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Chromophore Interactions in Flavocytochrome c_{552}: A Resonance Raman Investigation†

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ABSTRACT: Resonance Raman spectra with both Soret and visible excitation have been obtained for *Chromatium* flavocytochrome c_{552} and its isolated diheme subunit under varying conditions of pH and inhibitor binding. The spectra are generally consistent with previously established classification schemes for porphyrin ring vibrations. The presence of covalently bound flavin in the protein was apparent in the fluorescent background it produced and in flavin-mediated photoeffects observed in heme Raman spectra obtained at high laser power. No flavin modes were present in the Raman spectra, nor was any evidence of direct heme–flavin interaction found by using this technique; however, a systematic perturbation of heme B_{1g} vibrational frequencies was found in the oxidized holoprotein. The heme vibrational frequencies of c_{552} are compared to those of the diheme peptide and of other *c*-type cytochromes. They are consistent with an interpretation that involves pH-dependent changes in axial ligation and treats the hemes and flavin as isolated chromphores communicating via protein-mediated interactions.

Multicenter redox enzymes are necessary for proper function in a variety of biological systems. The importance of this class of proteins is underscored by the fact that often there are unusually severe kinetic or thermodynamic barriers to rapid and efficient progress in the reactions they catalyze. Examples include the role of nitrogenase in nitrogen fixation, of cytochrome oxidase in mitochondrial oxygen reduction, and of the manganese-containing protein involved in photosynthetic water oxidation. These proteins have attracted considerable recent interest because of the complex nature of the reactions in which they are involved and because of the likely occurrence of electronic- and protein-mediated interactions between the various intramolecular redox centers.

Flavocytochrome c_{552}, isolated from the purple photosynthetic bacterium *Chromatium vinosum*, is representative of this class of proteins. The protein has a molecular weight of 72 000 daltons, distributed among two dissimilar subunits, and contains two mesohemes and one covalently bound flavin (at the FAD[1] level) per molecule (Bartsch & Kamen, 1960). Both heme species appear to be bound to one subunit while the flavin chromophore is associated with the second (Fukumori & Yamanaka, 1979). The protein has been postulated to function as the initial electron transport intermediate between reduced sulfur substrates and the membrane-bound components of the photosynthetic apparatus (Van Grondelle et al., 1977; Seibert & DeVault, 1970). In this role, its function is analogous to that of the water-oxidizing, manganese-containing complexes of green plants. The less demanding requirements of sulfide oxidation ($E_0' = -230$ mV), however, apparently allow the organism to employ a more conventional set of biological redox centers.

Previous studies have explored the optical and magnetic properties of c_{552}; the results obtained from EPR (Strekas, 1976), Mössbauer (Moss et al., 1968), and circular dichroism (Vorkink, 1972) spectroscopies were interpreted to suggest electronic interaction among the chromophores of the protein. The circular dichroism spectra in the heme Soret region show derivative shape curves for the oxidized, reduced, and carbon monoxide bound enzyme. Bartsch et al. (1968) suggested that the derivative shape may result from exciton interaction between the two hemes. Flavin–heme interaction was implicated in the same study by the observation that cyanide binding to the flavin moiety results in a loss in the derivative-shape heme CD curves. Strekas (1976) has also suggested heme–flavin interaction based on the results of his magnetic resonance studies. The EPR spectra were found to be composed of several low-spin heme resonances; the line shape of one of these was postulated to change as a result of low pH complexation of substrate analogues to the flavin chromophore.

We have undertaken a thorough physiochemical study of flavocytochrome c_{552} by using optical, fluorescence, magnetic, and resonance Raman spectroscopies. Here we report the

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[1] Abbreviations used: FAD, flavin adenine dinucleotide; EPR, electron paramagnetic resonance; CARS, coherent anti-Stokes Raman spectroscopy; Mes, 2-(*N*-morpholino)ethanesulfonic acid; CAPS, cyclohexylaminopropanesulfonic acid.

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results of the resonance Raman portion of our work. Resonance Raman spectroscopy has been shown to be a powerful tool for the elucidation of the structure and function of biological molecules, in particular those proteins which contain a heme moiety (Felton & Yu, 1978). The information obtained in a resonant scattering experiment is specific to the vibrations of the heme active site and thus can be used to characterize both radical changes in iron redox and spin states as well as the more subtle perturbations due to alterations of the protein environment surrounding the active site. The technique should be particularly sensitive to vibrational manifestations of chromophore interactions. In the experiments described here, we have found no evidence of direct heme–flavin or heme–heme interactions through either the heme axial positions or periphery. The resonance Raman spectra of c_{552} can be interpreted in terms of indirect, protein-mediated heme–flavin interactions.

Materials and Methods

C. vinosum strain D was grown in 10-L batches to stationary phase by using the heterotrophic medium of Cusanovich (1967). The isolation and purification of flavocytochrome c_{552} followed previously published methods (Bartsch & Kamen, 1960), from which protein purity criteria of $A_{475}/A_{525} = 1.25$ (flavin/heme) and $A_{280}/A_{410} = 0.53$ (protein/Soret) have been established. All spectra shown here were obtained with samples having $A_{475}/A_{525} \geq 1.17$ and $A_{280}/A_{410} \leq 0.72$. Minor deviations in either flavin/heme or protein/Soret ratios had no apparent effect upon either the fluorescence or resonance Raman spectral properties of the protein. The diheme peptide of c_{552} was prepared by overnight incubation in either a 0.10 M CAPS buffer solution at pH 11.0 or an 8 M urea solution (Bartsch et al., 1968) and purified by using gel column chromatography (Sephadex G-100) to eliminate the dissociated flavin subunit.

