= mesityl). Thus the origin of the doubling of resonances in o-tolylmyoglobin must rest, as suggested by Ortiz de Montellano,[2] in o-tolyl-protein contacts and not in methyl-porphyrin interactions. From the spectrum of **1** (R = o-methyl, A = C_6H_5) we also conclude that the resonance corresponding to the upfield metaphenyl(iron) resonance is not detected in the o-tolylmyoglobin spectrum since it lies within the crowded diamagnetic region.

While the axial phenyl resonances in arylmyoglobin have been assigned in previous work,[1,2] the axial histidyl resonances have not been identified. On the basis of our observations on the six-coordinate adducts **2**, it can be expected that the 1-, 2-, and 4-imidazole histidine resonances will be shifted downfield from the crowded diamagnetic envelope into the 50–25 ppm region. Indeed the published spectra for phenylmyoglobin (Figure 1 of ref 1) and p- and m-tolylmyoglobin (Figure 2 of ref 2) do indeed show two resonances at ca. 30 ppm, which we believe can be assigned to the 2- and 4-protons of the axial histidine.

Experimental Section

Materials. The iron complexes **1** were prepared from the appropriate iron(III) porphyrin chloride and Grignard reagent by using an established procedure.[13] Unlike other derivatives, the o-tolyl complexes **1** were unstable particularly to column chromatography on silica gel. Therefore, these o-tolyl complexes (**1**, R = o-CH_3, A = C_6H_5 or mesityl) were generated by the reaction of o-tolylmagnesium bromide and the appropriate iron(III) complex directly in an NMR tube without further purification. All complexes were handled in a dioxygen-free, argon atmosphere in a glovebox. NMR spectra were run in deoxygenated chloroform-*d*. Imidazole adducts **2** were obtained by the addition (via a syringe) of a solution of the appropriate ligand in chloroform-*d* to a chloroform-*d* solution of **1**.

Instrumentation. NMR spectra were obtained on a Nicolet NT-360 FT spectrometer operating in the quadrature mode (^1H frequency 360 MHz). Between 200 and 1000 transients were accumulated over a 40-kHz bandwidth with 16K data points and a 6-μs 90° pulse. The signal-to-noise ratio was improved by apodization of the free induction decay, which introduced a negligible 3–10 Hz of line broadening. The peaks were referenced against tetramethylsilane.

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Registry No. $C_6H_5Fe(TpTP)$, 87607-84-9; $C_6H_5Fe(TMP)$, 99726-42-8; p-$CH_3C_6H_4Fe(TPP)$, 87621-56-5; m-$CH_3C_6H_4Fe(TPP)$, 99784-41-5; o-$CH_3C_6H_4Fe(TPP)$, 99726-43-9; 1,5-Me_2Im, 99726-44-0; 1,4-Me_2Im, 99726-45-1; 2-MeIm, 99726-46-2; 1-MeIm, 99726-47-3; Im, 99726-48-4.

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Resonance Raman Spectroscopic Evidence for Perturbation of Vinyl Modes in Copper(I)-Protoheme π-Complexes

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Copper(I) addition to aqueous micellar suspensions of ferriprotoporphyrin or its dimethyl ester causes perturbation of resonance-enhanced Raman bands associated with vibrational motions of the vinyl group substituents at the 3- and 8-positions of the porphyrin ring. No spectral perturbations occur upon adding cupric, zinc, or hexaaquochromic ions to these heme solutions or upon adding Cu(I) to ferrideuteroporphyrin. The data are interpreted to indicate Cu(I) coordination at the vinyl positions. Consistent with this view, intensity is lost in the vinyl carbon–carbon stretching region at 1615–1630 cm^{-1} and a new intensity appears at ~1530 cm^{-1}, assignable to the carbon–carbon stretch of Cu(I) π-complexed olefin bonds. Similar changes occur in this region upon reduction of oxidized cytochrome oxidases of both mitochondrial and microbial origin, suggesting that the peripheral vinyl substituents on the heme may form part of the Cu(I) ligation environment in the reduced copper–heme binuclear center comprising the oxygen reductase site.

Introduction

Protoporphyrin IX has two peripherally bound vinyl groups. The influence of these groups upon the physicochemistry of the porphyrin core is illustrated by comparing a series of deuteroporphyrins with zero, one, or two vinyl substituents. Such a series exhibits, for example, progressive red-shifting of electronic bands with increasing substitution (Figure 1).[1-3] This incremental shifting indicates that both vinyl groups conjugate with the porphyrin aromatic system; bathochromism indicates that they influence porphyrin electronic states in the expected $\pm E$ electromeric manner.[4,5] Vinyl groups also affect porphyrin vibrational properties in various ways that generally increase the number of detectable bands. Since vinyl vibrational modes are vibronically active in porphyrin electronic transitions, laser excitation within these transitions produces resonance Raman enhancement of both vinyl and porphyrin skeletal modes.[6-8] Resonance coupling occurs between some vinyl and porphyrin fundamental modes, causing band splitting and frequency shifts, which identify the coupled motions.[8,9] Attachment of the vinyl groups at the asymmetric 3,8-positions[10] in protoporphyrin IX causes symmetry lowering, which can induce Raman activity into porphyrin E_u modes forbidden under D_{4h} symmetry. Several such E_u bands have been assigned in the spectra of nickel(II) and iron(III) protoporphyrin IX.[9,11]

The modification of porphyrin physicochemistry by vinyl substituents may be important to the function of many protoheme proteins, e.g. in modulating the oxygen affinities of O_2-binding

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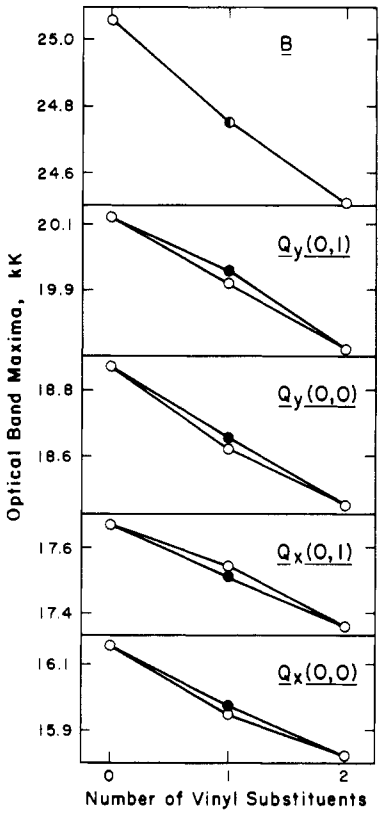

