Resonance Raman Evidence for Intramolecular Electron Transport from Flavin to Heme in Flavocytochrome *c*-552 and Nature of Chromophoric Interactions†

Teizo Kitagawa,* Yoshihiro Fukumori, and Tateo Yamanaka

ABSTRACT: Resonance Raman spectra of *Chromatium* flavocytochrome *c*-552 were measured for its oxidized and reduced states and also in the presence of cyanide, sulfite, or EDTA. The Raman lines due to the isoalloxazine and iron porphyrin were both observed and were used to monitor the oxidation states of the flavin and heme separately. Oxidized cytochrome *c*-552 was completely reduced by extensive irradiation of laser light in the presence of EDTA but scarcely in its absence. This was also confirmed by absorption spectroscopy. The efficiency of photoreduction was wavelength dependent, being highest at 457.9 nm among the emission lines of an argon ion laser. The split flavoprotein subunit was also photoreduced in the presence of EDTA but not in its absence. However, under the same irradiation conditions, the split cytochrome subunit was not photoreduced at all. Consequently, it is presumed for intact cytochrome *c*-552 that the reduction of heme takes place through an intramolecular electron transport from flavin to heme after the photoreduction of the flavoprotein subunit occurred. Upon binding of cyanide or sulfite to the oxidized cytochrome, the Raman lines of flavin disappeared but the spin-state marker Raman lines of heme remained at the characteristic frequency of the ferric low-spin state. The Raman spectrum of the heme moiety of intact cytochrome was distinguishable from the spectra of its cyanide or sulfite adduct and of the split cytochrome subunit, particularly upon excitation at 514.5 nm. The spectral difference is characterized by intensity enhancement of $\nu_{12}(B_{1g})$, $\nu_{19}(A_{2g})$, $\nu_{20}(A_{2g})$, and $\nu_{21}(A_{2g})$ in the intact cytochrome but without a change of their frequencies. All these modes involve atomic displacements of methine carbon and hydrogen perpendicular to the heme radius in the heme plane. Therefore, the Raman spectral feature suggests that an intramolecular chromophoric interaction is present, and it perturbs specifically the vibronic coupling in the Q excited state but has little effect on the ground electronic state.

Flavocytochrome *c* is one of multicenter redox enzymes having flavin and heme as active sites and has so far been found in two genera of photosynthetic sulfur bacteria, *Chlorobium limicola f. thiosulfatophilum* and *Chromatium vinosum* (Bartsch et al., 1968). The former has sulfide–reductase activity (Kusai & Yamanaka, 1973) while the latter has additionally the catalytic activity in reduction of elementary sulfur to sulfide with reduced benzylviologen as an electron donor (Fukumori & Yamanaka, 1979). Physicochemical studies of such multicenter redox enzymes are important in understanding the complex nature of the reaction involved. Moreover, elucidation of intramolecular chromophoric interactions is of potential importance in its possible application to intermolecular chromophoric interactions present between various electron-transport proteins in biomembranes.

Cytochrome *c*-552 from *Chromatium vinosum* (M_r = 67 000) contains two hemes *c* and one FAD (Hendriks & Cronin, 1971). It consists of two dissimilar subunits, cytochrome subunit with M_r = 21 000 and flavoprotein subunit with M_r = 46 000 (Fukumori & Yamanaka, 1979). The FAD[1] is bound to a polypeptide through a thioether linkage at position 8 of isoalloxazine (Kenney & Singer, 1977). Cytochrome *c*-552 is known to react with thiosulfate, sulfite, cyanide, and mercaptans (Meyer & Bartsch, 1976), and its catalytic activities are inhibited by cyanide (Fukumori & Yamanaka, 1979). Detailed catalytic and inhibitive mechanisms are left unknown.

Various spectroscopic data of this cytochrome have been accumulated. According to the Mössbauer spectrum (Moss et al., 1968), the two heme groups of cytochrome *c*-552 are identical in iron coordination, whereas with EPR (Strekas, 1976) the two hemes are distinguishable and only one of the heme irons exists in two different low-spin environments in the pH range from 5.5 to 10.5. The pH-dependent ligand exchange was recently confirmed with resonance Raman spectroscopy, although no flavin modes were observed (Ondrias, et al., 1980). The ORD study suggested the existence of coupled heme interaction in the molecule (Yong & King, 1971).

Resonance Raman spectroscopy possesses a great advantage in selective exploration of the vibrational spectra of coexisting chromophores in a single biomolecule by excitation light tuning (Adar & Erecińska, 1978,; Spiro & Gaber, 1977; Carey, 1978). For various hemoproteins, this technique has revealed the molecular vibrations of the heme interacting with its immediate environments, providing information about structural details of heme (Spiro, 1975; Felton & Yu, 1978; Kitagawa et al., 1978b). On the other hand, the Raman spectra of flavoproteins have long been unknown under the veil of strong fluorescence, but recently they became observable (Dutta et al., 1977, 1978; Nishina et al., 1978; Benecky et al., 1979). The Raman lines of flavin are qualitatively assigned on the basis of the isotopic frequency shifts for the specific isotope substitutions of isoalloxazine (Kitagawa et al., 1979a), and

† From the Department of Molecular Physiological Chemistry, Medical School, Osaka University, Nakanoshima, Kita-ku, Osaka, 530 Japan (T.K.), and the Department of Biology, Faculty of Science, Osaka University, Toyonaka, Osaka, 560 Japan (Y.F. and T.Y.). *Received February 26, 1980.* Supported in part by a grant-in-aid for special project research on photophysiology (510304) from the Japanese Ministry of Education, Science, and Culture.

[1] Abbreviations used: FAD, flavin adenine dinucleotide; FMN, flavin mononucleotide; EPR, electron paramagnetic resonance; EDTA, disodium ethylenediaminetetraacetate.

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on the basis of the assignments, the site of charge-transfer interaction between FMN of old yellow enzyme and an inhibitor was determined from Raman spectroscopy (Kitagawa et al., 1979b). Therefore, this method might be a powerful tool for elucidating interactions between two redox centers of cytochrome *c*-552, that is, the flavin–heme interactions, if present.

Here we report the results of resonance Raman study of *Chromatium* cytochrome *c*-552. We could observe the Raman lines of both flavin and heme of intact cytochrome and some effects of cyanide and sulfite on the Raman lines. The present observation will bring about the first direct evidence for electron transport from flavin to heme.