Optical spectra were obtained on Cary 17 or McPherson EU-707D recording spectrophotometers. Total heme concentrations were determined by using the Soret extinction coefficients of Vorkink (1972). Protein fluorescence was monitored with a Perkin-Elmer MPF-2 fluorometer. Resonance Raman spectra were recorded by using instruments described earlier with either a helium–cadmium laser (Salmeen et al., 1978), $\lambda = 441.6$ nm, or an argon ion laser (Whitfield, 1977), $\lambda = 514.5$ nm, as a source of exciting radiation. A photon-counting detection mode was used with the helium–cadmium spectrometer while the argon ion instrument employed direct current detection. In both cases slit widths were used which provided a peak position accuracy of ± 2 cm^{-1}.

Samples were cooled during Raman spectroscopy by passing cold dry nitrogen gas through a copper housing which held either 5- or 10-mm optical cuvettes. The temperature was controlled at 5 ± 2 °C by regulation of the flow rate of the cooling gas. A bottom illumination geometry was employed in both spectrometers. Protein concentrations and incident laser power were varied to obtain optimal signal-to-noise ratios for each sample and are noted below each set of spectra. Chemically reduced samples were obtained by the addition of a small excess of sodium dithionite (Virgina Smelting) to sealed cuvettes under an argon gas atmosphere. Depolarization ratios were measured for reduced c_{552} by placing a Polaroid filter in the pathway of the scattered light.

Results

The heme moieties in flavocytochrome c_{552} display optical absorption spectra consistent with their assignment as low-spin hemes *c* (Adar, 1978). Soret maxima occur at 410 and 416 nm for the oxidized and reduced forms of the protein, respectively. The positions and relative intensities of the heme Q_{00} and Q_{01} transitions for the reduced protein are similar to those of other ferrous heme *c* spectra. There are, however, some deviations from typical heme *c* optical behavior evidenced by c_{552}. Its covalently bound flavin is manifest in the oxidized protein spectrum as pronounced shoulders on both the low- and high-energy sides of the Soret peak. The flavin absorption band at 475 nm bleaches upon flavin reduction and is sensitive to CN^-, SO_3^{2-}, and $S_2O_3^{2-}$ binding to oxidized c_{552} [for example, CN^- binding lowers A_{475}/A_{525} to 0.83 (Meyer & Bartsch, 1976)]. Optical spectra of oxidized, reduced, and $S_2O_3^{2-}$-bound c_{552} are shown in Figure 1, along with the positions of the laser lines used in our Raman studies.

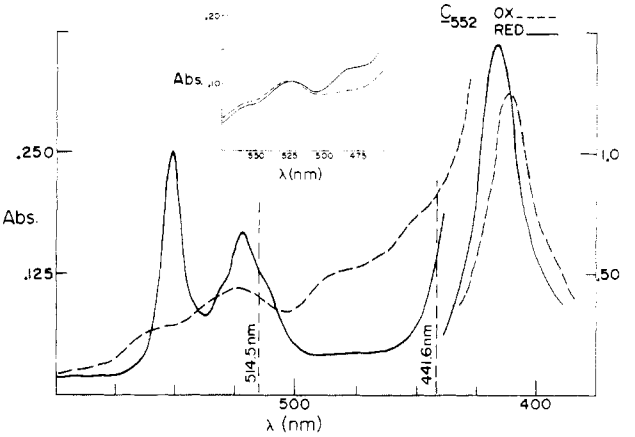

FIGURE 1: Absorption spectra of ~6 μM oxidized (---) and reduced (—) flavocytochrome c_{552} in 0.1 M Tris buffer, pH 7.5. Insert: ~6 μM oxidized c_{552} in 0.1 M Mes buffer, pH 6.1, before (—) and after (---) addition of 2 mM $Na_2S_2O_3$.

The fluorescence of the flavin moiety in c_{552} is strongly quenched relative to both free riboflavin and glucose oxidase, a flavoprotein which contains no heme groups. Figure 2 shows the fluorescence emission spectra of the oxidized forms of these molecules when excited by light at 442 nm. The maxima in the excitation spectra of all three were found to be 445 ± 3 nm. The fluorescence quantum efficiency of c_{552} is ~1% that of free riboflavin and ~33% that of glucose oxidase, after heme reabsorption is taken into account. Nonethless, even this small amount of fluorescence posed a significant obstacle to our resonance Raman studies and manifests itself as a rising base line in the spectra presented here.

Raman spectroscopy employing excitation in resonance with the heme Soret transition yields enhancement of polarized (depolarization ratio, $\rho = I_\perp/I_\parallel < 3/4$) heme vibrational modes, corresponding to Franck–Condon scattering. Excitation in resonance with heme visible absorption bands admits scattering for vibronically coupled as well as isolated states. Scattering from coupled states corresponds to a Herzberg–Teller scattering mechanism (Clark & Stewart, 1979), which allows enhancement of depolarized ($\rho = 3/4$) modes of B_{1g} and B_{2g} symmetry and anomalously polarized ($\rho > 3/4$) modes of A_{2g} symmetry in D_{4h} hemes. Spectra of flavocytochrome c_{552} have been obtained in both of the above-mentioned scattering regimes with 441.6- and 514.5-nm excitation.

The high- and low-frequency regions of resonance Raman spectra obtained with 441.6-nm excitation are shown in Figure 3. Scattering from the polarized heme modes of the ferric and ferrous forms of the protein is typical of other *c*-type cytochromes investigated at this wavelength (Yamamoto, 1974). Even at the extremely low laser power used (~10 mW) a small amount of heme photoalteration is evident as a low-