Figure 1. Optical absorption maxima of substituted deuteroporphyrin dimethyl esters in $CHCl_3$ as a function of number of vinyl substituents. Deuteroporphyrin and protoporphyrin data were obtained from ref 64; data for 3-vinyldeuteroporphyrin (open circles) and 8-vinyldeuteroporphyrin (closed circles) were obtained from ref 65. By treating the effects of peripheral substituents on the energies of the visible bands as a sum of perturbational elements (PE) upon the spectra of reference octaalkylporphyrins (OAP),[28] i.e. $[E(Q_x) + E(Q_y)] = [\sum(\text{PE})] + [E(Q_x) + E(Q_y)](\text{OAP})$, we calculate from the (0, 0) and (0, 1) data that PE(vinyl) = -332 cm^{-1}. The experimental data are exceptions to the empirical rule that only substituents on opposite pairs of pyrrole rings should be included in the summation,[28] since substitution of a second vinyl group on an adjacent ring gives a spectral shift of nearly identical magnitude.

hemes[12-14] and redox properties of cytochromes[15] and peroxidases.[16-18] A considerable body of physical evidence has accumulated which suggests that the cytochrome a_3 site in mammalian cytochrome oxidase contains a unique heme–copper binuclear center.[19] The possibility has been raised that olefinic substituents on heme *a* may form part of the Cu(I) ligand environment at this site in reduced and semireduced forms of the oxidase or otherwise mediate electron transfer between the metal ions.[20-22]

Our immediate interest is to obtain information on simple heme–copper(I) binuclear ions that might serve to identify structural features of the oxidase a_3 site. Specifically, the micellar ferri- and ferroprotoporphyrin–Cu(I) ions described here were chosen to evaluate the potential role of the heme *a* 8-vinyl substituent in Cu(I) coordination. Because the possibility also exists for Cu(I) coordination to the 1-hydroxyfarnesyl 3-substituent in heme *a*, these data are essential to identify the binding site(s) in heme *a*–Cu(I) π-ions.[23] Addition of Cu(I) to sodium dodecyl sulfate-solubilized suspensions of the protoporphyrin IX dication and other olefin-containing porphyrins and hemes gives rise to red-shifting of their optical bands.[23] Several lines of evidence implicate the peripheral vinyl substituents as the Cu(I) binding sites.[23] Such shifts are not seen for porphyrins and hemes possessing a variety of β-pyrrolic substituents, but lacking vinyl groups. Furthermore, the shifts are accompanied by the appearance of new bands in the near-ultraviolet region assignable to Cu(I) → olefin(π^*) charge-transfer excitation,[24] and other aquo metal ions are unable to induce the shifts.[23] Copper(I) coordination to olefins also causes large decreases in frequencies of their C=C stretching modes,[25,26] a consequence of increasing electron density in the olefin π^* orbitals and concomitant loss in the π-bonding orbitals attending formation of the metal–olefin bond.[27] Resonance Raman spectra reported here demonstrate this shift for the vinyl stretching mode of protoheme. Additionally, we find that Cu(I) binding perturbs virtually all of the other vibrational modes that are thought to contain some component of vinyl vibration, e.g. those sensitive to deuterium substitution of vinyl protons,[9,11] but leaves other modes unaffected. In examining published spectra of cytochrome oxidases containing reduced a_3 sites, we note that a new resonance-enhanced Raman band appears at the same frequency as measured for the C=C stretching frequency in the heme–copper(I) models, suggesting that similar Cu(I) coordination to the heme periphery may exist in the oxidase.

Materials and Methods

Except where indicated, all chemicals were reagent grade and used without further purification. Water was purified by reverse osmosis–ion exchange followed by glass distillation. Reagent solutions of Cu(I) were prepared by reduction of Cu(II) using either chromous or europous ions. Copper(I)–protoheme solutions were prepared by adding the Cu(I) reagent to micellar protoheme solutions using anaerobic syringe transfer techniques.[23,29] By the criteria of optical and Raman spectra, the two preparative methods generate chemically identical heme–Cu(I) solutions. To minimize the possibility of catalyzed Cu(I) disproportionation, glassware was soaked in concentrated nitric acid prior to use. Oxygen was removed from all reactant solutions to prevent oxidation of Cu(I) and photooxidation of the heme[30] by bubbling solutions with argon gas that had been passed over BASF Catalyst R 3-11.

Sodium dodecyl sulfate (SDS) was obtained from Aldrich and British Drug Houses, Ltd. (BDH); the Aldrich material was triply recrystallized from ethanol to remove impurities[31] and the "specially purified" BDH material was used without further treatment. Sodium dodecyl sulfate was added to aqueous acidic solutions at concentration levels above the critical micelle concentration of the detergent. Under these conditions, hemin is solubilized in its monomeric state within the hydrophobic region of the micelle.[32] This environment bears some resemblance to the physiological

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Table I. Raman Frequencies (cm^{-1}) of Protoheme and Cu(I)–Protoheme

