 0.05 M phosphate buffer, pH 7.4. HRG concentrations were determined spectrophotometrically using 55 mM^{-1} cm^{-1} at 277 nm. Fe^{3+}-mesoporphyrin (Porphyrin Products, Logan Utah) was dissolved in Me_2SO (Aldrich Chemical Co., Milwaukee, WI) and its concentration determined using 170 mM^{-1} cm^{-1} at 394 nm. The amount of Me_2SO in the mesoheme·HRG solution was $< 5\%$ (Muhoberac et al., 1988). $CuCl_2$ (Sigma Chemical Co., St. Louis, MO) was dissolved in pH 3.5 deionized water to prevent hydrolysis. The Cu^{2+} concentration was determined by the absorptivity of its chelate with bathocuproine sulfonate (GFS Chemicals, Columbus Ohio) using 13.9 mM^{-1} cm^{-1} at 479 nm (Welcher, 1958).

The interaction of Cu^{2+} with the 1:1 Fe^{3+}-mesoporphyrin·HRG complex was investigated by titrating the appropriate amount of Cu^{2+} solution into 200 μL of complex and incubating for ~5 min at room temperature. In this manner, a series of Fe^{3+}-mesoporphyrin·HRG·Cu^{2+} complexes with overall stoichiometries of 1:1:16 and greater were produced.

Two methods were employed to generate the dithionite reduced, 1:1:x Fe^{2+}-mesoporphyrin·HRG·Cu^{1+} complexes. The first method involved creating the 1:1 Fe^{3+}-mesoporphyrin·HRG complex and subsequent degassing with five cycles of vacuum/scrubbed N_2 in an anaerobic optical cell. A slight excess of solid sodium dithionite (Aldrich Chemical Co.) was added followed by the addition of the appropriate amounts of Cu^{2+}. Both of these additions were under an N_2 atmosphere. For the second method, Cu^{2+} was titrated directly into the 1:1 Fe^{3+}-mesoporphyrin·HRG complex using absorption spectra to verify the Cu^{2+} binding. The sample was then degassed with five cycles of vacuum/N_2 followed by addition of a slight excess of solid dithionite. Both methods yielded identical absorption and resonance Raman spectra for the fully reduced complexes. To prepare the CO-bound complex, Fe^{2+}-mesoporphyrin·HRG was flushed with five cycles of vacuum/CO and given a final CO atmosphere of ~60 Torr.

Because it was found that Cu^{1+} addition (i.e., addition of Cu^{2+} in conjunction with dithionite) had little effect on the resonance Raman spectra of the 1:1 Fe^{2+}-mesoporphyrin·HRG complex, a procedure was developed to verify that Cu^{1+} does indeed bind to the complex. The 1:1:20 Fe^{3+}-mesoporphyrin·HRG·Cu^{2+} complex was prepared and characterized by optical absorption spectroscopy. The sample (~0.5 mL) was reduced anaerobically, applied to the top of a N_2-equilibrated Sephadex G-50 column (5 × 1 cm), and eluted anaerobically with 0.05 M phosphate buffer, pH 7.4. The elutant was equilibrated with O_2, which reoxidized the complex, and again characterized by optical spectroscopy. The ratio of absorbances at 620 nm (high-spin) to 520 nm (low-spin) before and after column chromatography was used to estimate the amount of Cu^{1+} that remained bound to HRG as it passed through the column. This technically difficult procedure verified at least 85% Cu^{1+} retention.

Optical absorption spectra were recorded on a Hewlett-Packard HP8452A diode array UV/visible spectrometer. Resonance Raman spectra were obtained using instrumentation described in detail by Findsen and Ondrias (1988). Briefly, an N_2-pumped dye laser (Molectron UV-24/DL-14, Laser Photonics, Orlando, Florida) served as the excitation source. The scattered light was collected and dispersed through a SPEX 1403 double monochromator, and the signal was detected with a Hamamtsu R928 photomultiplier. All resonance Raman spectra are unsmoothed scans recorded at 0.5 cm^{-1} steps (2.5 s/cm^{-1}) with a spectral bandpass of approximately 10 cm^{-1}.

RESULTS

Oxidized mesoheme·HRG complexes

Fig. 1 shows the visible absorption spectra of the Fe^{3+}-mesoporphyrin·HRG complex titrated with varying

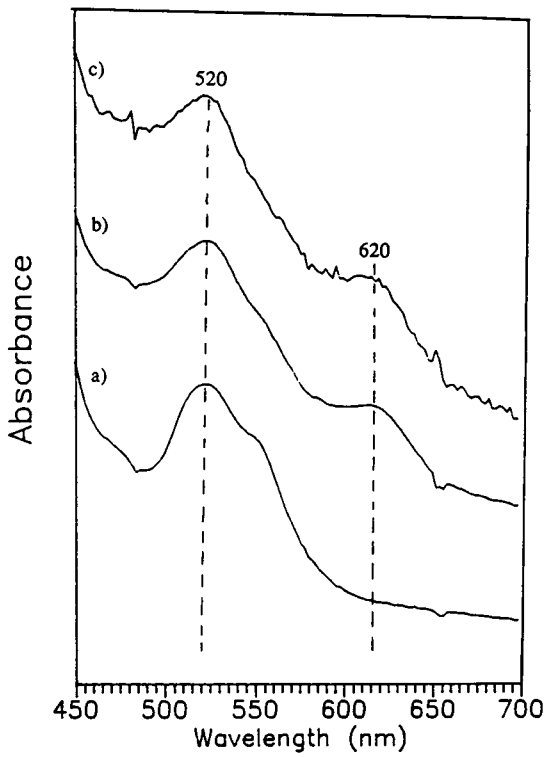

FIGURE 1 Visible absorption spectra of the 1:1 Fe^{3+}-mesoporphyrin·HRG complex titrated with (*a*) no Cu^{2+}, (*b*) 16 equivalents of Cu^{2+}, and (*c*) 24 equivalents of Cu^{2+}. Sample concentration was ~100 μM HRG in 0.05 M phosphate buffer, 7.4.