Table I: High-Frequency Raman Modes for Flavocytochrome c_{552} Species[a]

	ferrous				
mode symmetry (ρ)	cyt *c* (pH 7.5)	heme peptide (pH 7.5)	cyt c_{552} (pH 7.5)	cyt c_{552} (pH 6.05)	cyt c_{552} (pH 10.0)
B_{1g} (0.75)	1621 (m)[b]	1622 (m)	1621 (m)	1621 (m)	1621 (m)
A_{2g} (3.18)	1584 (vs)	1585 (s)	1586 (vs)	1585 (vs)	1586 (s)
B_{1g} (0.74)	1547 (s)	1541 (m)	1544 (s)	1542 (s)	1548 (m)
B_{1g} (0.83)	1401 (s)	1401 (m)	1402 (s)	1399 (s)	1402 (m)
A_{1g} (0.29)	1363 (s)	1362 (m)	1363 (vs)	1362 (s)	1363 (s)
A_{2g} (1.93)	1313 (vs)	1310 (vs)	1311 (vs)	1312 (vs)	1311 (vs)
B_{1g} (0.60)	1229 (m)	1228 (s)	1230 (m)	1229 (m)	1230 (m)
B_{2g} (0.70)	1172 (m)	1173 (s)	1172 (m)	1174 (m)	1173 (m)
A_{2g} (1.10)	1127 (m)	1126 (s)	1127 (m)	1126 (m)	1124 (m)
	ferric				
mode symmetry	cyt *c* (pH 7.5)	heme peptide (pH 7.5)	cyt c_{552} (pH 7.5)	cyt c_{552} (pH 6.05)	cyt c_{552}·$S_2O_3^{2-}$ (pH 6.05)
B_{1g}	1635 (vs)	1642 (vs)	1640 (vs)	1642 (vs)	1643 (vs)
A_{2g}	1585 (s)	1587 (s)	1586 (s)	1588 (s)	1589 (vs)
B_{1g}	1562 (s)	1567 (m)	1571 (s)	1565 (s)	1567 (s)
B_{1g}	1412 (m)	1409 (m)	1407 (m)	1407 (m)	1408 (m)
A_{1g}	1372 (m)	1373 (m)	1372 (m)	1370 (m)	1372 (m)
A_{2g}	1315 (s)	1315 (m)	1315 (w)	1314 (s)	1315 (vs)
B_{1g}	1238 (m), 1250 (m)	1252 (w)	1244 (m)	1246 (m)	1243 (s)
B_{2g}	1170 (s)	1172 (m)	1169 (m)	1173 (m)	1172 (m)
A_{2g}	1127 (s)	~1122 (vw)	1126 (s)		1128 (s)

[a] All spectra were obtained with 514.5-nm excitation. Vibrational frequencies are in units of cm^{-1}. [b] vs = very strong, s = strong, m = medium, w = weak, and vw = very weak.

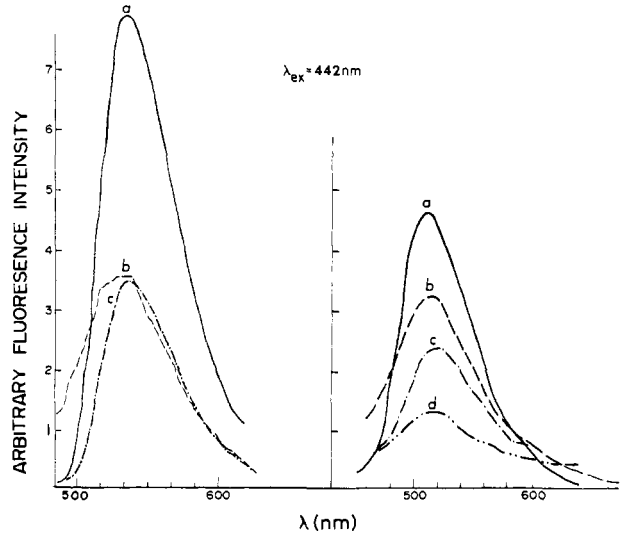

FIGURE 2: (Left panel) Fluorescence emission intensities of (a) 0.1 μM riboflavin (—), (b) 2 μM glucose oxidase (---), and (c) 6 μM flavocytochrome c_{552} (-·-) all in 0.1 M Tris buffer, pH 7.5. (Right panel) Fluorescence emission intensities of (a) 6 μM c_{552} in 0.1 M Mes, pH 6.1, 0.5 h after addition of 2 mM KCN (—), (b) 6 μM c_{552} in 0.1 M Mes, pH 6.1 (---), (c) 6 μM c_{552} in 0.1 M Tris, pH 7.5 (-·-), and (d) 6 μM c_{552} in 0.1 M Mes, pH 6.1, 0.5 h after addition of 2 mM $Na_2S_2O_3$ (-··-). The excitation wavelength was 442 nm for both sets of spectra. Concentrated solutions of KCN and $Na_2S_2O_3$ were prepared in 0.1 M Mes and adjusted to pH 6.1 prior to addition to the protein sample.

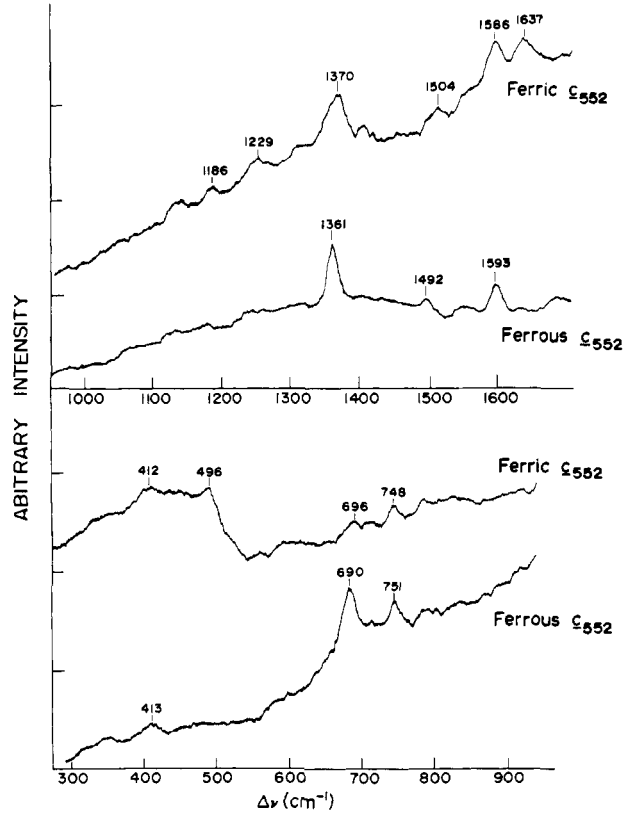

FIGURE 3: Resonance Raman spectra of flavocytochrome c_{552} obtained with 441.6-nm excitation. The power was 10 mW, and the c_{552} concentration was 75 μM in 0.1 M Tris, pH 7.5.

energy shoulder on the 1370-cm^{-1} band of the oxidized protein. This band has been used as an indication of heme redox state (Spiro & Strekas, 1974) and photoreduction (Adar & Yonetani, 1978; Salmeen et al., 1978), although some ambiguity exists in interpretations based on its position since the mode is also sensitive to the basicity of heme axial ligands (Salmeen et al., 1973). No evidence of resonance enhancement of flavin vibrational modes is observed.