	296 K		90 K		90 K (+0.3 M ClO_4^-)	
assignment[a,b]	–Cu(I)	+Cu(I)	–Cu(I)	+Cu(I)	–Cu(I)	+Cu(I)
ν_{10}	1634 m	1634 m	1650 m	1650 m	1650 m	1649 m
			1633 sh	1636 sh		
ν(C=C)(1)*	1634 m	1634 m	1633 m	1636 m	1632 w	~1630 vw
ν(C=C)(2)	c	c	~1617	c	1617 vw	c
ν_2*	1572 s	1572 s	1571 s	1575 s	1577 s	1577 s
ν(Cu···C=C)[d]		1528 w		1529 m		1531 m
ν_{38}	1526 vw		1529 w		1532 w	
ν_3	1503 w	1501 w	1491 m	1491 w	1513 w	1509 w
δ_s(=CH_2)(1)*	1434 m	1435 m	1435 vw	1420 vw	1435 vw	1424 vw
$\nu_4(Fe^{3+})$	1373 vs	1371 vs	1371 vs	1369 s	1371 s	1368 s
$\nu_4(Fe^{2+})$		~1360 sh				~1365 sh
δ_s(=CH_2)(2)*	c	c	c	~1335 vw	c	1335 vw
ν_{21}, δ(CH=)*	1304 w	c	1300 vw	c	1295 vw	c
ν_{13}	1225 w	1222 w	1221 w	1221 w	1224 w	1221 w
$\nu(C_b–C_\alpha)$(1)*	c	c	1168 w	1161 vw	1165 vw	1157 vw
$\nu_6 + \nu_8$	1122 w	1123 w	1119 w	1119 w	1116 w	1116 w
δ_{as}(=CH_2)*	1066 w	1066 w	1075 vw	1072 vw	1076 vw	1076 vw
γ(CH=)*	c	1009 w	c	1010 w	1004 vw	1010 vw
	756 m	756 m	752 m	~752 m	752 w	752 w
*	~722 vw	~722 vw	~726 vw	c	714 w	714 w
ν_7*	681 m	681 m	677 m	679 w	676 m	676 w
ν_{48}*	c	c	604 m	604 m	603 w	603 w
$\delta_{C_bC_\alpha C_\beta}$(1)*	c	c	418 vw	c	c	c
γ_{C_bS}	c	c	~380 vw	~380 vw	c	c
ν_8	~339 vw	~339 vw	~346 w	~346 vw	345 vw	345 vw
$\delta_{C_bC_\alpha C_\beta}$(2)*	c	c	310 w	~310 vw	313 w	294 w
ν_9	c	c	251 m	250 w	~251 w	~251 w

[a] Assignments taken from ref 11. [b] Asterisks indicate bands that shift upon vinyl deuteration (ref 11, 45, and 46). [c] Not observed. [d] Attributed to Cu(I)-coordinated vinyl stretching mode.

milieu of hemes within proteins. The SDS micellar structure is maintained upon freezing the suspensions.[33] Acidities and ionic strengths were adjusted by adding trifluoroacetic acid (TFA) and/or its sodium salt.

Room-temperature optical and Raman spectra were obtained from samples in a 1-cm optical cuvette. To minimize introduction of adventitious oxygen, the cuvette was fitted with a double-septum antechamber, which was swept with argon. Oxygen introduced while the outer septum was being pierced was purged before piercing the inner septum. The bottom face of the cuvette was polished, permitting Raman spectra to be obtained in a 90° scattering geometry by laser illumination from beneath the cell. To reduce self-absorption,[34] the distance of travel of scattered light through the solution was minimized by focusing the laser beam near the cuvette sampling window. Low-temperature Raman spectra were obtained from samples in 4-mm (o.d.) septum-sealed EPR tubes. Temperature was controlled by placing these tubes in the quartz Dewar insert of a Varian E-4540 variable temperature controller. Scattered light was sampled from the top-forward quadrant of the frozen solution in a sample tube aligned perpendicular to the scattering plane, a configuration approximating a 90° scattering geometry. The computer-controlled Raman spectrophotometer has been described previously.[35] Excitation was provided by a Spectra-Physics Model 164-01 krypton ion laser equipped with an ultrahigh-field magnet. The scattered light signal was detected on an RCA C31034A photomultiplier tube and amplified by an Ortec Model 9302 amplifier/discriminator prior to storage in the computer.

Results

Solutions of Cu(I) prepared in aqueous 0.1 M TFA contribute a single band at 1434 cm^{-1} to the room-temperature Raman spectrum.[36] In frozen solutions (~90 K) this band is absent, and is therefore also considered to be absent in the resonance Raman spectra of frozen heme–copper solutions.

The resonance Raman spectra of micellar ferri- and ferroprotoporphyrin are given in Figures 2 and 3. The ferriprotoporphyrin spectrum is typical of high-spin heme, as indicated by the positions of the vibrational bands in the core size (1550–1600 cm^{-1}) marker region. Low-temperature EPR studies have con-

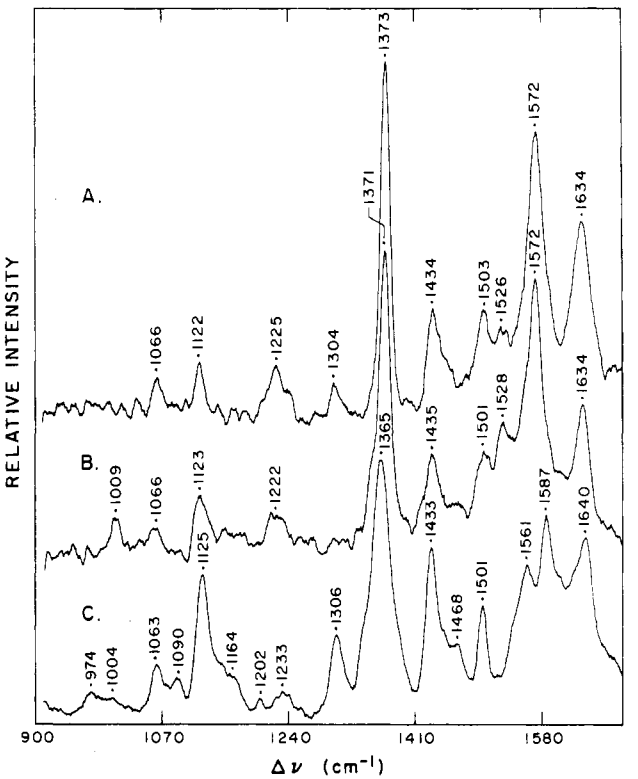

Figure 2. Resonance Raman spectra of iron protoporphyrin in 2.2% SDS and 0.1 M HTFA at 296 K: (A) 1.4×10^{-5} M ferriprotoporphyrin chloride, 6×10^{-4} M Cu(II), 1 scan at 1 cm^{-1} s^{-1}, 32 mW incident power; (B) same conditions as for part A except 3.8×10^{-4} M Cu(II), 2.2×10^{-4} M Cu(I), 2.2×10^{-4} M Cr(III); (C) ferroprotoporphyrin chloride obtained by addition of slight stoichiometric excess of Eu(II) to 2.4×10^{-5} M ferriprotoporphyrin chloride, in 0.11 M glycine, pH 2.6, 4 scans, 19 mW incident power. Laser excitation was at 406.7 nm; a 25-point smooth was applied to each spectrum.