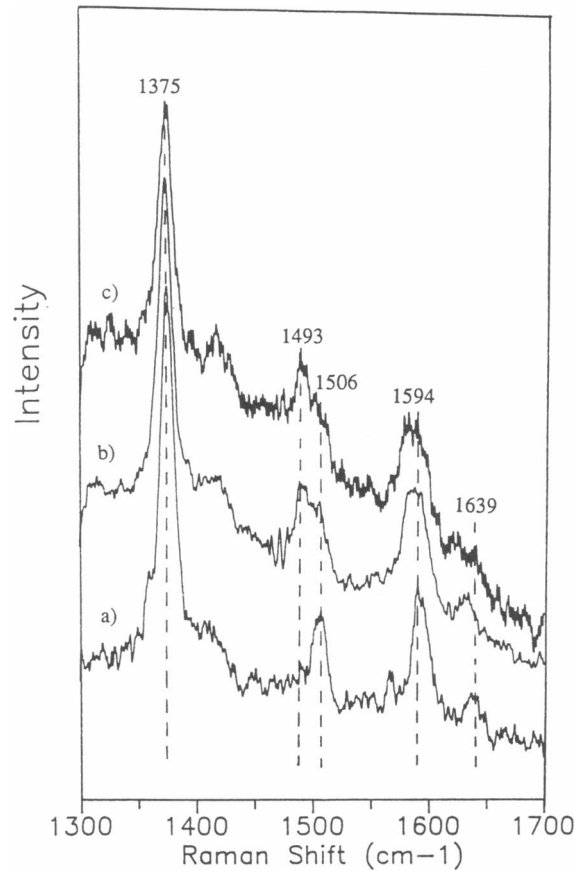

FIGURE 2 High frequency resonance Raman spectra of the 1:1 Fe^{3+}-mesoporphyrin·HRG complex titrated with (*a*) no Cu^{2+}, (*b*) 16 equivalents of Cu^{2+}, and (*c*) 24 equivalents of Cu^{2+}. Excitation frequency was 406 nm (0.3 mJ/pulse). Sample conditions were the same as those in Fig. 1.

amounts of Cu^{2+}. The spectrum of the complex without Cu^{2+} exhibits a band at 520 nm and is similar to those of low-spin heme proteins with bis-histidine coordination (Smith and Williams, 1970). Addition of 16 equivalents of Cu^{2+} to Fe^{3+}-mesoporphyrin·HRG produces a band at 620 nm. An absorption band in this region can be induced in cytochrome b_5 and cytochrome *c* upon replacement of the sixth ligand with H_2O (Sugiyama et al., 1980; Kaminsky et al., 1972) and is considered a marker for a low- to high-spin transition.

Fig. 2 shows the high frequency resonance Raman spectra of the Fe^{3+}-mesoporphyrin·HRG complex titrated with Cu^{2+}. The spectrum of the complex in the absence of Cu^{2+} is consistent with other low-spin ferric heme complexes with ν_4 ~ 1,375 cm^{-1}, ν_3 ~ 1,506 cm^{-1}, ν_2 ~ 1,594 cm^{-1} and ν_{10} ~ 1,639 cm^{-1}. The positions of the spin-state sensitive modes, ν_3, ν_2 and ν_{10}, are very similar to those in heme proteins with low-spin, six-coordinate iron (Spiro and Strekas, 1974). The spectrum of the Fe^{3+}-mesoporphyrin·HRG complex with 16 equivalents of Cu^{2+} exhibits a mixture of high- and low-spin vibrations with a broad ν_3 at ~1,493 cm^{-1} (high-spin) and a shoulder at ~1,506 cm^{-1} (low-spin). The shift of ν_2 to ~1,583 cm^{-1} is also indicative of high-spin ferric hemes. Upon addition of 24 equivalents of Cu^{2+} to the complex, the high-spin vibrations become more pronounced and the low-spin intensities at 1,594 cm^{-1} (ν_2) and 1,506 cm^{-1} (ν_3) decrease further. A prominent shift in ν_{10} (from 1,639 to 1,624 cm^{-1}) also accompanies the addition of 24 equivalents of Cu^{2+}.

The resonance Raman spectra of the intermediate frequency region (320–520 cm^{-1}) with 0, 16, and 24 equivalents of Cu^{2+} added are shown in Fig. 3, and they are dominated by the prominent band at ~350 cm^{-1} (ν_8). No bands in the region of iron-ligand vibrations (450 to 520 cm^{-1}) were resolved, however such bands are often weakly enhanced (Spiro, 1983).

Reduced mesoheme·HRG complexes

The visible absorption spectra of the Fe^{2+}-mesoporphyrin·HRG complex titrated with increasing amounts of Cu^{1+} are shown in Fig. 4. Unlike the oxidized spectra, the reduced absorption bands display no signifi-

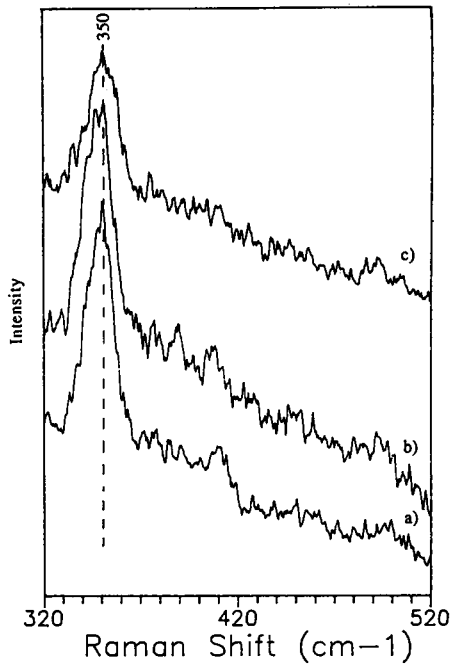

FIGURE 3 Resonance Raman spectra of the intermediate frequency region of the 1:1 Fe^{3+}-mesoporphyrin·HRG complex titrated with (*a*) no Cu^{2+}, (*b*) 16 equivalents of Cu^{2+}, and (*c*) 24 equivalents of Cu^{2+}. Sample and spectral conditions were the same as those in Figs. 1 and 2.

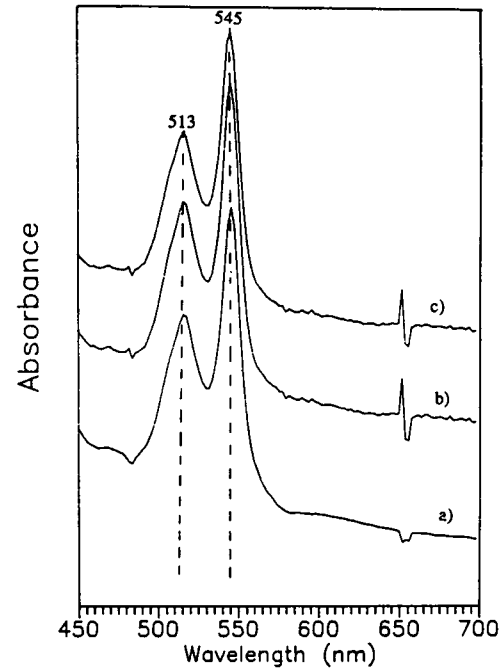

FIGURE 4 Visible absorption spectra of the dithionite-reduced, 1:1 Fe^{2+}-mesoporphyrin·HRG complex titrated with (*a*) no Cu^{1+}, (*b*) 16 equivalents of Cu^{1+}, and (*c*) 24 equivalents of Cu^{1+}. Sample conditions were the same as those in Fig. 1.

cant Cu^{1+} dependence and remain indicative of a low-spin ferrous heme complex (Smith and Williams, 1970). However, the binding of Cu^{1+} to HRG was verified by the inability of column chromatography to remove more than 15% of the copper. (See Materials and Methods.)