Resonance Raman spectra of reduced c_{552} and its diheme subunit, obtained with 514.5-nm excitation and shown in Figure 4, display the variety of mode symmetries expected for heme visible band resonance scattering. The resonance Raman spectrum of reduced horse heart cytochrome *c* is included for ease of comparison. Vibrational symmetries have been assigned (see Table I) on the basis of the depolarization ratios obtained from the reduced protein under the assumption of

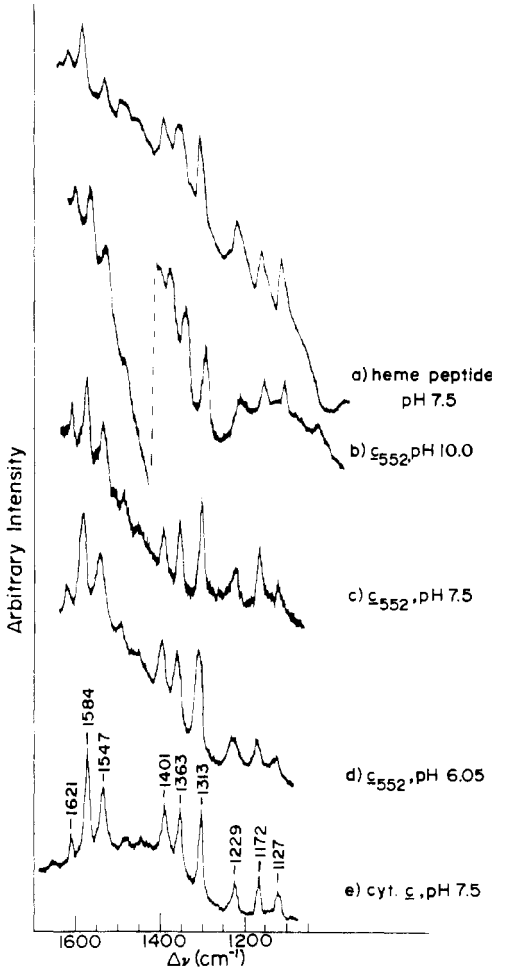

FIGURE 4: Resonance Raman spectra obtained with 514.5-nm excitation of (a) 70 μM ferrous flavocytochrome c_{552} diheme peptide in 0.1 M Tris, pH 7.5, with 350 mW of laser power, (b) 100 μM ferrous flavocytochrome c_{552} in 0.1 M CAPS, pH 10.0, with 180 mW of laser power, (c) 80 μM ferrous flavocytochrome c_{552} in 0.1 M Tris, pH 7.5, with 250 mW of laser power, (d) 100 μM ferrous flavocytochrome c_{552} in 0.1 M Mes, pH 6.05, with 250 mW of laser power, and (e) 200 μM ferrous horse heart cytochrome c in 0.1 M Tris, pH 7.5, with laser power equal to 250 mW. Frequency positions of the principal bands are given in Table I. The fluorescence background of the diheme peptide spectrum arises from a small amount of residual flavin peptide which could not be separated from the sample.

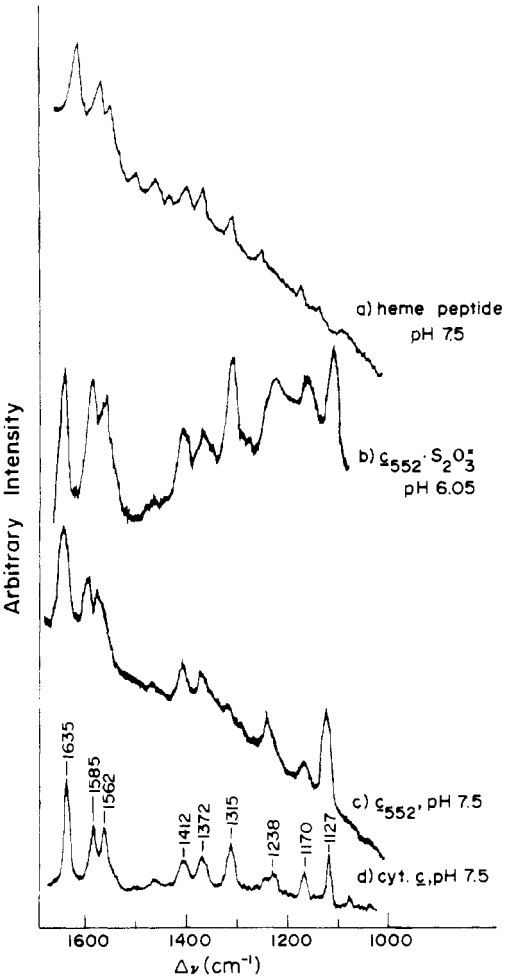

FIGURE 5: Resonance Raman spectra obtained with 514.5-nm excitation of (a) 70 μM ferric c_{552} diheme peptide in 0.1 M Tris, pH 7.5, with 250 mW of laser power, (b) 100 μM ferric flavocytochrome c_{552} in 0.1 M Mes, pH 6.05, + 2 mM $Na_2S_2O_3$ with 200 mW of laser power, (c) 80 μM ferric flavocytochrome c_{552} in 0.1 M Tris, pH 7.5, with 95 mW of laser power, and (d) 200 μM ferric horse heart cytochrome c in 0.1 M Tris, pH 7.5, with 250 mW of laser power. Frequency positions of the principal bands are given in Table I.