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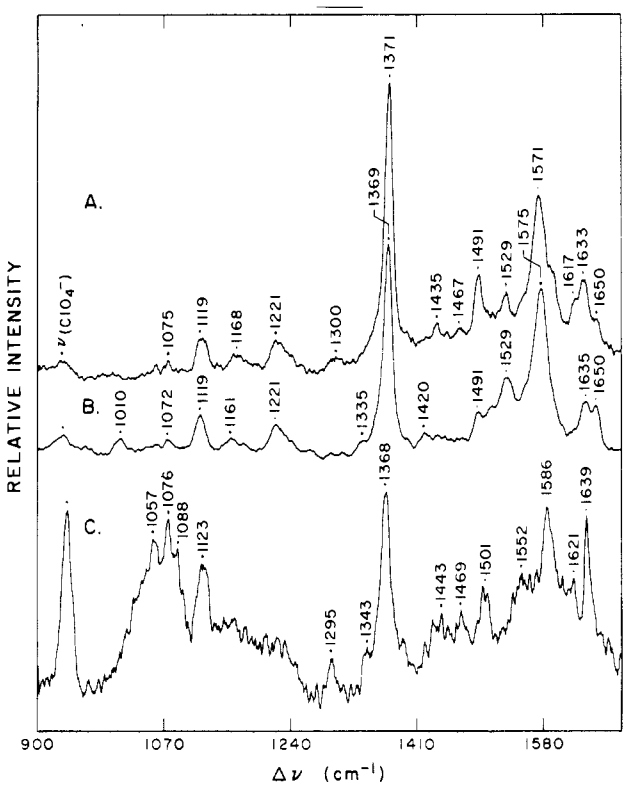

Figure 3. Resonance Raman spectra of iron protoporphyrin in 2.2% SDS at 90 K: (A) 1.0×10^{-4} M ferriprotoporphyrin chloride, 1.9×10^{-3} M Cu(II), 0.1 M HTFA, 0.06 M $NaClO_4$, 8 scans at 1 cm^{-1} s^{-1}, 34 mW incident power; (B) same conditions as for part A except 1.7×10^{-3} M Cu(II), 1.9×10^{-4} M Cu(I), 1.9×10^{-4} M Eu(III); (C) ferroprotoporphyrin chloride obtained by addition of slight stoichiometric excess of Eu(II) to 4.8×10^{-5} M ferriprotoporphyrin chloride in 0.3 M $NaClO_4$, pH 3.0 (HTFA), 8 scans at 1 cm^{-1} s^{-1}, 30 mW incident power. Laser excitation was at 406.7 nm; a 9-point smooth was applied to each spectrum.

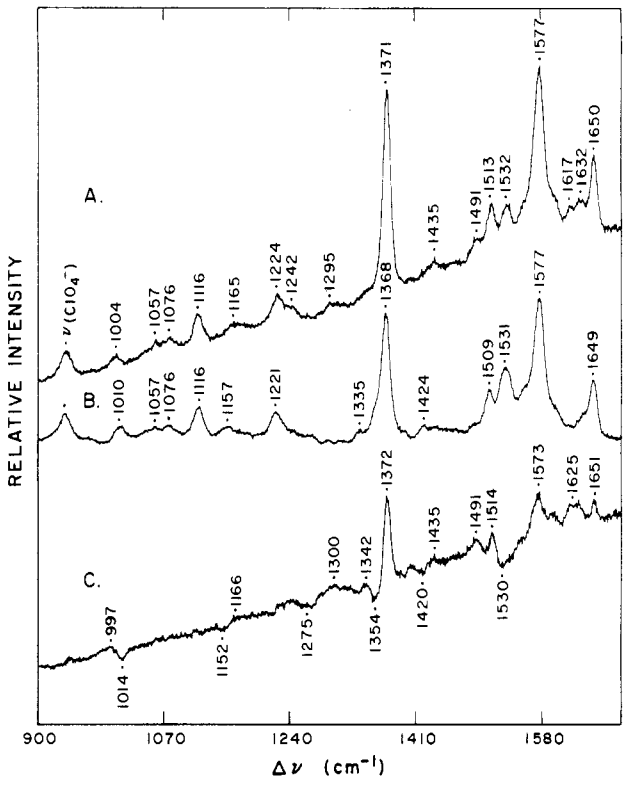

Figure 4. Resonance Raman spectra of iron protoporphyrin in 2.2% SDS at 90 K: (A) same as for Figure 3A except 0.3 M $NaClO_4$; (B) same as for Figure 3B except 0.3 M $NaClO_4$; (C) subtraction of middle spectrum from upper spectrum. Laser excitation was at 406.7 nm; spectra are presented without smoothing.

firmed this electronic spin-state assignment.[22] Ferriprotoporphyrin bands shown in Figures 2–4 are listed in Table I, using assignments taken from Spiro and co-workers.[11] The ν_{10} and vinyl symmetric stretching modes are apparently not well resolved; assuming that ν_{10} contributes to the band at 1634 cm^{-1}, the positions of the skeletal modes ν_{10}, ν_2 (1572 cm^{-1}), and ν_3 (1503 cm^{-1}) are consistent with 5-coordinate ligation of the SDS-solubilized ferriheme.[37] The band at 418 cm^{-1} that we assign to a vinyl bending mode may also contain a pyrrole folding mode;[11] however, we do not observe bands reported[11] at 1260 ($\nu_5 + \nu_9$), 694 (ν_{47}), and 373 cm^{-1} ($2\nu_{35}$), and the glass band obscures bands reported at 555 (ν_{49}) and 492 cm^{-1} (pyrrole fold). The ferroprotoporphyrin spectra (Figures 2C and 3C) are very nearly identical with the previously reported spectrum taken in aqueous cetyltrimethylammonium micelles.[38] Positions of the bands at 1640, 1501, and 1365 cm^{-1} are consistent with the SDS-solubilized ferroheme existing as the tetracoordinate intermediate spin ($S = 1$) species.[38,39]