Materials and Methods

A strain of *Chromatium vinosum* was kindly given by Drs. R. G. Bartsch and T. Meyer (University of California, San Diego). The organism was cultured in large scale, and cytochrome *c*-552 was purified according to the method of Bartsch & Kamen (1960). Biochemical characterization of the cytochrome used and the preparation procedure for the split cytochrome and flavoprotein subunits are described elsewhere (Fukumori & Yamanaka, 1979). The cytochrome solutions at pH 8.5 and 6.5 were obtained through dialysis against 0.1 M Tris-HCl and 0.1 M phosphate buffers, respectively. The "resting" cytochrome means the sample stored for several months in a frozen state after the dialysis.

Chemical reduction of the cytochrome was performed with 54 mM Na_2S or sodium dithionite fresh solutions in 0.1 M buffer in the Raman cell, and the Raman cell was evacuated to 0.05 mmHg immediately after the reduction and was stopped. In the photoreduction experiments, 4 μL of freshly prepared EDTA solution in 0.1 M Tris-HCl buffer, pH 7.5, was added to ca. 60 μL of the 67 μM cytochrome solution, and the cell was evacuated to 0.05 mmHg and kept at 15 °C. The irradiation power was 60 mW at 457.9 nm and 120 mW at 514.5 nm, and its duration time was standardized to 0.5 h. As the irradiation was performed in the Raman spectrometer with the same disposition as for measurement of Raman scattering, the probe beam for Raman scattering could be introduced precisely to the irradiated point of the sample. Accordingly, even though the whole sample was not illuminated by laser light, the present Raman experiments can detect the effect of laser irradiation on the cytochrome much more effectively than in the experiments for absorption spectroscopy. The power of the probe beam was 40 mW at 514.5 nm and 20 mW at 457.9 nm. It was confirmed that prolonged irradiation (4 h) of the probe beam did not cause any change of Raman spectra. The photoreduction experiment with absorption spectroscopy was performed by illuminating a 100-W tungsten lamp to a cuvette placed at 30 cm from the lamp. A 10-cm-long water layer was placed between the lamp and the cuvette.

In the Raman experiments with sulfite and cyanide, the cytochrome solution at pH 6.5 was titrated with 34 mM solutions of $NaHSO_3$, pH 6.7, and 27 mM solutions of KCN, pH 8.5, respectively, in a unit of 1 μL against 50 μL of the 70 μM cytochrome solution. The results about the 514.5- and 457.9-nm excitations (Figure 6) are from independent titration experiments. As the fluorescent background varied in every step of the titration and significantly differed between the two excitations, a precise intensity evaluation of Raman lines was quite difficult.

Raman spectra were excited with an argon or argon–krypton mixed gas laser (Spectra Physics, model 164) and recorded on a JEOL-400D Raman spectrometer equipped with an electronically cooled HTV-R649 photomultiplier. Frequency calibration of the spectrometer was performed with indene (Hendra & Loader, 1968) for every experiment.

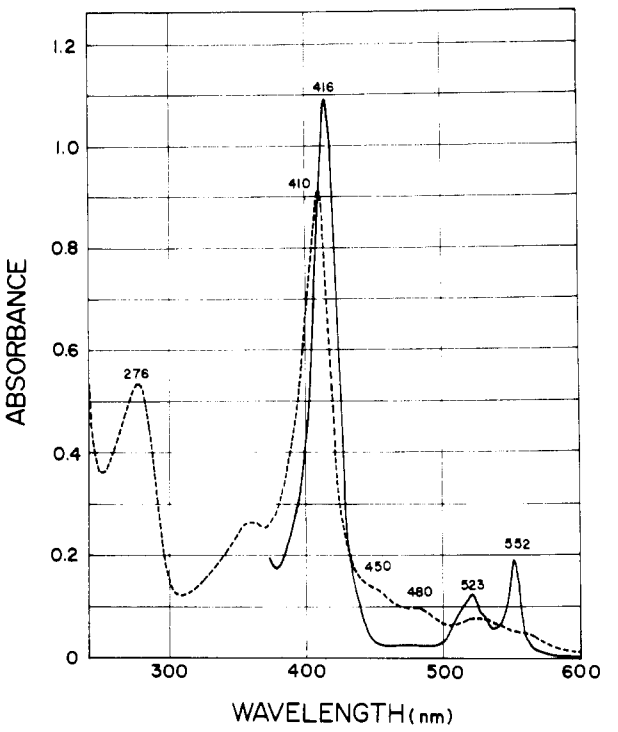

FIGURE 1: Absorption spectra of *Chromatium* flavocytochrome *c*-552 in 0.1 M Tris-HCl buffer, pH 8.5. Solid line, reduced form; broken line, oxidized form.

Results

The absorption spectra of the present preparations of oxidized and reduced cytochrome *c*-552 at pH 8.5 are shown in Figure 1. The spectral features are almost identical with those reported by Bartsch et al. (1968). The absorbance ratios for the oxidized form are $A_{276}/A_{410} = 0.58$ and $A_{450}/A_{410} = 0.15$, indicating high purity of the cytochrome.

The resonance Raman spectra of dithionite-reduced cytochrome *c*-552 excited at 530.9, 520.8, 514.5, and 488.0 nm are shown in Figure 2, where polarization components are displayed with a solid line ($I_{\parallel}$) and a broken line ($I_{\perp}$) only for the 514.5-nm excitation. The spectra were indistinguishable from those of Na_2S reduced ones. The number of Raman lines and their polarization properties are almost identical with those of other ferrocytochromes *c* (Spiro & Strekas, 1972; Kitagawa et al., 1975). Reduced riboflavin with the similar concentration did not show any Raman lines under the present instrumental conditions. Accordingly, all the Raman lines in Figure 2 are conclusively due to heme. If the heme groups lay in appreciably different environments, the Raman lines would be broader because various *c*-type cytochromes give rise to the corresponding Raman lines at slightly different frequencies; for example, the lines which correspond to those at 1588 and 1316 cm^{-1} of ferrocytochrome *c*-552 are spread in ranges 1582–1588 cm^{-1} and 1311–1315 cm^{-1}, respectively, with other *c*-type cytochromes (Kitagawa et al., 1978b). The sharp and symmetric features of the two lines of cytochrome *c*-552 suggest that the two hemes are indistinguishable in the reduced state at pH 8.5. The Raman spectra were unaltered by addition of excess cyanide.