D_{4h} heme symmetry. Some caution must be exercised in the use of single excitation frequency ρ values for this purpose, as reduction of chromophore symmetry can lead to dispersion in ρ (Collins et al., 1973). Nonetheless, for c-type heme proteins the assignment of heme vibrational symmetries provides a valuable means of systematizing their behavior. Both the holoprotein and heme peptide spectra are quite similar to those of horse heart cytochrome c and other small molecular weight monoheme c proteins (Spiro & Strekas, 1974; Kitagawa et al., 1977a), the only substantial difference being the flavin fluorescence background (Ghisla et al., 1974). The anomalously polarized (ap) band at 1586 cm^{-1} and the depolarized (dp) band at 1621 cm^{-1}, which are sensitive to heme spin state (Spaulding et al., 1975), appear at frequencies consistent with low-spin heme c. This confirms the assignment made from previous magnetic studies (Strekas, 1976). The position of the polarized oxidation state marker at 1363 cm^{-1} offers no evidence of anomalous heme axial ligation such as that seen with the P_{450} cytochromes (Ozaki et al., 1978).

Figure 5 displays spectra of the ferric forms of c_{552}, its diheme peptide, and horse heart cytochrome c, in the high-frequency region, obtained with 514.5-nm excitation. As with the ferrous spectra, the positions and intensities of the Raman bands of the holo- and apoproteins are generally typical of monohemes c. Two major departures from this "typical" behavior are evident. The first is a small but consistent deviation in the positions of the high-frequency modes with B_{1g} symmetry, as may be noted from Table I. These bands are characterized by depolarization ratios equal to 3/4 and occur at 1635, 1562, 1412, and 1250 cm^{-1} in horse heart cytochrome c. In ferric c_{552} these modes, and only these modes, show wavenumber shifts relative to horse heart cytochrome c. The shifts range from +9 cm^{-1} (for the 1571-cm^{-1} band) to −5 cm^{-1} (for the 1407-cm^{-1} band). The observation that the positions of only a specific symmetry class of resonance Raman active heme vibrations are anomalous is suggestive of some specific perturbation of the heme environment in c_{552}. Moreover, this perturbation of the B_{1g} modes is not observed in the ferrous form of the protein (see Table I).

The second case of anomalous behavior lies in the relative ease with which the heme spectrum in the holoprotein is altered in the laser beam. The small amount of photolability observed with Soret excitation increases dramatically with the higher laser powers used for visible excitation. Figure 6 shows a spectrum of resting c_{552} at 315 mW of incident laser power,

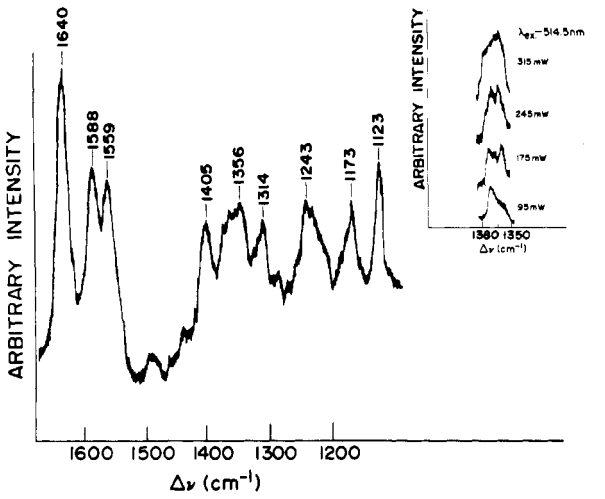

FIGURE 6: Resonance Raman spectrum of 80 μM ferric flavocytochrome c_{552} in 0.1 M Tris, pH 7.5, obtained with 315 mW of 514.5-nm laser light incident upon the sample. Insert: the position and intensity dependence of the oxidation state marker band in ferric c_{552} as a function of 514.5-nm intensity upon the sample.

plus an inset displaying spectra in the oxidation state marker band region (Spiro & Strekas, 1974) as a function of incident laser power. It is apparent that at high laser intensity this band shifts from 1370 to 1356 cm^{-1}. At intermediate powers a double peak is clearly visible. Neither horse heart cytochrome *c* nor the heme peptide displays this behavior; indeed, spectra of the ferric forms of these proteins were routinely obtained with 200 mW of laser power.

The binding of thiosulfate and cyanide to the oxidized protein resulted in an initial lowering of flavin fluorescence. However, CN^- binding was found to be short-lived. Subsequent to cyanide binding, the ratio A_{475}/A_{525} returned to its initial value and flavin fluorescence increased greatly. Thiosulfate, on the other hand, remained bound and continued to quench flavin fluorescence. Relative fluorescence levels of these species are contrasted in Figure 2. The instability of the c_{552}·CN^- complex precluded observation of its resonance Raman spectrum. The spectrum obtained with 514.5-nm excitation of c_{552}·$S_2O_3^{2-}$ is included in Figure 5. It differs from the unbound holoprotein only in the substantial increase in intensity exhibited by the 1315- and 1589-cm^{-1} anomalously polarized modes.

The pH dependence of the holoprotein Raman spectra was also investigated. Fluorescence levels increased markedly at both the high pH ($\sim$10.0) and low pH ($\sim$6.0) limits of c_{552} stability. This resulted in a deterioration of Raman spectral quality, and we were unable to obtain a spectrum of the ferric protein at high pH. As with other cytochromes, c_{552} band positions display some pH dependence. In particular, the band which appears at 1544 and 1571 cm^{-1} in the ferrous and ferric holoproteins, respectively (at pH 7.5), was sensitive to pH changes. Figure 4 exhibits the pH dependence of scattering from ferrous c_{552} and attests to the dramatic rise in background fluorescence as the high pH limit of heme–flavin subunit binding stability is reached. A summary of band positions, intensities, and depolarization ratios obtained for the various forms of flavocytochrome c_{552} with 514.5-nm excitation is given in Table I.