Figs. 5 and 6 display the high and low frequency regions of the resonance Raman spectra of the Fe^{2+}-mesoporphyrin·HRG complex with varying amounts of added Cu^{1+}. The high frequency region of the spectrum is characteristic of low-spin hemes, regardless of the amount of Cu^{1+} with $\nu_4 \sim 1{,}357\ cm^{-1}$, $\nu_3 \sim 1{,}493\ cm^{-1}$ and $\nu_2 \sim 1{,}600\ cm^{-1}$. Interestingly, ν_2 in Fe^{2+}-mesoporphyrin·HRG titrated with 24 equivalents shifts to 1,605 cm^{-1} with a shoulder at 1,600 cm^{-1}. The low frequency region of the complex is also unaffected by the addition of Cu^{1+} and exhibits modes at $\sim 265\ cm^{-1}$ (ν_9), $\sim 348\ cm^{-1}$ (ν_8), $\sim 387\ cm^{-1}$ and $\sim 420\ cm^{-1}$ (both pyrrole folding modes). Furthermore, this region displays no iron-histidine stretching mode between 200 and 240 cm^{-1} regardless of the amount of Cu^{1+} added.

CO-bound mesoheme·HRG complexes

The visible absorption spectra of the CO-bound Fe^{2+}-mesoporphyrin·HRG complex with 0, 16, and 24 equivalents of Cu^{1+} added are shown in Fig. 7. As with the reduced complex, the CO-bound form of Fe^{2+}-mesoporphyrin·HRG displays no Cu^{1+}-dependent spectral alterations, and the spectrum is consistent with those of other low-spin, CO-bound ferrous heme complexes (Tsubaki et al., 1982). The small absorption at 620 nm with 24 equivalents of Cu^{1+} is likely due to a minor component of high-spin Fe^{3+}-mesoporphyrin·HRG caused by the presence of excess copper.

Fig. 8, *a* and *b*, shows the high frequency region of the resonance Raman spectra of the CO-bound Fe^{2+}-mesoporphyrin·HRG complex with 0 and 24 equivalents of added Cu^{1+}. The CO-bound complex exhibits vibrational modes consistent with other low-spin, CO-bound hemes with $\nu_4 \sim 1{,}367\ cm^{-1}$ and $\nu_2 \sim 1{,}600\ cm^{-1}$. Fig. 9, *a* and *b*, displays the intermediate frequency region of the CO-bound complex with 0 and 24 equivalents of added Cu^{1+}, and the mode(s) at $\sim 505\ cm^{-1}$ is consistent with those found in other CO-bound heme proteins (Tsubaki et al., 1982). The absence of the vibrational mode(s) at $\sim 505\ cm^{-1}$ in the CO-free complex (Fig. 9 *e*) provides additional evidence that this vibration is due to CO coordination to the iron. The low frequency spectrum is given in Fig. 10, *a* and *b*. No

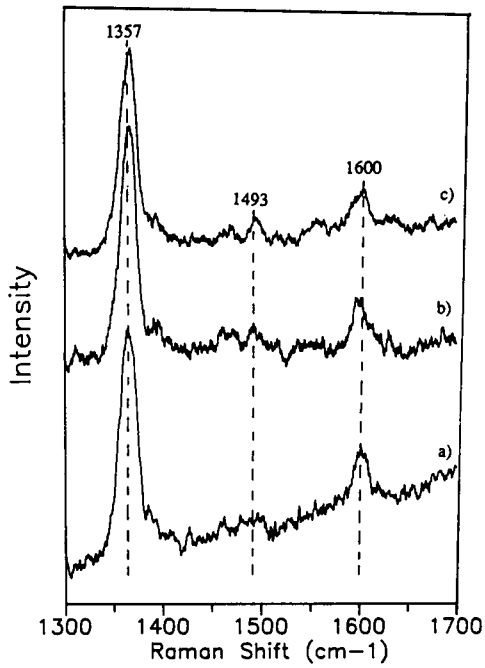

FIGURE 5 High frequency resonance Raman spectra of the dithionite-reduced, 1:1 Fe^{2+}-mesoporphyrin·HRG complex titrated with (*a*) no Cu^{1+}, (*b*) 16 equivalent of Cu^{1+}, and (*c*) 24 equivalents of Cu^{1+}. Excitation frequency was 410 nm (0.3 mJ/pulse). Sample conditions were the same as those in Fig. 1.

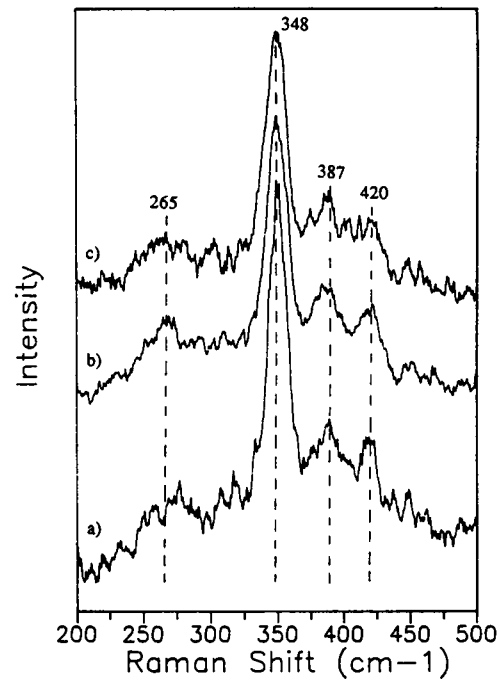

FIGURE 6 Low frequency resonance Raman spectra of the dithionite-reduced, 1:1 Fe^{2+}-mesoporphyrin·HRG complex titrated with (*a*) no Cu^{1+}, (*b*) 16 equivalents of Cu^{1+}, and (*c*) 24 equivalents of Cu^{+}. Sample and spectral conditions were the same as those in Figs. 1 and 5.

significant changes are observed for any of the ferrous heme modes upon the addition of Cu^{1+}.