The resonance Raman spectra of oxidized cytochrome *c*-552 excited at 514.5, 501.7, 496.5, 488.0, and 476.5 nm are displayed in Figure 3 where the Raman line of SO_4^{2-} can serve

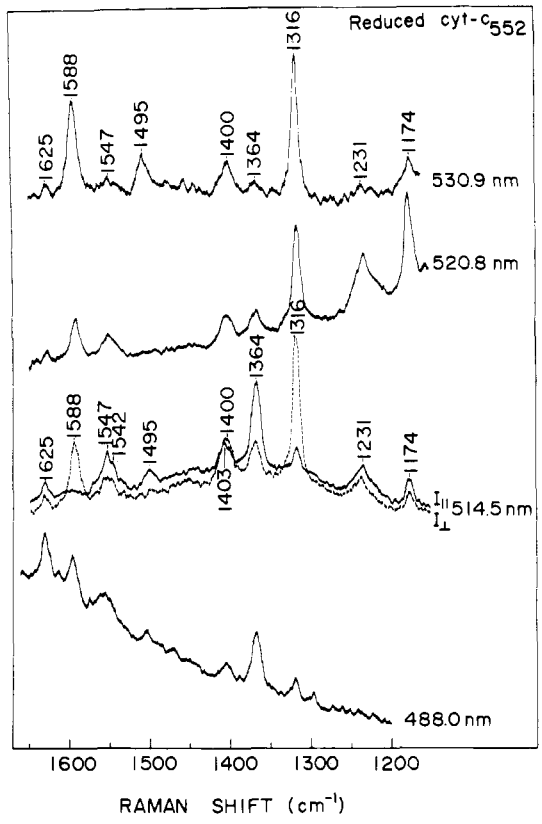

FIGURE 2: Resonance Raman spectra of dithionite reduced cytochrome *c*-552 at pH 8.5, excited at 530.9, 520.8, 514.5, and 488.0 nm. The spectra for the 514.5-nm excitation are represented by each polarization components; the solid line ($I_{\parallel}$) and broken line ($I_{\perp}$) indicate that the electric vector of the scattered radiation is parallel and perpendicular to that of incident radiation, respectively.

as an intensity standard. It is known that some of the Raman lines of oxidized flavin are strongly resonance enhanced upon excitation at 476.5 nm (Nishina, et al., 1980a), whereas the nontotally symmetric modes of heme are more intensified upon excitation around 500–530 nm (Spiro & Strekas, 1972; Nafie et al., 1973). In fact, upon excitation at shorter wavelengths, the Raman lines at 1645, 1566, and 1316 cm^{-1} which arise from the cytochrome subunit lose intensity and conversely the lines at 1629 and 1346 cm^{-1} which arise from the flavoprotein subunit gain intensity.

This feature is more clearly demonstrated in Figure 4 where the intensity of a few selected lines relative to that of the SO_4^{2-} ion at 983 cm^{-1} are plotted vs. the excitation wavenumbers. Because of band overlapping, the band intensity was evaluated as the peak height from a base line shown by broken lines in Figure 3. Although the intensity evaluation may involve large errors, it would be sufficient for a qualitative use.

The 1629-, 1346-, and 1244-cm^{-1} lines of cytochrome *c*-552 are strongly intensified at 476.5 nm, as are the 1631-, 1355-, and 1252-cm^{-1} lines of oxidized riboflavin (Nishina et al., 1980a). Low-spin ferriheme *c* has never given Raman lines at 1629 and 1346 cm^{-1}. Therefore, these two lines are undoubtedly assigned to the flavin modes and will be used, hereafter, as an indicator of oxidized flavin. The 1588- and 1403-cm^{-1} lines for the 476.5-nm excitation are also likely due to flavin because the 1584- and 1407-cm^{-1} lines of oxidized riboflavin were strongly resonance enhanced upon excitation at 476.5 nm. However, the 1592- and 1404-cm^{-1} lines for the 514.5-nm excitation (top trace in Figure 3) are presumably associated with the heme moiety as judged from its spectral similarity to other ferricytochromes *c*. Thus these two lines involve contributions from both flavin and hemes and therefore

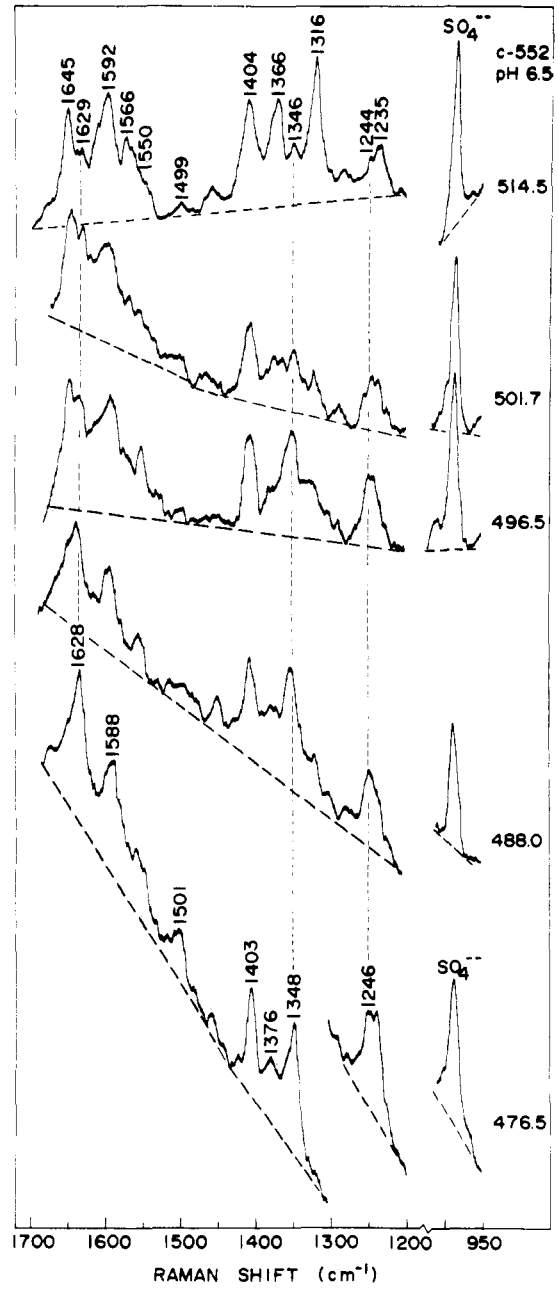

FIGURE 3: Resonance Raman spectra of oxidized cytochrome *c*-552 at pH 6.5, excited at 514.5, 501.7, 496.5, 488.0, and 476.5 nm. The concentration of cytochrome is 73 μM. The preparation contains 1.2% (w/w) $(NH_4)_2SO_4$ as the intensity standard. The Raman line marked as SO_4^{2-} denotes the Raman line of the SO_4^{2-} ion at 983 cm^{-1} and is measured with the same instrumental conditions as that for the higher frequency region. The broken lines as the spectral base line are used for the intensity estimation.

are not suitable to any indicators.