At room temperature the Soret excitation Raman spectrum of ferriprotoporphyrin (Figure 2A) is perturbed upon adding Cu(I) (Figure 2B). Although ferriheme remains high spin, intensity is lost in the vinyl stretching mode at 1634 cm^{-1} and gained at 1528 cm^{-1}; the vinyl rocking mode at 1304 cm^{-1} disappears, and the vinyl trans wagging motion appears at 1009 cm^{-1}; the oxidation state marker band, ν_4, loses intensity at 1373 cm^{-1} but gains a shoulder at approximately 1360 cm^{-1}. No changes are detected in low-frequency bands. Oxygenation of the solution causes reversion to the original ferriprotoporphyrin spectrum.[40]

Copper(I)–protoheme spectra obtained in frozen suspensions at ~90 K demonstrate other perturbations in addition to those noted above (Figure 3A,B: Table I); line width narrowing may explain some of these effects. With added Cu(I), a shoulder at 1650 cm^{-1} is resolved; this appears to be a consequence of loss of a weak shoulder at ~1617 cm^{-1} and loss of intensity at ~1633 cm^{-1}. In addition, the core size marker at 1571 cm^{-1} (ν_2) shifts +4 cm^{-1}, the vinyl-ring stretch at 1168 cm^{-1} shifts −7 cm^{-1} and loses intensity, the vinyl asymmetric rocking mode at 1075 cm^{-1} shifts −3 cm^{-1}, and intensity is lost in the vinyl bending modes (418 and 310 cm^{-1}) and in three fundamentals [ν_7 (677 cm^{-1}), ν_8 (~346 cm^{-1}), ν_9 (251 cm^{-1})]. Since at liquid-nitrogen temperature interference from the 1434-cm^{-1} TFA solvent band is eliminated, the influence of Cu(I) on the weak vinyl scissors mode[11] is detected as a −15-cm^{-1} shift. Identical spectral shifts are obtained with the dimethyl ester of ferriprotoporphyrin.

To obtain an accurate difference spectrum relative to an internal standard, aqueous perchloric acid was added to ±Cu(I)–heme solutions and the spectra were recorded at ~90 K (Figure 4A,B). The ferriprotoporphyrin minus ferriprotoporphyrin–Cu(I) difference spectrum (Figure 4C) was obtained by subtracting the corresponding Raman spectra normalized to $\nu_1(ClO_4^-)$ at ~930 cm^{-1}. At room temperature, the ferriheme spectrum is unchanged upon addition of 0.3 M $NaClO_4$, but when these solutions are frozen, several spectral shifts occur in the high-frequency region. Specifically, below 180 K intensity is lost in the 1634-cm^{-1} region, a new band appears at 1650 cm^{-1}, and the bands at 1572, 1526, and 1503 cm^{-1} shift to slightly higher frequencies, i.e. 1577, 1532, and 1513 cm^{-1}, respectively. The ClO_4^--modified protoheme can also be detected in the ~90 K spectrum of solutions containing

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(38) Nagai, K.; Kitagawa, T.; Morimoto, H. *J. Mol. Biol.* **1980**, *136*, 271–289.
(39) Spiro, T. G.; Burke, J. M. *J. Am. Chem. Soc.* **1976**, *98*, 5482–5489.
(40) Oxygenation of Cu(I)–protoheme solutions also diminishes the absolute intensity of Raman scattering across the entire spectral region. This is most probably a consequence of degradation of a portion of the porphyrin chromophore by coupled oxidation[41] or photooxidation.[30]
(41) Bonnett, R.; McDonagh, A. F. *J. Chem. Soc. D* **1970**, 237–238. O'Carra, P. "Porphyrins and Metalloporphyrins"; Smith, K. M., Ed.; Elsevier: Amsterdam, 1975; pp 123–153.

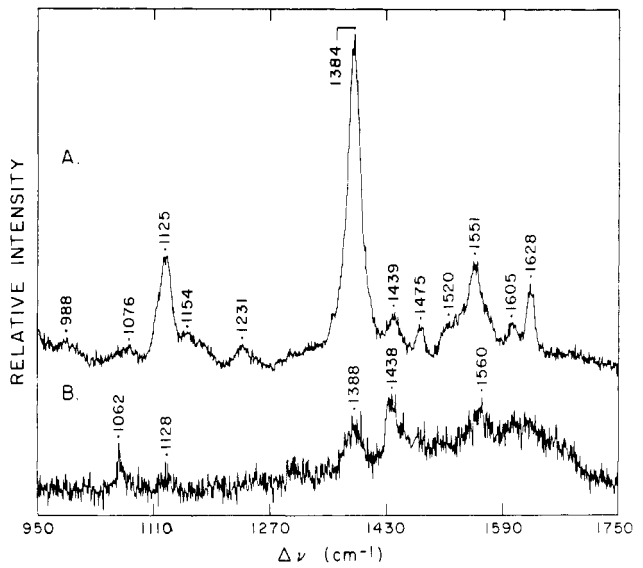

Figure 5. Resonance Raman spectra of tetraprotonated protoporphyrin in 1% SDS at 298 K: (A) 4.8×10^{-6} M protoporphyrin, 6.0×10^{-4} M Cu(II), 0.1 M HTFA, 1 scan at 1 cm^{-1} s^{-1}, 25 mW incident power at 406.7-nm excitation; (B) same conditions as in part A except 3.8×10^{-4} M Cu(II), 2.2×10^{-4} M Cu(I). Spectra are presented without smoothing at the correct relative scattering intensities.

0.06 M ClO_4^- ion (Figure 3A,B), although here the predominant species is clearly the unperturbed heme.

Although high concentrations of perchlorate ion alter the low-temperature protoheme spectra, the difference spectrum (Figure 4C) demonstrates a set of Cu(I) perturbations similar to those in the absence of perchlorate ion. Most prominently, intensity is lost in the region of the vinyl symmetric stretching band (~1630 cm^{-1}) and gained at ~1530 cm^{-1}. Negative shifts also occur for components of the vinyl scissors (1435 cm^{-1}) and vinyl group stretching modes (~1168 cm^{-1}), and the vinyl wagging mode (~1300 cm^{-1}) and a component of the vinyl stretching mode (~1617 cm^{-1}) both lose intensity, whereas a component of the vinyl scissors mode (1335 cm^{-1}) gains intensity.