Fig. 8, *c* and *d*, shows the high frequency region of the CO-photolyzed Fe^{2+}-mesoporphyrin·HRG complex with 0 and 24 equivalents of Cu^{1+} added. Upon CO photolysis, at least two distinct photolytic transients are present in the spectra. Vibrational modes at ~1,367 cm^{-1} (ν_4), ~1,503 cm^{-1} (ν_3) and ~1,603 cm^{-1} (ν_2) correspond to the low-spin, CO-bound heme complex, whereas modes at ~1,357 cm^{-1} (ν_4), ~1,470 cm^{-1} (ν_3) and ~1,585 cm^{-1} (ν_2) are indicative of a high-spin, 6-coordinate heme ion. Low frequency spectra of the CO-photolyzed Fe^{2+}-mesoporphyrin·HRG complex with 0 and 24 equivalents of added Cu^{1+} are shown in Fig. 10, *c* and *d*. These spectra are nearly identical to that of Fe^{2+}-mesoporphyrin·HRG without exposure to CO, further indicating that the photolytic transients contain a large population of low-spin, six-coordinate heme iron. The intermediate frequency region of the photolytic transient with 0 and 24 equivalents of Cu^{1+} is displayed in Fig. 9, *c* and *d*. The spectra are similar to those of the equilibrium CO-bound species in the iron-carbon stretching region, except that the mode located at ~505 cm^{-1} in the ligated species is shifted to ~500 cm^{-1} in the photolytic transient. In general, there are no clear differences between the spectra of the photolyzed complex with and without added Cu^{1+}.

DISCUSSION

Oxidized mesoheme·HRG complexes

The binding of a single equivalent of ferric mesoheme to HRG has been shown to produce a low-spin complex with bis-histidine coordination (Muhoberac et al., 1988). Studies of rose bengal (a halogenated fluorescein derivative) displacement by heme demonstrated that the 45-kDa, histidine-normal domain contains two thermodynamically preferred heme binding sites which are spectroscopically equivalent (Burch and Morgan, 1985). This equivalency is confirmed by resonance Raman spectra of both the oxidized and reduced 1:1 mesoheme·HRG complexes without bound copper, which display vibrational modes whose bandwidths are consistent with a single environment of low-spin heme. Low equivalents of Cu^{2+} bound to HRG produce type II copper EPR resonances that originate from the 30-kDa, histidine-rich domain (Muhoberac et al., 1988). The binding of >5 equivalents of Cu^{2+} initiates interactions that directly influence the environment of the heme sites

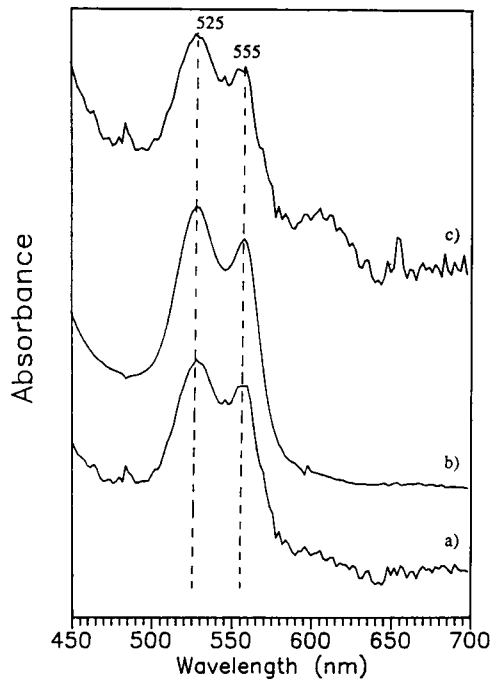

FIGURE 7 Visible absorption spectra of the dithionite-reduced, CO-bound, 1:1 Fe^{2+}-mesoporphyrin·HRG complex titrated with (*a*) no Cu^{1+}, (*b*) 16 equivalents of Cu^{1+}, and (*c*) 24 equivalents of Cu^{1+}. Sample conditions were the same as those in Fig. 1.

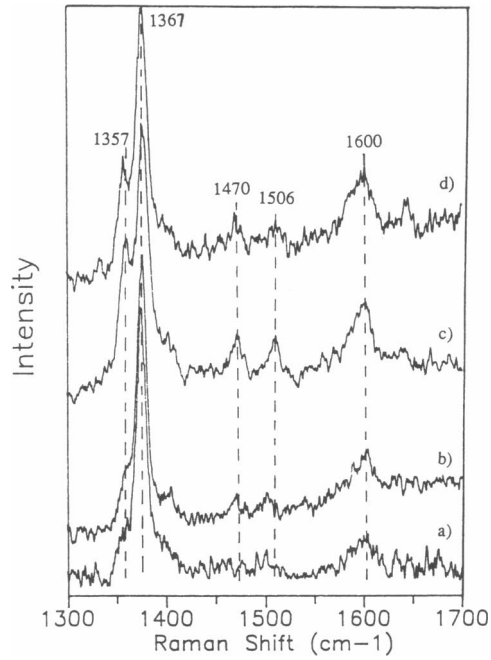

FIGURE 8 High frequency resonance Raman spectra for the CO-bound and photolytic transient forms of the 1:1 Fe^{2+}-mesoporphyrin·HRG complex: (*a*) CO-bound complex without Cu^{1+}, (*b*) CO-bound complex titrated with 24 equivalents of Cu^{1+}, (*c*) photodissociated complex without Cu^{1+}, and (*d*) photodissociated complex titrated with 24 equivalents of Cu^{1+}. Excitation frequency was 410 nm (0.1 mJ/pulse for CO-bound form and 0.8 mJ/pulse for photolytic transient). Sample conditions were the same as those in Fig. 1.

on the 45-kDa domain (Burch et al., 1988). The Cu^{2+}-induced increase in the 620-nm high-spin marker band is generally consistent with a low- to high-spin heme transition, and this increase is paralleled by a decrease in the Soret absorbance which continues throughout the addition of 20 equivalents. However, the low- to high-spin transition in hemoglobin and myoglobin is accompanied by an increase in Soret absorbance (Smith and Williams, 1970) contrasting that found in the 1:1 mesoheme·HRG complex. Furthermore, the Cu^{2+}-induced changes in the heme EPR spectra are complicated (as discussed in more detail below) and result in signals with poorly understood origins. Thus, only the axial coordination of the Cu^{2+}-free protein is clearly defined.