The Raman lines at 1645, 1566, and 1316 cm^{-1} for the 514.5-nm excitation (top trace in Figure 3) correspond to those of horse ferricytochrome *c* at 1637, 1563, and 1316 cm^{-1}, respectively (Lanir et al., 1979), and, accordingly, are assigned to the heme moiety. Note that the marker lines of the oxidized flavin and hemes are most intense at the shortest and longest wavelengths available, respectively.

Figure 5 illustrates the change of absorption spectrum when cyanide or sulfite is added to oxidized cytochrome *c*-552 at pH 6.5. When the concentrations of cyanide or sulfite are increased, the absorbance at 450 nm is decreasing and a new band appears at longer wavelengths while the band around 530 nm changes little. The concentration required for complete

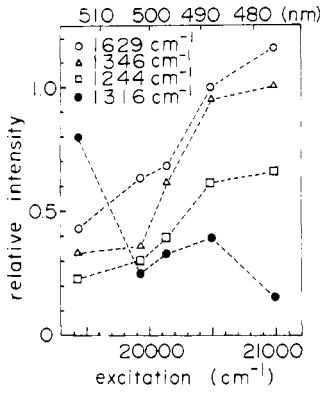

FIGURE 4: Excitation wavenumber dependence of Raman intensity of a few selected lines of oxidized cytochrome *c*-552. The intensity is evaluated as the relative peak height to the line of the SO_4^{2-} ion.

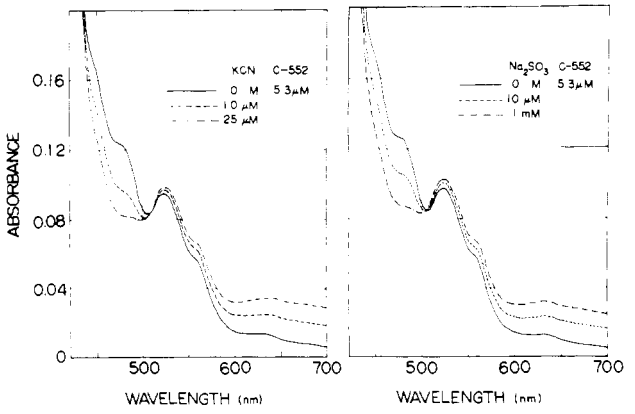

FIGURE 5: Change in absorption spectra when cyanide (left) or sulfite (right) is added to oxidized cytochrome *c*-552 in 0.1 M phosphate buffer, pH 6.5. The concentrations of the added ligands are specified in the figure. Although KCN and Na_2SO_3 are added to the solution, they are practically HCN and $NaHSO_3$, respectively, at this pH. The concentration of cytochrome *c*-552 is commonly 5.3 μM.

disappearance of the 450-nm band was lower for cyanide than for sulfite, 25 μM and 1 mM, respectively, for 5.3 μM cytochrome *c*-552. The similar spectral change occurred upon addition of $Na_2S_2O_3$, although 10 mM $Na_2S_2O_3$ was necessary to the complete disappearance of the 450-nm band in the same cytochrome solution (Fukumori, 1980).

The Raman spectral change upon addition of cyanide or sulfite is illustrated in Figure 6. The effect of the cyanide or sulfite addition upon flavin and hemes is more explicitly seen in the spectra of the 457.9- (right) and 514.5-nm (left) excitations, respectively. The spectra displayed were arbitrarily selected from those obtained in the titration experiments. When a very small amount of cyanide (0.05 mM CN^-) was added, the fluorescent background was considerably diminished without any significant change in frequencies of Raman lines. For more concentrated solutions, the Raman lines at 1318 and 1592 cm^{-1} relatively decreased in intensity and new lines appeared at 1412 and 1377 cm^{-1} (see left of Figure 6). The latter change may mislead us to think that the 1404- and 1366-cm^{-1} lines of intact cytochrome *c*-552 were shifted to 1412 and 1377 cm^{-1}, respectively, but this is not the case because of different polarization properties of the Raman lines. The Raman lines at 1348 and 1628 cm^{-1} which arise from FAD (see right of Figure 6) vanished upon addition of cyanide while the oxidation state marker line of ferriheme at 1376 cm^{-1} was relatively intensified.

When $NaHSO_3$ was added, essentially the identical spectral change occurred except for the 1368-cm^{-1} line. The depolarized line at 1645 cm^{-1} has been acknowledged as a spin-state marker (band F in Spiro & Strekas, 1974; band I in Kitagawa et al., 1976; ν_{10} in Abe et al., 1978). Accordingly, invariance of the 1645-cm^{-1} line upon addition of sulfite or cyanide represents the unaltered low-spin state of the heme iron, in good agreement with the EPR results (Strekas, 1976).

Figure 7 illustrates the EDTA-promoted photoreduction of cytochrome *c*-552. The second trace displays the spectrum