Discussion

The general features of the resonance Raman spectra of flavocytochrome c_{552} presented here conform well to the classification methodology developed for monoheme proteins by Spiro & Strekas (1974). Upon closer examination, however, several distinguishing aspects of the c_{552} spectra become apparent. These can be divided into two categories: general effects involving the flavin moiety of the protein and specific perturbations of heme vibrational modes. The former have the more obvious impact on the spectra, whereas the interpretation of the latter provides insight into the multicomponent nature of flavocytochrome c_{552}.

Flavin Effects. The most salient of the flavin effects is the broad fluorescence background it produces in the resonance Raman spectra c_{552}. This is unusual not because of its existence but rather because it is weak enough to permit the observation of resonance Raman scattering. Free flavins are strongly fluorescent, some having quantum efficiences as high as 58% (Kotaki & Yagi, 1970). The quenching of flavin fluorescence upon binding to peptides is well established. Flavin interactions with aromatic amino acid residues in both flavoproteins and model complexes have been shown to quench flavin fluorescence, and quantum efficiencies as low as 3% have been reported (Chance et al., 1971). The fluorescence quenching evident in flavocytochrome c_{552} results in a quantum yield of $\sim$0.7% that of riboflavin in the same solvent or $\sim$0.2% absolute efficiency [based on a value of 26% for the quantum efficiency of free riboflavin (Kotaki & Yagi, 1970)]. We attribute the anomalously low fluorescence level to two effects. The first and more important is the quenching due to protein tyrosine residues. Sequencing studies by Kenney et al. (1973) on c_{552} digestive peptide fragments containing the covalently bound flavin have established that the flavin is intimately associated with at least one and probably two tyrosine residues of the protein. It was found that the fluorescence efficiency of the flavopeptides prepared by trypsin–chymotrypsin digestion was 1% that of riboflavin, only marginally larger than that of the apoprotein. Heme reabsorption is the second factor which influences the apparent fluorescence yield; this results in a 15% decrease in apparent flavin fluorescence at 525 nm (based on a 5-mm path length in a 6 μM solution of c_{552}). These two considerations appear to be sufficient to explain the quenching of flavin fluorescence in c_{552} and obviate the necessity of invoking direct heme–flavin energy transfer of the Förster type (Förster, 1959).

An indication of heme–flavin communication is apparent in the heme photolability at high incident laser power. The shift in the "oxidation state marker" frequency from 1370 to 1360 cm^{-1} has been used as an indication of increased electron density located on the heme iron (Kitagawa et al., 1975). Such a shift occurs in flavocytochrome c_{552} spectra at moderate laser powers but it is absent in the heme peptide and horse heart cytochrome *c* spectra, implicating the flavin moiety as the source of the effects in c_{552}. Under aerobic conditions the photoeffects described here are most evident for the 1372-cm^{-1} band. At the highest laser power used, two of the B_{1g} heme modes (at 1571 and 1407 cm^{-1}) begin to decrease in frequency; however, the systematic lowering of heme vibrational frequencies known to result from chemical reduction is largely absent despite the fact that the oxidation state marker appears at 1356 cm^{-1} in the "photoreduced" protein (7 cm^{-1} lower in wavenumber than in chemically reduced c_{552}). In particular, the 1640-cm^{-1} (dp) mode which undergoes large changes in both position and intensity depending on the iron redox state clearly retains its oxidized character. This indicates that the lability of the oxidation state marker is predicated, at least in part, upon factors independent of the formal iron redox state. Anomalous oxidation state marker behavior has also been observed in (carbon monoxy)- and oxyhemoglobin and myo-

globin resonance Raman spectra (Salmeen et al., 1973) and has been interpreted as resulting from cis back-bonding between porphyrin π^* and iron d orbitals (Shelnutt et al., 1979).

The resonance Raman spectra obtained with Soret excitation are noteworthy for the absence of any bands attributable to flavin scattering despite the fact that the 441.6-nm exciting radiation is in resonance with the flavin absorption at ~450 nm in c_{552}. This absorption arises from an in-plane flavin $\pi \rightarrow \pi^*$ transition (Sun et al., 1972). Thus, resonance enhancement of in-plane isoalloxazine vibrational modes would be expected to result in several peaks in the 1000–1600-cm^{-1} region of the resonance Raman spectrum. These modes have recently been observed in resonance Raman spectra of protein-bound FAD (Kitagawa et al., 1979) and in CARS spectra of FAD and glucose oxidase (Dutta et al., 1977). The absence of flavin bands in our spectra can be readily explained by the fact that at 441.6 nm heme scattering dominates the spectrum because of its greater extinction, leaving the flavin modes unobservable at the laser power used. Resonance enhancement via simple Franck–Condon scattering is proportional to the quantity R given below (Albrecht & Hutley, 1971; Adar et al., 1976), although this is recognized as only a first approximation (Champion & Albrecht, 1979):

$$R = \epsilon_m^2/[(\nu - \nu_0)^2 + \Gamma^2]$$

where ϵ_m = extinction coefficient at the peak of the chromophore absorption band, ν_0 and ν = energies of the absorption peak and exciting light, respectively, and Γ = natural line width of the absorption band. For the following conditions, ν = 22 645 cm^{-1} (441.6 nm), ν_0(flavin) = 22 422 cm^{-1} (446 nm), ν_0(heme) = 24 390 cm^{-1} (410 nm), Γ(flavin) ≈ 1500 cm^{-1}, Γ(heme) ≈ 1200 cm^{-1}, ϵ_m(heme) = 125 mM^{-1} cm^{-1}, and ϵ_m(flavin) = 10 mM^{-1} cm^{-1}, we calculate that the ratio R(heme)/R(flavin) is more than 100. Thus, any flavin band would be at least an order of magnitude less intense than the c_{552} heme bands and undetectable with a conventional spectrometer.