Burch et al. (1988) observed two optical transitions in the Soret region of the 1:1 Fe^{3+}-mesoporphyrin·HRG complex with binding of Cu^{2+} which were paralleled by distinct changes in the heme EPR spectrum. Addition of the first 10 equivalents of Cu^{2+} produces a monotonic decrease in the low-spin, $g = 3$ resonance concomitant with a similar increase in the high-spin rhombic, $g = 6$ resonance, and this transition is marked by a Soret isosbestic point at 392.5 nm. Addition of ~ 14 equivalents of Cu^{2+} produces maximum intensity of the $g = 6$ resonance with 30% of the $g = 3$ resonance remaining. A second optical transition is observed with the addition of > 14 equivalents of Cu^{2+} which yields a decrease in the $g = 6$ resonance, a continued decrease in the $g = 3$ resonance, and new unusual signals at $g = 9.7$, 7.7, and 4.8. By addition of 20 equivalents, no $g = 3$ resonance remains. This transition occurs over the range of Cu^{2+} concentration that produces a second isosbestic point at 386.5 nm, and thus, the optical and EPR spectra were interpreted in terms of at least three kinds of heme axial coordination (Burch et al., 1988). Still, the origin(s) of these unusual EPR signals is (are) unclear and may involve specific coordinations and/or conformations at the heme site including iron-copper interactions. Similar unusual EPR signals are found with the mammalian cardiac cytochrome *c* oxidase (Aasa et al., 1976; Brudvig et al., 1981; Armstrong et al., 1983) which contains an iron-copper bridging ligand of uncertain identity (Scott, 1989).

The resonance Raman data from the 1:1 Fe^{3+}-mesoporphyrin·HRG complex titrated with varying amounts of Cu^{2+} differentiate between only two heme species, which suggests the complicated optical absorption and EPR changes of Burch et al. (1988) may not

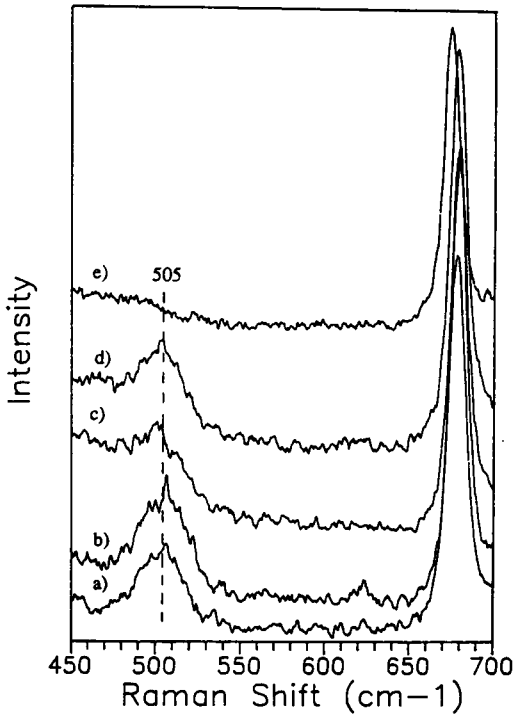

FIGURE 9 Intermediate frequency resonance Raman spectra for the CO-bound, photolytic transient, and CO-free forms of the 1:1 Fe^{2+}-mesoporphyrin·HRG complex: (*a*) CO-bound complex without Cu^{1+}, (*b*) CO-bound complex titrated with 24 equivalents of Cu^{1+}, (*c*) photodissociated complex without Cu^{1+}, (*d*) photodissociated complex titrated with 24 equivalents of Cu^{1+}, and (*e*) CO-free complex without Cu^{1+}. Sample and spectral conditions were the same as those in Figs. 1 and 8.

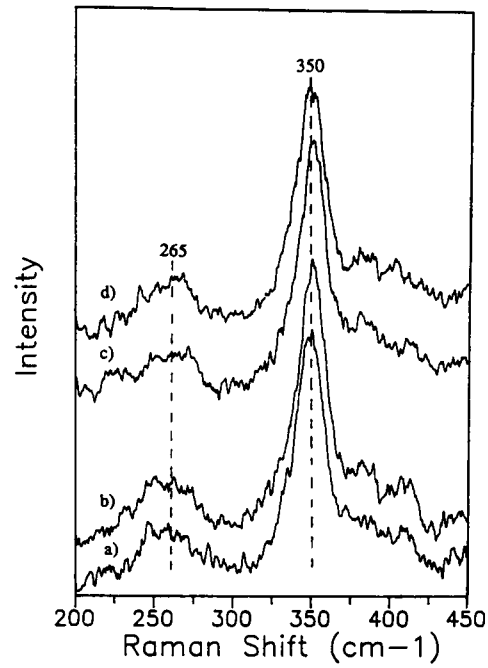

FIGURE 10 Low frequency resonance Raman spectra for the CO-bound and photolytic transient forms of the 1:1 Fe^{2+}-mesoporphyrin·HRG complex: (*a*) CO-bound complex without Cu^{1+}, (*b*) CO-bound complex titrated with 24 equivalents of Cu^{1+}, (*c*) photodissociated complex without Cu^{1+}, and (*d*) photodissociated complex titrated with 24 equivalents of Cu^{1+}. Sample and spectral conditions were the same as those in Figs. 1 and 8.

require the three kinds of axial coordination that they outlined. The high frequency Raman spectrum of the complex without bound Cu^{2+} is indicative of low-spin, six-coordinate heme iron consistent with bis-histidine coordination. Upon addition of 16 equivalents of Cu^{2+}, high-spin bands appear in the core-size sensitive region of the spectrum (1,500–1,650 cm^{-1}). Both ν_3 and ν_2 show broadening associated with the formation of a high-spin, five-coordinate heme site consistent with iron bound to a single histidine. This coordination is in contrast to six-coordinate iron of hemoglobin and myoglobin, and explains the decreasing Soret absorbance as the iron in the HRG complex becomes high-spin. More specifically, the results are similar to those found with the unusual invertebrate myoglobin isolated from *Aplysia limacina* which contains five-coordinate iron (Bolognesi et al., 1985), displays a prominent 1,491 cm^{-1} marker band (Rousseau et al., 1989), and exhibits a broad, low-absorptivity Soret that contrasts six-coordinate, vertebrate myoglobins (Giacometti et al., 1981).

After binding of 16 equivalents of Cu^{2+}, significant intensity is still observed for low-spin Raman bands in the HRG complex indicating that complete conversion to high-spin heme is not achieved. This incomplete conversion (as well as the dominance of high-spin vibrational intensity over that of low-spin) is in general agreement with the simultaneous presence of $g = 6$ and 3 EPR resonances near 14 equivalents outlined above. However, the presence of low-spin modes in the Raman spectra after addition of 24 equivalents of Cu^{2+}, which exceeds the binding capacity of the protein, shows that a population of low-spin, six-coordinate heme sites remains after the disappearance of the resonance at $g = 3$. Two explanations for these changes can be suggested with the simplest involving only the two kinds of axial coordination described above (bis- and mono-histidine). Here, the observation by Raman of low-spin heme at high equivalents of Cu^{2+} is consistent with a population of bis-histidine remaining throughout the titration and with dipolar coupling either broadening away the $g = 3$ resonance or converting it into one of the unusual EPR signals. It follows that the unusual signals may arise at least in part from dipolar interactions between low-spin heme and copper without axial ligand exchange. Thus, these signals should not be exclusively assigned to

high-spin iron even though the low-spin, $g = 3$ resonance has disappeared by addition of 20 equivalents.