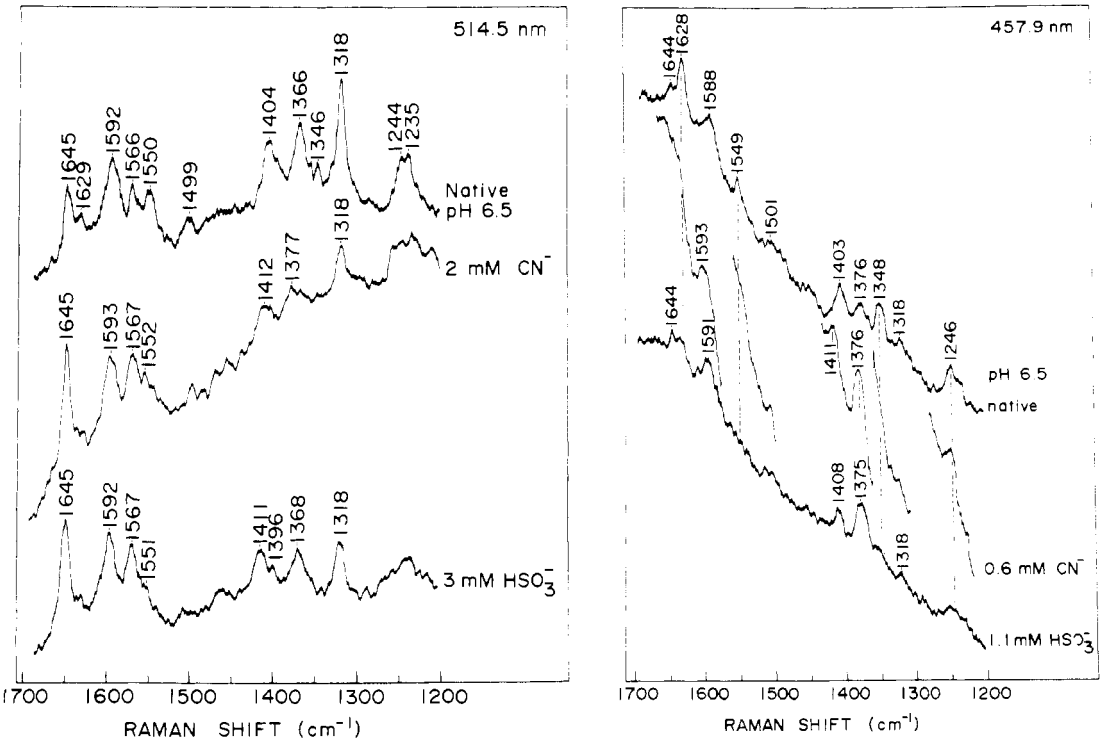

FIGURE 6: Effects of cyanide and sulfite upon the resonance Raman spectra of oxidized cytochrome *c*-552 at pH 6.5. The left and right figures represent the spectra for the 514.5- and 457.9-nm excitations, respectively. The top traces in both are for intact cytochrome *c*-552. The concentrations of cyanide and sulfite are specified at the right side of individual spectra.

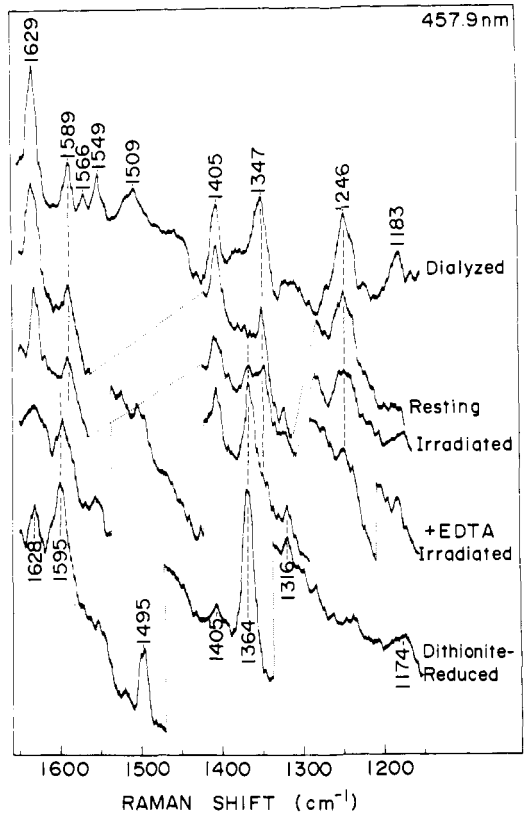

FIGURE 7: Effect of laser irradiation at 457.9 nm upon the Raman spectra of oxidized cytochrome *c*-552. Laser power is common to the five spectra, 60 mW for irradiation (0.5 h) and 20 mW for measurement. Top, the preparation dialyzed just before the Raman measurement (37 μM, pH 8.5); second, the resting cytochrome (70 μM, pH 8.5); third, irradiated for the resting cytochrome in the reduced pressure in the absence of EDTA; fourth, irradiated in the reduced pressure in the presence of 5 mM EDTA; bottom, dithionite reduced (final dithionite concentration, 3.5 mM) and kept in the reduced pressure.

of resting cytochrome *c*-552. When this sample was irradiated as described under Materials and Methods, the 1629- and 1347-cm^{-1} lines lost intensity, and instead a new line appeared at 1364 cm^{-1} as shown in the third trace. After addition of EDTA to this sample at the final concentration of 5 mM, it was subjected to the same irradiation condition again. Then the fourth trace was obtained. The 1347-cm^{-1} line disappeared completely, and an intense line appeared at 1364 cm^{-1}. When this sample was exposed to air for one night, the 1347-cm^{-1} line was restored and the 1364-cm^{-1} line disappeared. Therefore, the light-induced spectral change is reversible and is turned back by oxidation.

The bottom trace in Figure 7 was obtained after the cytochrome was chemically reduced. The intense 1364-cm^{-1} line corresponds to the oxidation-state marker of ferrohemoproteins (Yamamoto et al., 1973; Spiro & Strekas, 1974). The fourth trace is close to the spectrum shown at the bottom. Accordingly, the spectral change upon laser irradiation in the presence of EDTA is most reasonably interpreted in terms of disappearance of oxidized flavin and appearance of ferroheme. In other words, photoreduction took place. The irradiation of laser light at 514.5 nm did not result in the photoreduction.

The top spectrum in Figure 7 was obtained immediately after the 12-h dialysis against 0.1 M Tris-HCl buffer, pH 8.5. This preparation shows no fluorescent background. It is emphasized that when this was subjected to the same irradiation conditions as above in the absence of EDTA, the spectrum remained quite unaltered, but when it was irradiated in the presence of EDTA, a spectral change identical with that for the photoreduction took place.

The strong fluorescence for the resting cytochrome suggests the presence of a small amount of free EDTA dissociated from the flavoprotein subunit during the storage, and the thorough dialysis resulted in removal of the free FAD from the cytochrome solution. Partial reduction of the resting cytochrome was presumably caused by photoreduction of the free FAD. For the examination of this idea, the phenomenon was monitored by absorption spectroscopy.

The right figure of Figure 8 illustrates the change of absorption spectra during the light illumination to oxidized cytochrome *c*-552 in the presence of EDTA. Since the illuminated light in this experiment is extremely weak compared with the case of laser irradiation (see Materials and Methods), the spectral change is very slow. However, it is evident that the absorbance at 450 nm is diminished and the absorption bands at 523 and 552 nm grow as time goes. This change was not observed in the absence of EDTA. When 0.29 μM free FAD was added to the 3.7 μM solution of cytochrome *c*-552, a pronounced spectral change took place in a shorter time as illustrated in the left of Figure 8. The spectrum observed 200 min after the initiation of illumination was practically identical with that of the dithionite–reduced cytochrome *c*-552. Consequently, absorption spectroscopy also confirmed that the photoreduction of cytochrome *c*-552 occurs in the presence of EDTA and is much promoted by the presence of free FAD.