Solutions of Cu(I)–protoheme are unstable, undergoing slow demetalation as indicated by changes in Raman spectra from that shown in Figure 2B to that of the tetraprotonated protoporphyrin dication (Figure 5A). Parallel changes occur in the heme optical absorption spectrum, also leading to the spectrum of the dication.[23] Heme demetalation is prevented by freezing Cu(I)–protoheme solutions. Samples stored at ~90 K appear to be stable indefinitely as judged by their Raman spectra.

No changes occur in the resonance Raman spectrum of ferriprotoporphyrin upon adding Cu(II) ion. Moreover, no changes occur in the resonance Raman spectrum of 3,8-diethyldeuteroheme with added Cu(I). However, dramatic changes appear in the resonance Raman spectrum of tetraprotonated protoporphyrin upon adding Cu(I). The multibanded porphyrin spectrum (Figure 5A) converts to an atypical, largely featureless spectrum (Figure 5B). Bands are observed at 1560, 1388, 1128, and 1062 cm^{-1}. These changes are largely reversed upon ferricyanide addition except that there is no return in intensity of the 1628-cm^{-1} band, and there is a −9-cm^{-1} shift in the 1560-cm^{-1} band.

We are presently unable to account for the observed behavior of the demetalated porphyrin. An explanation that overall weak Raman scattering is a consequence of electronic band shifts induced by Cu(I) appears untenable because their magnitude is small.[23] The high acidity of the porphyrin solution (pH ≃1) precludes metalation of the porphyrin core by Cu(I) or Cu(II), as is apparent from the optical absorption spectrum[23] and fluorescence emission intensity, which remains high following addition of the Cu(I) reagent.

Discussion

Resonance Raman spectra of protoheme solutions are perturbed by Cu(I) (Figure 2). Previous optical studies[23] suggest that these perturbations originate predominantly from Cu(I) π-bonding with vinyl substituents, with a minor contribution arising from reduction of some ferriheme by Cu(I).

Heme Reduction by Cu(I). When Cu(I) is added to protoheme, the ferriheme oxidation state marker band at 1373 cm^{-1} loses intensity and shifts to slightly lower energy (Figure 2B). Also, a weak shoulder develops at ~1365 cm^{-1}. By comparison with the spectrum of ferroprotoporphyrin (Figure 2C), whose oxidation state marker band is at 1365 cm^{-1}, these spectral changes are attributed to the reduction of a minor portion of the total ferriheme. This reduction is governed by the equilibrium

$$Cu(I) + Fe^{III}PPIX \rightleftharpoons Cu(II) + Fe^{II}PPIX \quad (1)$$

Establishment of equilibrium conditions is indicated by the following points:

(i) Manipulating the Cu(II)/Cu(I) ratio of protoheme solutions varies the intensities of the ferriheme and ferroheme oxidation state marker bands in a reciprocal manner.

(ii) In acidic media, solutions of ferriheme are stable, whereas ferroheme is unstable toward demetalation:

$$Fe^{II}PPIX + 4H^+ \rightarrow H_4PPIX^{2+} + Fe(II) \quad (2)$$

Ferroheme generated under acidic conditions via eq 1 undergoes demetalation. Subsequent redox equilibration provides further reactant for this demetalation, which goes to completion if Cu(I) is initially in excess over ferriheme. A resonance Raman spectrum of the final product from such an experiment is identical with one of the tetraprotonated porphyrin dication (Figure 5A). The rate law for demetalation is quantitatively in accord with a mechanism comprising reactions 1 and 2.[22,23]

When Cu(I) is added to protoheme, a number of changes occur in the vibrational spectra in addition to those in the oxidation state marker region. At room temperature (Figure 2), Cu(I) affects bands at 1634, 1526, 1304, and 1009 cm^{-1}; at liquid-nitrogen temperature (Figure 3 and Table I), additional bands are perturbed at 1571, 1168, 1075, 677, ~346, 310, and 251 cm^{-1}. The extent to which Cu(I) perturbs the protoheme spectrum is illustrated by the difference spectrum given in Figure 4C. Although the entire set of perturbations can be reversed by oxygen, the new Cu(I)-induced features do not resemble ferroheme (Figures 2C and 3C) and thus cannot be attributed to heme reduction. This conclusion is consistent with our kinetic study of demetalation[22,23] from which we determined the equilibrium constant for reaction 1 to be $K_1 = 1.8$. From this value we calculate for the experimental conditions of Figures 3B and 4B that ferroheme comprises less than 20% of the total heme. As a minor constituent, ferroheme should introduce bands only in the oxidation state marker region (~1365 cm^{-1}), where Raman scattering is exceptionally efficient.

It is also incorrect to attribute the various spectral changes to interactions involving non-vinyl heme substituents, e.g. propionyl groups,[42] or to incidental constituents of the Cu(I)–protoheme solutions, e.g. association of metal ions with SDS micelles.[44] This can be deduced from the tendencies of related systems to demonstrate a protoheme-like Cu(I) sensitivity. With added Cu(I), esterified protoheme shows the same Raman spectral changes as protoheme, but not 3,8-diethyldeuteroheme, which is unaltered in its resonance Raman spectrum. Also, protoheme spectra are unaffected by Cu(II), whether added as an authentic solution or

(42) Carboxylate coordination of Cu(I) is known.[43] However, carboxylic acids that contain olefin unsaturation preferentially bind Cu(I) at the olefin site without evidence of participation from the carboxylate group.[24]

(43) See, e.g.: Temussi, P. A.; Vitagliano, A. *J. Am. Chem. Soc.* **1975**, *97*, 1572–1575. Weber, P.; Hardt, H.-D. *Inorg. Chim. Acta* **1981**, *64*, L51–L53. Lukas, B.; Miller, J. R.; Silver, J.; Wilson, M. T.; Morrison, I. E. G. *J. Chem. Soc., Dalton Trans.* **1982**, 1035–1040.