An alternative explanation involves three kinds of axial coordination as suggested by Burch et al. (1988) mainly through Soret band changes. Here, the low-spin heme at high equivalents of Cu^{2+} observed by Raman is the end product of a progression from a low-spin (bis-histidine) through high-spin (mono-histidine) back to low-spin iron. This progression is in agreement with the maximization of the $g = 6$ resonance and its subsequent decrease. The empty sixth coordination site could rebind histidine becoming low-spin, but the $g = 3$ resonance is absent. Furthermore, this rebinding is stastically unlikely because Cu^{2+} competes strongly for histidines and since the 45-kDa domain to which the heme is bound is relatively low in histidine content versus that of carboxylate. Indeed, the 45-kDa domain contains a high percentage of Asx and Glx residues relative to the 30-kDa domain and to other proteins (Muhoberac et al., 1988; Morgan, 1985). Coordination of the carboxylate-containing axial ligand formate to myoglobin causes a spin-state equilibrium at room temperature with a population of low-spin heme (15%) that can be quantitated optically (Smith and Williams, 1968). However at EPR temperatures, formate produces only a $g = 6$ resonance (Sono and Dawson, 1982). Furthermore, because a carboxylate-containing ligand is more likely to bridge metal ions at high Cu^{2+} equivalents, the unusual EPR signals in HRG may involve an iron-copper exchange interaction. Unfortunately, the resonance Raman data do not identify precisely the sixth ligand. Specifically, neither the low frequency (data not shown) nor the intermediate frequency regions display vibrational bands that can be assigned to a particular axial ligand. Still with either explanation, the spin state transition characterized by Burch et al. (1988) must be extended to include low-spin mesoheme at 20 equivalents and this low-spin heme must be taken into account in further analysis of the optical and EPR spectra.

Reduced mesoheme·HRG complexes

When the 1:1:16 and copper-saturated Fe^{3+}-mesoporphyrin·HRG·Cu^{2+} complexes are reduced with dithionite, the high-spin resonance Raman bands found in the oxidized forms are no longer evident. Indeed, the spectra in the high frequency region are characteristic of a low-spin ferrous heme complex, regardless of the amount of copper bound. The low frequency region of the Raman spectrum of the Fe^{2+}-mesoporphyrin·HRG·Cu^{1+} complex supports the low-spin heme assignment. The spectral region between 200 and 250 cm^{-1} is of particular interest in heme-containing proteins since it may exhibit the iron-histidine stretching vibration. In general, this vibration is strongly enhanced through Soret excitation for high-spin heme complexes, whereas for low-spin complexes the resonance enhancement decreases considerably (Choi et al., 1983). The low frequency Raman spectrum of the Fe^{2+}-mesoporphyrin·HRG·Cu^{1+} complex displays no vibrational modes that may be attributable to an iron-histidine stretch. Thus, it is probable that the mesoheme has bis-histidine coordination in this complex. Such coordination indicates that the conformational changes induced at the heme site by copper binding to the ferric mesoheme·HRG complex are predicated upon the oxidation states of the metal centers. The high- to low-spin transition at the mesoheme could arise directly from a change in ligand affinity between ferric and ferrous heme. Alternatively, the mesoheme coordination change may result from more global conformational changes of the protein that are coupled to the copper oxidation state. A similar interaction has been observed in cardiac cytochrome c oxidase in which modification of the ligand environment of Cu_A (the low potential copper) is consistent with a high- to low-spin conversion at the ligand binding heme a_3 (Larsen et al., 1989). In addition, Thomson et al. (1985) suggested that reduction of Cu_A and/or heme a causes changes in the distance between heme a_3 and Cu_B.

CO-bound mesoheme·HRG complexes

Incubation of dithionite-reduced, 1:1 Fe^{2+}-mesoporphyrin·HRG with CO produces changes in the optical absorption spectra indicative of a CO-bound mesoheme complex. Furthermore, the CO-bound complex displays no changes upon addition of up to 24 equivalents of Cu^{1+}. The high and low frequency regions of the resonance Raman spectrum of the CO-bound complex are also independent of mesoheme:copper ratio and are quite similar to those of other CO-bound heme proteins (Tsubaki et al., 1982) such as hemoglobin and myoglobin. The spectra are consistent with a low-spin heme complex and a π-acceptor ligand at the sixth position.

The iron-CO vibrational modes provide an informative spectroscopic probe of the distal heme pocket as well as of the nature of the fifth axial ligand (Tsubaki et al., 1982; Rousseau and Friedman, 1988). The iron-CO stretching mode has been examined in a variety of heme proteins and ranges from 537 cm^{-1} for horseradish peroxidase (at low pH) to 464 cm^{-1} for P-450_{cam} from *P. putida* (Li and Spiro, 1988). The CO-bound Fe^{2+}-mesoporphyrin·HRG complex displays a broad band at $\sim$505 cm^{-1} independent of the amount of bound Cu^{1+} that is tentatively assigned as the $\nu_{Fe\text{-}CO}$ stretch. The position of this band is similar to the $\nu_{Fe\text{-}CO}$ stretch found

in hemoglobin (507 cm^{-1}) and differs from that of the CO-bound Fe^{2+}-protoporphyrin dimethylester imidazole complex (Li and Spiro, 1988). The fact that the band at 505 cm^{-1} is quite broad ($\Delta\nu \sim 30$ cm^{-1}) relative to other heme proteins ($\Delta\nu \sim 15$–20 cm^{-1}) indicates a pronounced inhomogeneity in mesoheme-CO bonding in the complex. However, it is unlikely that this broadening arises from differences between heme binding sites in HRG because this would affect other heme modes as well (see above). More likely, the linewidth of the iron-CO band reflects variability in distal pocket interactions present at both preferred mesoheme binding sites. This suggests that significant constraints exist within the distal heme pocket of the Fe^{2+}-mesoporphyrin·HRG complex.