For the determination of which subunit was first photoreduced, cytochrome *c*-552 was split into flavoprotein subunit and cytochrome subunit. The split flavoprotein subunit was too fluorescent for the Raman spectrum to be measured. However, when the sample was irradiated at 457.9 nm in the presence of EDTA, the fluorescence completely disappeared and the spectrum of urea which was added to the cytochrome solution to prepare the subunit was identified. This observation indicates that photoreduction of flavin occurred because the oxidized flavoproteins are known to be usually extremely fluorescent but the reduced ones much less fluorescent, and, moreover, the dithionite-reduced split flavoprotein subunit was nonfluorescent. The photoreduced flavoprotein subunit did not give any Raman lines assignable to flavin even upon excitation at 514.5 nm.

The resonance Raman spectra of the reduced and oxidized forms of the split cytochrome subunit are shown in Figure 9. The Raman spectrum of the split ferrocytochrome subunit is close to the third spectrum in Figure 2, although the composite spectrum of the two polarization components should be compared with the former. Therefore, at this pH at least, there would exist no significant difference in environments of the heme between the intact cytochrome and the split cytochrome subunit.

In the ferric state, on the other hand, the 1366- and 1404-cm^{-1} lines of the intact cytochrome (top trace in Figure 3) are relatively diminished, new lines appear at 1376 and 1410 cm^{-1} in the split cytochrome subunit, and the relative intensities of Raman lines, I_{1320}/I_{1410} and I_{1594}/I_{1643}, clearly differ between the two species. Note that the spectrum of the oxidized form of the cytochrome subunit is closely similar to those of other ferricytochromes *c* (Kihara et al., 1978; Kitagawa et al., 1977). When the cytochrome subunit was irradiated in the presence of EDTA, no spectral change was observed.

Discussion

Photoreduction. The present Raman and absorption spectra clearly demonstrated that both flavin and heme of cytochrome *c*-552 were photoreduced in the presence of EDTA but not

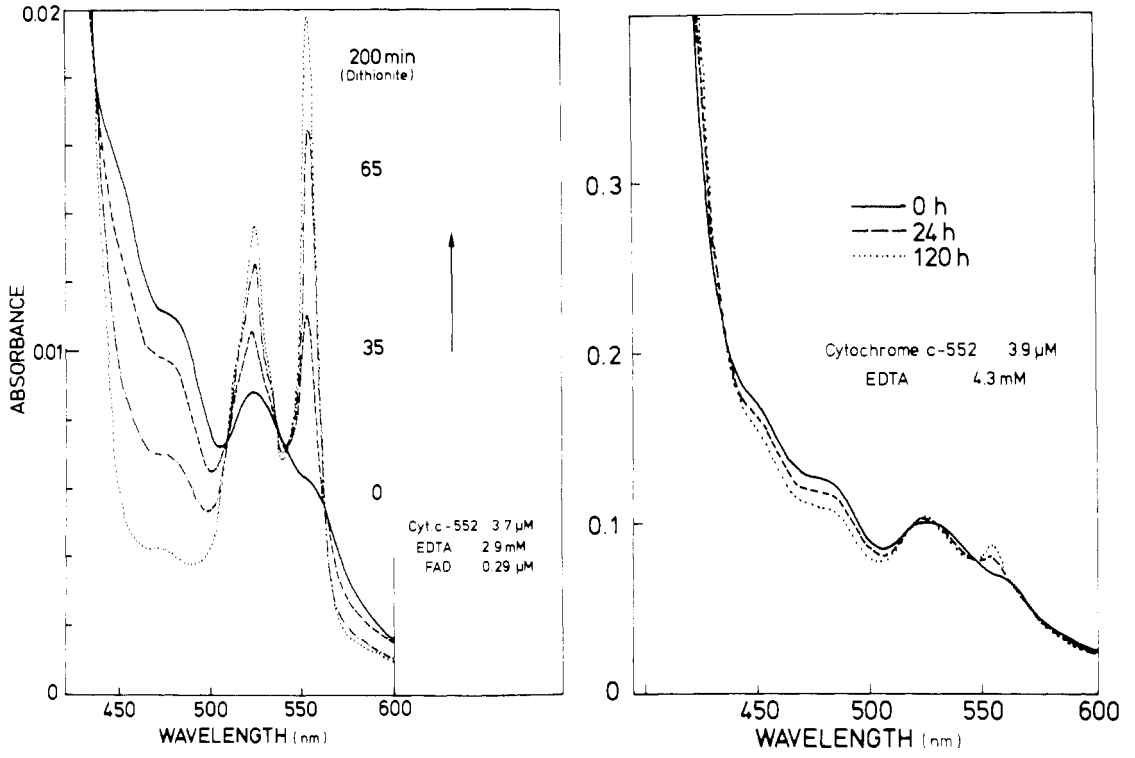

FIGURE 8: Change in absorption spectra of oxidized cytochrome *c*-552 during illumination of light (right) and the effect of the free FAD upon it (left). Both preparations were illuminated at 30 cm from a 100-W tungsten lamp, and a 10-cm-long water layer was placed between the cuvette and the lamp. The time after the initiation of illumination is specified in the figures. (Right) Concentration of cytochrome *c*-552, 3.9 μM; concentration of EDTA, 4.3 mM; buffer, 0.1 M Tris-HCl, pH 8.5. (Left) Concentration of cytochrome *c*-552, 3.7 μM; concentration of EDTA, 2.9 mM; concentration of free FAD, 0.29 μM; buffer, 0.1 M Tris-HCl, pH 8.5. The spectrum after 200 min was identical with that obtained after reduction by dithionite.

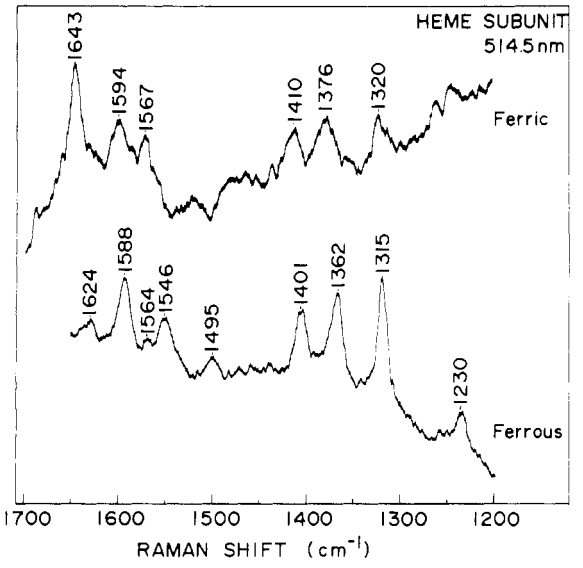

FIGURE 9: Resonance Raman spectra of the split cytochrome subunit in the ferric (upper) and ferrous (lower) states. The heme concentration is 16 μM. Excitation, 514.5 nm at 40 mW.

in its absence. The split flavoprotein subunit was also photoreduced under the same conditions as those for the intact cytochrome, and their photoreduction was similarly more effective with irradiation at shorter wavelengths. However, the split cytochrome subunit was never photoreduced even in the presence of EDTA.