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Table II. Raman Frequencies (cm^{-1}) for the Vinyl Modes of Protoheme and Other Selected Olefins[a]

	ν(C=C)		δ_s(=CH_2)		δ(CH=)		δ_{as}(=CH_2)		γ(CH=)	
	−Cu(I)	+Cu(I)	−Cu(I)	+Cu(I)	−Cu(I)	+Cu(I)	−Cu(I)	+Cu(I)	−Cu(I)	+Cu(I)
protoheme	1633 w	1529 w	1435 vw *f*	1420 vw 1335 vw	1300 vw	*f*	1074 vw	1072 vw	*f*	1010 w
pentaaquachromium(III) fumarate[b]	1660 m	1526 m	1436 vw	*f*	1275 w	1236 m	...	...	...	...
allylpyridine[c]	1640 m	1553 m	1435 vs	1445 s 1420 s	1297 w	*f*	1110 m	1095 m	993 s	1016 ms 1007 w
allyl alcohol[d]	1645 w	1550 w	1425 m	1420 m	1235 w	1230 w	1115 m	1107 m	995 s	995 s
acrolein[e]	1620 m	1530 s	1425 m	1430 m	1275 m	1240 w	1160 s	1150 s	975 s	960 s

[a] See also: Thompson, J. S.; Swiatek, R. M. *Inorg. Chem.* **1985**, *24*, 110–113 and references therein. [b] References 22 and 24. [c] Yingst, R. E.; Douglas, B. E. *Inorg. Chem.* **1964**, *3*, 1177–1180. [d] Ogura, T.; Furuno, N.; Kawaguchi, S. *Bull. Chem. Soc. Jpn.* **1967**, *40*, 1171–1174. [e] Kawaguchi, S.; Ogura, T. *Inorg. Chem.* **1966**, *5*, 844–846. [f] Not observed.

as the oxygenated product of a Cu(I)-containing solution. The foregoing circumstantial evidence suggests that the various protoheme spectral changes are due to specific interactions between Cu(I) and porphyrin vinyl substituents. This conclusion is in accord with previous arguments based on optical studies.[23]

Cu(I)–Vinyl Bonding. Direct evidence for Cu(I)–vinyl bonding is provided by the Cu(I) perturbation of vinyl-related modes. Vinyl modes have been identified by resonance Raman studies on protoheme and reconstituted protoheme proteins[11,45,46] from frequency shifts that accompany deuteration of vinyl carbons and are denoted by asterisks in Table I. Nearly all of the modes affected by Cu(I) are also vinyl-related modes.[47] Conversely, the modes not perturbed by Cu(I) contain neither local nor porphyrin-coupled vinyl vibrational components.

Vibrational data on several Cu(I)–olefins are collected in Table II. Upon Cu(I) binding, a large negative shift in frequency of the C=C stretching mode is observed. For many metal–olefin coordination compounds this shift is diagnostic of π-complex bond formation.[25,26] When Cu(I) is added to the protoheme solutions, there occurs a loss of intensity at 1615–1630 cm^{-1} and a concomitant increase at ~1530 cm^{-1} (Table II, Figures 3 and 4). The intensity increase occurs on top of an underlying weak band (ν_{38}) at 1526 cm^{-1}. However, this increase is readily observed as a prominent feature in the difference spectrum (Figure 4C). By analogy to the simple olefins (Table II), the intensity increase at 1530 cm^{-1} is anticipated if π-bonding occurs to vinyl substituents on the porphyrin. The change in intensity does not correlate with any Cu(I)-induced spin-state changes since EPR spectra of protoheme and Cu(I)–protoheme solutions indicate Fe^{3+} high-spin heme as the predominant species.[22]

Since it was shown in earlier studies[23] that Cu(I)–vinyl complexation occurs with 1:1 binding stoichiometry, only one of the two potential olefinic coordination sites is occupied in the associated ion. The apparent splitting of the 1633-cm^{-1} band into bands at 1635 and 1650 cm^{-1} observed in the ~90 K spectrum of the protoheme–Cu(I) binuclear ion (Figure 3B) may also arise as a consequence of diminished intensity in the C=C symmetric stretching mode, thereby revealing the underlying contribution at 1650 cm^{-1}. The frequency of the 1650-cm^{-1} band is high for assignment to vinyl C=C stretching modes, and instead may be ν_{10}. The ClO_4^- concentration and temperature dependence observed in the resonance Raman spectra suggest that axial ClO_4^- ligation of the heme can occur at the lower temperatures. A band assigned to ν_{10} has been observed at ~1650 cm^{-1} in an intermediate-spin ($S = {}^3/_2$) five-coordinate (perchloratoferri)octaethylporphyrin complex;[48] the ClO_4^--induced shift from 1503 to 1513 cm^{-1} in the ν_3 band (Figures 2A and 4A) and the insensitivity of the ν_4 band (1373 cm^{-1}) are also consistent with assignment of the new species as a perchlorate-bound five-coordinate intermediate-spin heme.[48]

Table III. Raman Bands of Cytochrome Oxidase[a] from 1500 to 1540 cm^{-1}

excitation wavelength, nm	Raman band, cm^{-1} oxidized enzyme	Raman band, cm^{-1} reduced enzyme	ref
406.7	1506 vw	1520 m	57
	1506 vw	1519 m	54
	1506 vw	1519 m	55
	1509 vw	1518 w[b]	*c*
413.1	~1509 vw	~1520 m	*d*
	~1504 vw	~1520 m	57
	bands absent	1525 m	*c*
	1504 vw	1520 vw	53
	1507 vw	1520 m	*e*
441.6	bands absent	1520 w	56
	not reported	~1520 w	57
	not reported	~1520 w	58
	not reported	1519 w	59
	bands absent	1517 w[f]	59
	not reported	1519 w[g]	59
	not reported	1519 w[h]	55

[a] Mitochondrial enzyme, except where noted. [b] Partially reduced form. [c] Ondrias, M. R.; Babcock, G. T. *Biochem. Biophys. Res. Commun.* **1980**, *93*, 29–35. [d] Woodruff, W. H.; Kessler, R. J.; Ferris, N. S.; Dallinger, R. F.; Carter, K. R.; Antalis, T. M.; Palmer, G. *Adv. Chem. Ser.* **1982**, *No. 201*, 625–659. [e] Copeland, R. A.; Naqui, A.; Chance, B.; Spiro, T. G. *FEBS Lett.* **1985**, *182*, 375–379; oxidized species studied include the resting, 420 nm "pulsed", 420 nm "peroxy" and 428 nm "oxygenated"forms. [f] For PS3 thermophilic bacterium. [g] For yeast (*Saccaromyces cerevisiae*). [h] For *Thermus thermophilus*.