The behavior of the $\nu_{Fe\text{-}CO}$ (stretch) and $\delta_{Fe\text{-}C\text{-}O}\beta$ (bend) modes in heme-CO resonance Raman spectra are critically dependent upon bonding geometry (Li and Spiro, 1988; Yu and Kerr, 1988; Yu et al., 1983). The preferential perpendicular-linear binding of CO minimizes the resonance enhancement of the $\delta_{Fe\text{-}C\text{-}O}$ bending mode because of symmetry restrictions. Specifically, the $\delta_{Fe\text{-}C\text{-}O}$ mode is of Γ_2 symmetry and does not couple to the porphyrin π-π^* transitions. On the other hand, distal perturbations of heme-CO geometry that result in a bent configuration reduce the symmetry and allow enhancement of $\delta_{Fe\text{-}C\text{-}O}$. The absence of a $\delta_{Fe\text{-}C\text{-}O}$ mode in the 520–600 cm^{-1} region of the CO-bound mesoporphyrin·HRG complex suggests a linear geometry. This absence is in contrast to the modes found at 578 cm^{-1} in hemoglobin and myoglobin. The inhomogeneous broadening of $\nu_{Fe\text{-}CO}$ in the HRG complex most probably results from distal pocket constraints which produce tilting of a linear Fe-C-O (relative to the heme plane). Studies of heme-CO model complexes demonstrated that relatively small variations in tilt angle can produce significant shifts in the $\nu_{Fe\text{-}CO}$ frequency (Yu and Kerr, 1988).

Photolytic transients of mesoheme·HRG complexes

Transient resonance Raman spectroscopy provides a useful probe for investigating heme pocket and ligand binding dynamics in heme-containing proteins. Ligand photolysis of CO-bound heme complexes produces transient high-spin, five-coordinate heme iron on a picosecond timescale (Rousseau and Friedman, 1988). Several processes may occur subsequent to photodissociation. First, CO may rapidly recombine with the heme reforming the ligand-bound complex in a process known as geminate recombination. Depending upon the tertiary structure of the distal heme pocket, CO may diffuse into the surrounding solvent and hence recombine slowly in a bimolecular process. Finally, protein relaxation to an equilibrium, unliganded geometry may occur before the bimolecular religation.

The photolytic transients of the Fe^{2+}-mesoporphyrin·HRG complex contain a large population of CO-bound heme (regardless of the amount of Cu^{1+}) even under high incident laser flux ($\sim$110 mJ/pulse cm^2). This is evident in the intensity of ν_4 for the CO-bound form ($\sim$1,367 cm^{-1}). The apparently low quantum yield for CO photolysis of the complex may be attributed to very rapid (subnanosecond) geminate recombination occurring within the laser pulse (<10 ns). A similar low net quantum yield for CO photolysis has also been observed in a dimeric cytochrome *c'* from *Chromatium vinosum* and was attributed to a closely packed, hydrophobic distal heme pocket that inhibits ligand diffusion into the surrounding solvent (Hobbs et al., 1990). This is consistent both with the HRG fluorescence studies, which indicate that the preferred heme binding sites are hydrophobic (Burch and Morgan, 1985), and with the distribution of iron-CO frequencies, which may originate from hydrogen bonding of iron-coordinated CO to a nearby residue in the distal pocket (Li and Spiro, 1988).

The intermediate frequency region of the photolytic transients of the Fe^{2+}-mesoporphyrin·HRG complex (in the absence and presence of Cu^{1+}) is similar to that of the CO-bound complex. The photodissociated species displays a broad band at $\sim$505 cm^{-1} with a reduced intensity relative to the unphotolysed complex which was obtained under low laser flux. This indicates that the CO that geminately recombines within the laser pulse width binds to a heme with a distal pocket geometry and trans-axial coordination which are quite similar to that of the equilibrium, liganded species. This is consistent with time-resolved resonance Raman studies of CO recombination in hemoglobin which have shown that during the geminate phase of recombination (<500 ns), the iron-CO frequency (as well as band width) does not change in the rebound species. (unpublished results)

The photolytic transients of the Fe^{2+}-mesoporphyrin·HRG complex also display a population of high-spin, six-coordinate heme iron. This is most evident in the high frequency region of the resonance Raman spectrum in which vibrational bands at 1,358 cm^{-1} (υ_4 for unliganded ferrous heme) and 1,470 cm^{-1} (υ_3 for high-spin, six-coordinate heme) are clearly distinguished. The low frequency Raman spectra support this assignment (i.e., that of a high-spin, six-coordinate heme) because they show no evidence for high-spin, five-coordinate, transient heme iron. Both hemoglobin and myoglobin as well as other heme proteins exhibit well enhanced iron-histidine stretching vibrations in the low frequency region of their respective photolytic transients (Rous-

seau and Friedman, 1988). The lack of any vibrational mode attributable to an iron-histidine stretch may be due to the presence of a weak field ligand that rapidly binds to the mesoheme (< 10 ns) before CO recombination.

CONCLUSION

This resonance Raman study characterizes the iron spin state and coordination in oxidized and reduced mesoheme·HRG·Cu complexes, and provides models for both heme binding sites and protein-mediated, heme-metal interactions. Mesoheme·HRG exhibits apparent copper-induced ligation state changes at the heme sites that may be related to the ligand environment of the copper and/or its oxidation state. The Fe^{2+}-mesoporphyrin·HRG complex contains high-spin, five-coordinate iron as found in *Aplysia* myoglobin, and is thus in contrast to the axial coordination in almost all other hemoglobins and myoglobins. In addition, the Fe^{2+}-mesoporphyrin·HRG complex displays extremely rapid geminate recombination indicative of a close-packed, hydrophobic distal heme pocket similar to that found in a dimeric cytochrome *c'* from *Chromatium vinosum*. The characteristics of this pocket may also be causal in providing a weakly coordinating transient after CO photolysis and in keeping the iron five-coordinate in high-spin Fe^{3+}-mesoporphyrin·HRG·Cu^{2+}. The variety of metals and hemes that can be complexed to HRG should provide a wide range of HRG-based models for continuing investigations of heme proteins.

This work was supported by National Institutes of Health grants GM33330 (M. R. Ondrias), HL37570 (W. T. Morgan), and an institutional grant (B. B. Muhoberac).

Received for publication 19 August 1991 and in final form 2 December 1991.

REFERENCES

Aasa, R., S. P. J. Albracht, K. Falk, B. Lanne, and T. Vänngård. 1976. EPR signals from cytochrome *c* oxidase. *Biochim. Biophys. Acta.* 422:260–272.

Armstrong, F., R. W. Shaw, and H. Beinert. 1983. Cytochrome *c* oxidase: time dependence of optical and EPR spectral changes related to the oxygen-pulsed form. *Biochim. Biophys. Acta.* 722:61–71.

Bolognesi, M., A. Coda, G. Gatti, P. Ascenzi, and M. Brunori. 1985. Crystal structure of ferric *aplysia limacina* myoglobin at 2.0 Å resolution. *J. Mol. Biol.* 183:113–115.

Brudvig, G. W., T. H. Stevens, R. H. Morse, and S. I. Chan. 1981. Conformations of oxidized cytochrome *c* oxidase. *Biochemistry.* 20:3912–3921.

Burch, M. K., and W. T. Morgan. 1985. Preferred heme binding sites of histidine-rich glycoprotein. *Biochemistry.* 24:5919–5924.