Vorkink & Cusanovich (1974) have examined the photoreduction of horse heart ferricytochrome *c* in detail. The photoreduction of cytochrome *c* is accelerated by an order of 3 in the presence of EDTA or Tris, and the rate of the photoreduction is 8 times faster upon irradiation at 410 nm than at 535 nm. Cytochrome oxidase is also known to be quickly photoreduced by laser irradiation (Adar & Yonetani, 1978; Salmeen et al., 1978; Kitagawa & Orii, 1978). Recently it was found that aquomethemoglobin is photoreduced in the T quaternary structure but not in the R quaternary structure (Kitagawa & Nagai, 1979). The photoreduction of cytochrome oxidase and aquomethemoglobin is prominent upon irradiation around 440–460 nm. In this regard the photoreduction of cytochrome *c*-552 and those hemoproteins are alike, and thus a possibility for direct photoreduction of hemes of cytochrome *c*-552 may have to be taken into consideration. This may lead to the assumption that the photoreduction takes place under a particular tertiary structure such as in the intact cytochrome *c*-552.

However, there is a significant difference in photoreductions of cytochrome *c*-552 and aquomethemoglobin: the photoreduction of cytochrome *c*-552 occurs only in the presence of EDTA (or free FAD and EDTA), but photoreduction of aquomethemoglobin is not affected by addition of EDTA. The effects of EDTA upon the photoreduction of intact cytochrome *c*-552 and split flavoprotein subunit are alike. Therefore, it is more likely that the flavoprotein subunit of intact cytochrome *c*-552 is photoreduced first in the presence of EDTA and the electron is transported to the cytochrome subunit. Although this was anticipated from the lower oxidation–reduction potential of flavin than that of heme (Fukumori, 1980), the present results may serve as the first direct experimental evidence for an intramolecular electron transport from flavin to heme.

It is interesting to determine whether the photoreduced flavin of intact cytochrome *c*-552 and the split flavoprotein subunit is a semiquinone or a completely reduced form. Nishina et al. (1980b) produced the semiquinone form of riboflavin by illumination of light in an anaerobic condition

and observed an intense line at 1617 cm^{-1} besides other weak lines upon excitation at 514.5 nm. They pointed out that the 1617-cm^{-1} line could serve as a marker band of the semiquinone form because the 1631-cm^{-1} line of the oxidized form was replaced by the 1617-cm^{-1} line in the semiquinone form. In the present spectra of the photoreduced form, we failed to observe the marker band of semiquinone, even excitation at 514.5 nm. Therefore the photoreduced flavins of the intact cytochrome *c*-552 and the split flavoprotein subunit are similarly likely to adopt the completely reduced form.

The resting cytochrome *c*-552 with fluorescent background was partially photoreduced even in the absence of EDTA, but the dialyzed preparation without fluorescent background was never photoreduced in the absence of EDTA. The difference is attributed to the presence of a trace amount of free FAD which was dissociated from cytochrome *c*-552 during storage, because a free FAD is quite fluorescent and its presence considerably promotes photoreduction of cytochrome *c*-552. Exposure of FAD from the pocket of flavoprotein subunit upon partial denaturation would also cause the fluorescence, and the exposed FAD may contribute to photoreduction in the same way as did the added free FAD.

It is noteworthy that even the split cytochrome subunit as well as the intact cytochrome and the split flavoprotein subunit was photoreduced in the presence of free FAD and EDTA when it was monitored by absorption spectroscopy. This implies that the reduced free FAD can transfer electrons to either flavoprotein subunit or cytochrome subunit. Therefore it is exceedingly important to remove a trace amount of free FAD in the experiment of photoreduction. In our attempt to elucidate the inhibition mechanism of cyanide, the photoreduction of the cyanide and sulfite adducts of cytochrome *c*-552 was examined. The samples were relatively fluorescent, and the level of fluorescence of this preparation was altered by an amount of cyanide or sulfite added. This may indicate partial exposure of FAD from the pocket of flavoprotein subunit by addition of them. Although the two kinds of adducts were both photoreduced, this may be caused by the exposed FAD which can presumably transfer an electron to the heme. Since more detailed experiments are in progress, we withhold drawing any conclusion about the photoreduction of the two adducts at the present stage.

Adducts with Cyanide and Sulfite. Reaction of cytochrome *c*-552 with sulfite and cyanide is accompanied by loss of the near-ultraviolet and visible absorbance of flavin and development of new bands at 310–320 nm and 600–700 nm (Kusai & Yamanaka, 1973; Meyer & Bartsch, 1976), and the change is reversible. This is confirmed in the present experiment (see Figure 5). Thus two possibilities were taken into consideration, reduction of flavin and formation of addition compound. Figure 6 shows that the spin-state marker line of hemes remains at the characteristic frequency of ferric low-spin state after reaction with KCN or $NaHSO_3$, although the Raman lines of oxidized flavin are absent. This is consistent with unalteration of the 530-nm band and disappearance of the 450-nm band in absorption spectra.

Müller & Massey (1971) demonstrated the complex formation of free flavin with sulfite at N(5) of isoalloxazine. The addition of sulfite at N(5) disrupts the π conjugation of isoalloxazine, causing significant blue shift of the visible absorption band.

The term of molecular polarizability tensor responsible for the resonance Raman scattering is given by (Placzek, 1934)

$$\alpha_{mn} = \frac{1}{h}\sum_e \frac{\langle m|\mu|e\rangle\langle e|\mu|n\rangle}{\nu_{em} - \nu_0 - i\Gamma_e}$$

where $\langle m|\mu|e\rangle$ is the transition dipole moment for transition from $|m\rangle$ to $|e\rangle$, ν_{em} and ν_0 are the transition and excitation frequencies, respectively, and Γ_e is the damping constant at the $|e\rangle$ state. If the visible band were shifted from 453 to 315 nm upon formation of adduct, α_{mn} of the adduct for the 457.9-nm excitation would be 0.02 of that for the intact cytochrome if the magnitude of transition dipole moments were common to both. As Raman intensity is proportional to the square of α_{mn}, the Raman intensity of the adduct would be 10^{-4} times of the intact cytochrome. Consequently, the disappearance of resonance Raman lines of oxidized flavin upon formation of adduct is reasonable.