Vibrational Coupling. For protoheme, π-bonding may be expected to perturb vibrational doublets that derive from coupling[49] between vinyl modes and other degenerate vibrations of like symmetry.[8,50] These degenerate vibrations are probably from the porphyrin macrocycle. Although vinyl–vinyl coupling is also conceivable,[9] it is not likely since seven bonds intervene between the two protoporphyrin vinyl groups. A similar separation distance in conjugated polyenes weakens vibrational coupling between such groups.[51] Self-consistent HMO calculations also predict a rapid decrease in vibrational coupling between two groups as the number

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(46) Three deuteration-sensitive modes of nickel(II) protoporphyrin (ν_{38}, ν_{29}, ν_{20})[9] and one of myoglobin(F^-) (ν_{44})[11] do not appear to be resonance enhanced in Soret excitation spectra of iron protoporphyrin. The glass band obscures two deuteration-sensitive modes that have been reported for myoglobin(F^-) (ν_{49}, pyrrole ring fold at 472 and 501 cm^{-1}) and for the bis(imidazole) complex of iron protoporphyrin (pyrrole ring fold at 488 and 507 cm^{-1}).

(47) There appear to be only a few exceptions to this generalization. Cu(I) addition shifts the oxidation state marker band by ~ −3 cm^{-1}, presumably owing to partial reduction of heme according to eq 1. In addition, intensity changes are observed in bands at 346 and 251 cm^{-1} not known to be associated with vinyl modes. However, these intensity changes are not reproduced in spectra obtained at higher ClO_4^- concentrations (0.3 M), possibly due to axial ligation effects. On the other hand, a dramatic shift of the 310-cm^{-1} vinyl bending mode (−19 cm^{-1}) was observed only at the higher ClO_4^- concentrations (Table I).

(48) Teraoka, J.; Kitagawa, T. *J. Phys. Chem.* **1980**, *84*, 1928–1935.

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(50) Adar, F.; Erecinska, M. *Arch. Biochem. Biophys.* **1974**, *165*, 570–580.

(51) Curry, B.; Broek, A.; Lugtenberg, J.; Mathies, R. *J. Am. Chem. Soc.* **1982**, *104*, 5274–5286.

of intervening bonds increases.[52]

Cu(I) π-Bonding in Cytochrome Oxidase. The vinyl stretching mode of hemes *a* and a_3 in oxidized cytochrome oxidase has been assigned to a band at 1626 cm^{-1}.[53] Since other bands overlap in this region, it is difficult to quantitate the intensity change of the 1626-cm^{-1} band upon reducing the enzyme. However, in the region 1500–1540 cm^{-1}, the appearance of a moderately intense band at ~1520 cm^{-1} is readily observed in reduced oxidases of both mitochondrial and microbial origin (Table III). This new band is similar to the Cu(I)–protoheme band at ~1530 cm^{-1} arising from the shifted vinyl stretching mode. Examination of resonance Raman spectra of a large number of heme proteins reveals that a redox-dependent spectral behavior of this sort is unique to cytochrome oxidase. The behavior of the oxidase appears to be analogous to Cu(I)–protoheme. On this evidence, we suggest Cu(I)–olefin π-bonding may occur in the reduced oxidase and should be considered in structural descriptions of the reduced $Cu_B \cdots a_3$ site.

The evidence in support of this notion is only valid insofar as copper-free heme *a* models do not exhibit analogous spectral behavior. Published spectra are somewhat ambiguous with regard to this point. Reduced heme *a* model compounds in a variety of organic and aqueous solvents show a band in the 1500–1520-cm^{-1} region, whose intensity and frequency are strongly dependent on excitation wavelength. For example, resonance Raman spectra of the bis(imidazole) adduct of heme a^{2+} show a medium-intensity band at 1518 cm^{-1} with 406.7-nm excitation.[53-55] As the excitation wavelength increases, however, this band appears to shift gradually in frequency and intensity, becoming a weak feature at 1504 cm^{-1} with 457.9-nm excitation.[53] During the shift of this band, no new feature appears in the region between 1520 and 1540 cm^{-1}. Analogous behavior is not observed for cytochrome oxidase. Resonance Raman spectra of reduced cytochrome oxidase using 441.6–457.9-nm excitation lines have been reported by several investigators to contain a band of moderate intensity at 1520 cm^{-1}.[53,55-59] The presence of this band in the reduced oxidase supports our proposed assignment.

Recent structural evidence from EXAFS spectroscopy is also consistent with this interpretation.[60] For some preparations of the resting form of the oxidized enzyme, scattering from the cytochrome a_3 site suggests that the metal ions are separated by a distance of about 3.7 Å.[61] However, in the reduced particle, EXAFS gives no indication of close proximity of the metal centers,[62] suggesting that the copper has moved to a site at a distance greater than 4 Å from the heme iron. Binding at the 8-vinyl substituent would place the heme–copper distance at 5.5–6.5 Å, depending upon the orientation of the vinyl group with respect to the heme plane. Much evidence exists indicating that relatively large conformational changes accompany changes in oxidation state of the oxidase;[19,63] the notion that the copper binding site might also change is therefore reasonable.

The micellar Cu(I)–protoheme binuclear ions described here are models for the oxygen binding site of cytochrome a_3.[19,22] We are currently investigating Cu(I)–heme *a* ions, for which optical evidence also indicates π-bonding.[23] Comparing the physical properties and dynamic behavior of heme *a* with those of protoheme will provide a basis for evaluating the physiological role of the heme *a* farnesyl substituent in Cu(I) binding and electron transfer.

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Registry No. Cu, 7440-50-8; ClO_4^-, 14797-73-0; cytochrome oxidase, 9001-16-5; tetraprotonated protoporphyrin, 493-90-3; iron protoporphyrin, 14875-96-8.

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