Heme Vibrational Bands. All the features found in the resonance Raman spectra of c_{552} can be readily interpreted as monoheme scattering coupled with the flavin effects discussed above. The applicability of previous classification schemes (Spiro & Strekas, 1974) to the heme scattering of c_{552} is obvious and confirms their assignment as low-spin heme *c*. This is particularly striking in the spectra of reduced c_{552} and its peptide under visible excitation. For both the holo- and apoproteins all bands are within ±2 cm^{-1} of their horse heart cytochrome *c* values, except the depolarized band at ~1545 cm^{-1} which displays a pH-dependent position. Investigations of *Desulfovibrio vulgaris* cytochrome c_3 by Kitagawa et al. (1977a) indicate that this band is sensitive to the nature of heme axial ligands. In fact, they used its position to monitor a pH-dependent heme ligand change: a shift in frequency from 1541 to 1536 cm^{-1} was interpreted as resulting from the replacement of histidine by lysine in the protein at high pH.

The existence of an analogous pH dependence in the heme axial ligation of ferric c_{552} has been indicated in a previous EPR study of the protein by Strekas (1976). The situation is somewhat more complicated in c_{552} than in cytochrome c_3 because only one of the protein's two hemes displays pH-dependent behavior. We interpret the EPR spectra of ferric c_{552} obtained by Strekas (1976) and reproduced in our laboratory to arise from a heme axial ligation scheme involving one pH-stable heme with methionine–histidine ligands and one pH-labile heme favoring lysine–histidine ligands at low pH and histidine–histidine ligands at high pH.[2] The pH-dependent behavior of the 1545-cm^{-1} Raman band in ferrous c_{552} indicates that a similar axial ligation scheme is obtained for the reduced protein. If this is the case, the resonance Raman spectra of ferrous c_{552} at neutral pH should, in principle, exhibit three peaks for the ligand-sensitive band at ~1545 cm^{-1}: one at 1547 cm^{-1}, another at 1541 cm^{-1}, and a third at 1536 cm^{-1}, corresponding to methionine–histidine (as in ferrous horse heart cytochrome *c*), histidine–histidine, and lysine–histidine ligation, respectively. In practice, a single asymmetric peak appears in this region: at 1542 cm^{-1} in the holoprotein at pH 6.05, 1544 cm^{-1} at pH 7.50, and 1548 cm^{-1} at pH 10.0, suggesting that the pH-dependent ligand shift is also operative for the reduced protein. For the ferrous heme peptide at pH 7.5 the band is observed at 1541 cm^{-1}, indicating a preference for the low pH ligand (lysine) in the apoprotein.

The spectra of ferric c_{552} holo- and apoproteins, while retaining the general characteristics of monoheme *c* spectra, display a larger deviation from horse heart cytochrome *c* behavior than do the ferrous spectra. Two effects are most likely responsible for this behavior: ligand effects seen in both c_{552} and the heme peptide and a general perturbation of heme modes of B_{1g} symmetry observed only in the oxidized holoprotein. The ligand effects which were limited to a single mode in reduced c_{552} are more widespread in the ferric spectra. Kitagawa et al. (1977a) have found that the bands at 1635, 1562, and 1372 cm^{-1} are pH (ligand) dependent in ferric horse heart cytochrome *c*, changing to 1641, 1568, and 1375 cm^{-1} upon replacement of the axial methionine by lysine at high pH. The Raman data presented here confirm that this situation also applies to flavocytochrome c_{552}. The heme peptide frequencies parallel the high pH values of horse heart cytochrome *c* (i.e., lysine–histidine ligands), indicating the effect of the pH-labile heme. For the ferric holoprotein, however, the positions of all of the high-frequency B_{1g} modes are shifted (relative to horse heart cytochrome *c*). Particularly evident is the 9-cm^{-1} change in the position of the 1562-cm^{-1} band. The band positions of the ferric heme peptide B_{1g} modes are, with the exception of the 1642-cm^{-1} band, intermediate between the values for horse heart cytochrome *c* and holo cytochrome c_{552}. On the basis of axial ligation, it would be expected that the situation would be reversed; the holoprotein with its mixture of lysine and histidine ligands would have band positions closer to those of horse heart cytochrome *c* than to those of the heme peptide. The expected situation holds for the low pH holoprotein but is not the case at pH 7.5. Thus, relative wavenumber shifts in the spectra cannot be explained as arising solely from axial ligand changes; the changes in frequency of the B_{1g} modes must also be diagnostic of some other protein influence.

Normal coordinate calculations (Abe et al., 1978) have indicated that B_{1g} modes in general and the ~1565-cm^{-1} mode in particular involve out-of-phase stretching of atoms at the porphyrin periphery (either C_β–C_β or C_m–H stretches), whereas the 1372-cm^{-1} mode is closely associated with C–N symmetric stretching. In fact, studies of metalloetioporphyrins by Spaulding et al. (1975) have stressed the importance of contributions from the core expansion of the inner 16-membered ring to both the oxidation state (A_{1g}) and spin state (A_{2g}) marker bands. The depolarized modes appearing at ~1565 and ~1250 cm^{-1} in ferric hemes have been shown to be particularly sensitive to substituent effects. The former shifts from 1547 cm^{-1} in ferrous cytochrome *c* to 1538 cm^{-1} in

[2] M. R. Ondrias, G. E. Leroi, and G. T. Babcock, unpublished experiments.

protoheme reconstituted ferrous cytochrome b_5, indicating the presence of the two peripheral vinyl groups in the protoheme (Adar, 1975). A similar effect was noted in ferric heme *a* by Kitagawa et al. (1977b). The 1250-cm^{-1} mode has been shown to be sensitive to deuteration of the methine hydrogens in ferrous mesoporphyrin IX dimethyl ester complexes (Spiro & Burke, 1976). These observations indicate that the depolarized modes of the heme are more sensitive to peripheral influences on the porphyrin than are the high-frequency A_{2g} or A_{1g} modes. In flavocytochrome c_{552} these B_{1g} modes all experience frequency shifts (with respect to horse heart cytochrome *c*) in the oxidized protein that are absent in the reduced protein. The effect is obscured by the axial ligand dependence of the 1640- and 1571-cm^{-1} bands but is clearly independent of it since the 1407-cm^{-1} band shows no axial ligand effect in reduced or oxidized c_{552} or in any of the ferric bacterial cytochromes *c* studied by Kitagawa et al. (1977a).