Burch, M. K., B. B. Muhoberac, and W. T. Morgan. 1988. Characterization of Cu^{2+} and Fe^{3+}-mesoporphyrin complexes with histidine-rich glycoprotein: evidence for Cu^{2+}–Fe^{3+}-mesoporphyrin interaction. *J. Inorg. Biochem.* 34:135–148.

Findsen, E. W., and M. R. Ondrias. 1988. The design of a versatile nanosecond transient and time-resolved Raman spectrometer. *App. Spectros.* 42:445–448.

Giacometti, G. M., P. Ascenzi, M. Brunori, G. Rigatti, G. Giacometti, and M. Bolognesi. 1981. Absence of water at the sixth co-ordination site in ferric aplysia myoglobin. *J. Molec. Biol.* 151:315–319.

Hobbs, J. D., R. W. Larsen, T. E. Meyer, J. H. Hazzard, M. A. Cusanovich, and M. R. Ondrias. 1990. Resonance Raman characterization of *chromatium vinosum* cytochrome *c'*: effect of pH and comparison of equilibrium and photolyzed carbon monoxide species. *Biochemistry.* 29:4166–4174.

Kaminsky, L. S., M. J. Byrne, and A. J. Davison. 1972. Iron ligands in different forms of ferricytochrome *c*: the 620 nm band as a probe. *Arch. Biochem. Biophys.* 150:355–361.

Kutty, R. K., and M. D. Maines. 1982. Oxidation of heme *c* derivatives by purified heme oxygenase. *J. Biol. Chem.* 257:9944–9952.

Larsen, R. W., M. R. Ondrias, R. A. Copeland, P. M. Li, and S. I. Chan. 1989. Resonance Raman studies of Cu_A-modified cytochrome *c* oxidase. *Biochemistry.* 28:6418–6422.

Li, W. Y., and T. G. Spiro. 1988. Is bound CO linear or bent in heme proteins? Evidence from resonance Raman and infrared spectroscopic data. *J. Am. Chem. Soc.* 110:6024–6033.

Maltempo, M. M., P.-I. Ohlsson, K.-G. Paul, L. Petersson, and A. Ehrenberg. 1979. Electron paramagnetic resonance analyses of horseradish peroxidase in situ and after purification. *Biochemistry.* 18:2935–2941.

Morgan, W. T. 1981. Interactions of the histidine-rich glycoprotein of serum with metals. *Biochemistry.* 20:1054–1061.

Morgan, W. T. 1985. The histine-rich glycoprotein of serum has a domain rich in histidine, proline, and glycine that binds heme and metals. *Biochemistry.* 24:1496–1501.

Muhoberac, B. B., M. K. Burch, and W. T. Morgan. 1988. Paramagnetic probes of the domain structure of histidine-rich glycoprotein. *Biochemistry.* 27:746–752.

Rousseau, D. L., Y.-c. Ching, M. Brunori, and G. M. Giacometti. 1989. Axial coordination of ferric *aplysia* myoglobin. *J. Biol. Chem.* 264:7878–7881.

Rousseau, D. L., and J. M. Friedman. 1988. Transient and cryogenic studies of photodissociated hemoglobin and myoglobin. *In* Biological Applications of Raman Spectroscopy. T. G. Spiro, editor. John Wiley and Sons, Inc., New York. Vol. 3. 133–214.

Scott, R. A. 1989. X-ray absorption spectroscopic investigation of cytochrome *c* oxidase structure and function. *Annu. Rev. Biophys. Biophys. Chem.* 18:137–158.

Smith, D. W., and R. P. J. Williams. 1968. Analysis of the visible spectra of some sperm-whale ferrimyoglobin derivatives. *Biochem. J.* 110:297–301.

Smith, D. W., and R. P. J. Williams. 1970. The spectra of ferric haems and haemoproteins. *Struct. Bonding.* 7:1–45.

Sono, M., and J. H. Dawson. 1982. Formation of low spin complexes of ferric cytochrome *P*-450-CAM with anionic ligands. *J. Biol. Chem.* 257:5496–5502.

Spiro, T. G. 1983. The resonance Raman spectroscopy of metalloporphyrins and heme proteins. *In* Iron porphyrins. A. P. B. Lever, and H. Gray, editors. Addison-Wesley, New York. 89–159.

Spiro, T. G., and X Y. Li. 1988. Resonance Raman spectroscopy of metalloporphyrins. *In* Biological Applications of Raman Spectroscopy. T. G. Spiro, editor. John Wiley and Sons, Inc., New York. 1–38.

Spiro, T. G., and T. C. Strekas. 1974. Resonance Raman spectra of heme proteins: effects of oxidation and spin state. *J. Am. Chem. Soc.* 96:338–345.

Sugiyama, T., R. Miura, T. Yamano, K. Shiga, and H. Watari. 1980. A reversible spin conversion of cytochrome b_5 at high temperatures. *Biochem. Biophys. Res. Commun.* 97:22–27.

Thomson, A. J., C. Greenwood, P. M. A. Gadsby, J. Peterson, D. G. Eglinton, B. C. Hill, and P. Nicholls. 1985. The structure of the cytochrome a_3-Cu_B site of mammalian cytochrome *c* oxidase as probed by MCD and EPR spectroscopy. *J. Inorg. Biochem.* 23:187–197.

Trumpower, B. L. 1990. Cytochrome bc_1 complexes of microorganisms. *Microbiol. Rev.* 54:101–129.

Tsubaki, M., R. B. Srivastava, and N. T. Yu. 1982. Resonance Raman investigation of carbon monoxide bonding in (carbon monoxy)hemoglobin and -myoglobin: detection of Fe-CO stretching and Fe-C-O bending vibrations and influence of the quaternary structure change. *Biochemistry.* 21:1132–1140.

Welcher, T. J. 1958. The Analytical Uses of Ethylenediaminetetracedic Acid. Van Nostrand, Princeton, New Jersey. 242 pp.

Yoshinaga, T., S. Sassa, and A. Kappas. 1982. Purification and properties of bovine spleen heme oxygenase. *J. Biol. Chem.* 257:7778–7785.

Yu, N. T., and E. A. Kerr. 1988. Vibrational modes of coordinated CO, CN^-, O_2, and NO. *In* Biological Applications of Raman Spectroscopy. T. G. Spiro, editor. John Wiley and Sons, Inc. New York. 39–96.

Yu, N. T., E. A. Kerr, B. Ward, and C. Chaney. 1983. Resonance Raman detection of Fe-CO stretching and Fe-C-O bending vibrations in sterically hindered carbonmonoxy "strapped hemes": a structural probe of Fe-C-O distortion. *Biochemistry.* 22:4534–4540.