The binding of $S_2O_3^{2-}$ to the low pH form of the oxidized protein produces no appreciable change in the heme Raman frequencies, implying that there is very little perturbation of the local heme vibrational environment upon substrate binding. Thus, in its low pH form, the heme environment is already in a conformation amenable to substrate binding. However, the intensity of Herzberg–Teller active heme modes is dependent upon the extent to which they couple the porphyrin Q and B states. The large increase in the relative intensities of the two anomalously polarized bands (at ~1315 and ~1589 cm^{-1}) in going from c_{552} at pH 7.5 to the thiosulfate-bound protein may be indicative of an alteration in the electronic environment of the heme.

Conclusions

It is apparent that the redox state of the protein (and by implication the flavin) has a noticeable effect on the peripheral environment of at least one of the heme moieties. The effect is small. No extra bands occur in the heme spectra, nor are there any dramatic changes in electron density at the heme iron (as evidenced by the position of the 1372-cm^{-1} band). Substrate binding to the flavin produces a more subtle alteration of the heme environment that may be reflected in a change in the vibronic coupling between heme electronic states. We conclude that there is no direct heme–flavin interaction through either electronic resonance or the axial heme positions, and if heme–flavin interaction occurs it does so via a protein-mediated heme–flavin communication through the heme periphery. The heme vibrational mode frequencies are consistent with an interpretation which considers both heme axial ligation and an indirect, protein-mediated heme–flavin interaction.

A working hypothesis for c_{552} function may be formulated in which the two hemes are in distinctly different protein environments. One heme is in a relatively invariant protein environment similar to that found in cytochrome *c*; the other experiences a mutable protein environment. The flavin can be postulated to serve as the substrate binding site and as such would be the initial center to be reduced. Its excited electronic state is extensively delocalized by neighboring protein residues so that upon reduction there is a change in the protein configuration that is reflected in the Raman spectra of the pH-labile heme. That these changes in protein environment subsequently facilitate the flow of electrons from flavin to heme is still a matter of speculation; however, the flavin-mediated heme photolability which we have observed in c_{552} may be an indication of the pathway for flavin-to-heme electron transfer.

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A γ2b–γ2a Hybrid Immunoglobulin Heavy Chain Produced by a Variant of the MPC 11 Mouse Myeloma Cell Line†

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ABSTRACT: The IgG2b-producing MPC 11 mouse myeloma cell line has yielded a number of variants which synthesize heavy chains characteristic of a different immunoglobulin subclass, IgG2a, as shown initially by serology, peptide maps, and assembly profiles. Primary structural analysis of the immunoglobulin synthesized by one variant, ICR 9.9.2.1, showed that the Fc fragment was most probably identical with that of MOPC 173, an IgG2a protein of known sequence, and different from the parental γ2b Fc fragment. We report here our studies on the protein synthesized by a second γ2a-producing variant, ICR 11.19.3. The Fc fragment of ICR 11.19.3 differed from that of ICR 9.9.2.1 by comparative peptide mapping and was shown by partial sequence determination to contain a long C-terminal stretch of γ2a sequence and a short stretch of γ2b sequence at the amino terminus. Identification of additional residue positions which discriminate between the two subclasses have localized the junction of γ2b and γ2a sequences in ICR 11.19.3 between residues N-308 and N-331, some 8–32 residues N terminal to the C_H2/C_H3 domain boundary. This junction comprises 24 amino acids which, with one possible exception, are identical between γ2b and γ2a subclasses. Our isolation and characterization of CNBr fragments from the N-terminal region of the parental MPC 11 heavy chain allowed us to describe an additional γ2b constant region fragment. The ICR 11.19.3 variant protein contained a homologous fragment which seemed to be identical, thus confirming the presence of γ2b sequence in this region. From these studies, we conclude that ICR 11.19.3 synthesizes a γ2b–γ2a hybrid immunoglobulin heavy chain.

A number of variants synthesizing altered immunoglobulin heavy chains have been isolated from the MPC 11 mouse myeloma cell line. Some variants synthesize short heavy chains of 50 000 and 40 000 M_r (Birshtein et al., 1974), compared to the parental size of 55 000 M_r. Other variants have discontinued synthesis of heavy chains containing the parental γ2b serological markers and now make heavy chains with the serological, peptide, and assembly characteristics of a second subclass, γ2a (Preud'homme et al., 1975; Koskimies & Birshtein, 1976; Francus et al., 1978; Liesegang et al., 1978; Morrison, 1979). We have been especially interested in this latter group of variants since they reflect the activation and expression of previously silent genetic information. Some γ2a-producing variants have appeared in the MPC 11 cell line either spontaneously or after mutagenesis with ICR-191 or Melphalan, while others arose upon recloning of certain primary variants. Secondary variants synthesizing γ2a heavy chains of 55 000 M_r have been derived both from two primary variants synthesizing heavy chains of 50 000 M_r and from the single primary variant which synthesizes a large γ2a heavy chain of 75 000 M_r.

The γ2a variant proteins share the parental idiotype, indicating the retention of at least part of the original variable region gene (Francus et al., 1978). However, they can be distinguished by electrophoretic mobility, peptide maps, and assembly patterns. Several variant proteins could be grouped on the basis of similarities in these parameters. One group consists of the immunoglobulins synthesized by two primary variants derived after Melphalan mutagenesis and one derived after ICR-191 treatment. A second group consists of the immunoglobulins synthesized by four secondary variants derived by recloning primary variants which synthesize short heavy chains of 50 000 M_r. The variant protein synthesized by the ICR 11.19.3 cell line, the subject of this paper, is included in this group (Scheme I). Still other variant proteins did not seem to fall into either of these two groups.

To approach the formidable task of determining the structural defects in these variant proteins, we decided to

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