For cyanide, on the other hand, its adduct formation with flavin is not established yet; rather, the possibility of its coordination to the axial position of the heme iron comes to mind, as cyanide is a strong ligand for metmyoglobin and hemoglobin. If cyanide were bound to the axial position of the heme iron, however, some Raman lines should show an appreciable frequency shift, as was observed for the pH-induced ligand exchange of various *c*-type cytochromes (Kitagawa et al., 1977). Therefore, the possibility is less likely. If cyanide formed a charge-transfer complex with flavin, the Raman lines of oxidized flavin should be preserved in the adduct because the charge-transfer complex of FMN in old yellow enzyme with phenol derivatives exhibited all the Raman lines of oxidized flavin at the unshifted frequency but a different intensity (Kitagawa et al., 1979b). The changes in absorption spectra upon addition of cyanide and sulfite are very alike (Figure 5). Thus we are now inclined to think that cyanide is bound to N(5) of isoalloxazine.

Chromophoric Interactions. Raman spectral difference between the split cytochrome subunit and intact cytochrome would be caused by the interactions between the flavoprotein and cytochrome subunits, namely, direct or protein mediated chromophoric interactions. Comparison of the lower trace in Figure 9 with the third trace in Figure 2 shows the two spectra are very alike. This suggests that little difference exists in the heme environments between them. Accordingly, there is presumably no significant chromophoric interaction in the ferrous state at pH 8.5. Lack of spectral change upon addition of cyanide is also consistent with this presumption.

In the oxidized state, on the other hand, when the upper trace in Figure 9 is compared with the top one in Figure 3, one will notice that the intensity of Raman lines of intact cytochrome at 1366 and 1404 cm^{-1} is relatively reduced and the lines at 1376 and 1410 cm^{-1} are intensified. The 1316- and 1592-cm^{-1} lines are also weakened in the split cytochrome subunit. As noted previously, the spectrum of the split cytochrome subunit is normal compared with those of other *c*-type cytochromes.

The 1376-cm^{-1} line of the split cytochrome subunit is polarized but that of intact cytochrome at 1366 cm^{-1} is depolarized. Therefore they arise from different vibrational modes. Since all the resonance Raman lines of flavoproteins are found to be polarized (Kitagawa et al., 1979a), the 1366-cm^{-1} line cannot be attributed to the flavin moiety. Theoretically two Raman lines are expected to exist around 1350–1380 cm^{-1}, a polarized line (ν_4, A_{1g}) and a depolarized line (ν_{12}, B_{1g}) (Abe et al., 1978). When the ν_{12} line is unusually intensified in the intact cytochrome, the spectrum could be reasonably interpreted.

The same consideration is applied to the apparent frequency shift of the 1404-cm^{-1} line. Normal coordinate calculations

(Abe et al., 1978) predict the presence of two Raman lines at 1395–1410 cm^{-1}, a depolarized line (ν_{29}, B_{2g}) and an anomalously polarized line (ν_{20}, A_{2g}). For nickel octaethylporphyrin, ν_{20} is lower in frequency than ν_{29} (Kitagawa et al., 1978a). Thus it is inferred that the 1320-cm^{-1} (ν_{21}, A_{2g}) and 1592-cm^{-1} lines (ν_{19}, A_{2g}) besides ν_{12} and ν_{20} are formed under conditions which cause strong resonance enhancement of Raman intensity for the intact cytochrome.

A common feature of these modes is seen in the vibrational displacements of the methine carbon (C_m) and hydrogen perpendicular to the heme radius in the heme plane and thus in contributions of the C_mH in-plane bending modes (Abe et al., 1978).

Raman intensity of such nontotally symmetric modes of metalloporphyrins was shown to depend upon the vibronic coupling in a single excited state (QQ type in Nishimura et al., 1977). Presumably the excited state for the Q band receives a specific influence from the chromophoric interaction in the intact cytochrome, resulting in stronger vibronic coupling. Lack of frequency change between the intact cytochrome and the split cytochrome subunit indicates that the ground electronic state does not largely differ between them. This kind of phenomena characterized by intensity enhancement of Raman lines without appreciable change of their peak frequencies were previously observed for FMN–phenol charge-transfer complexes (Kitagawa et al., 1979b). Therefore it is highly possible that the porphyrin ring of heme is under charge-transfer interaction with an aromatic side chains of the surrounding amino acid residue or alternatively with an adenine ring of FAD side chain.

It is stressed that the Raman spectrum of the cyanide complex resembles that of the split cytochrome subunit, although there may be some difference about the sulfite complex as indicated by the 1368-cm^{-1} line (Figure 6). It is likely that the origin for the spectral change upon formation of the adducts is essentially identical with that for splitting of the cytochrome into the subunits, and accordingly is related with removal of the chromophoric interactions. This consideration is consistent with the EPR study which reported that little change occurs upon binding of thiosulfate to cytochrome *c*-552 at pH 5.5 (Strekas, 1976) because the EPR technique reveals the magnetic characteristics of the heme iron in the ground electronic state and the chromophoric interactions present would be significant only in the electronically excited state. The importance of the electronically excited state of the heme in electron transport was pointed out previously in the study of photoreduction of horse cytochrome *c* (Vorkink & Cusanovich, 1974), and the nature of chromophoric interaction in cytochrome *c*-552 mentioned above seems to be compatible with their conclusion.

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References

Abe, M., Kitagawa, T., & Kyoguku, Y. (1978) *J. Chem. Phys. 69*, 4526–4534.

Adar, F., & Erečinska, M. (1978) *Biochemistry 17*, 5484–5488.

Adar, F., & Yonetani, T. (1978) *Biochim. Biophys. Acta 502*, 80–86.

Bartsch, R. G., & Kamen, M. D. (1960) *J. Biol. Chem. 235*, 825–831.

Bartsch, R. G., Meyer, T. E., & Robinson, A. B. (1968) in *Structure and Function of Cytochromes* (Okunuki, K., Kamen, M. D., & Sekuzu, I., Eds.) pp 443–451, University of Tokyo Press, Tokyo.

Benecky, M., Li, T. Y., Schmidt, J., Frerman, F., Watters, K. L., & McFarland, J. (1979) *Biochemistry 18*, 3471–3476.

Carey, P. R. (1978) *Q. Rev. Biophys. II 3*, 309–370.

Dutta, P. K., Nestor, J. R., & Spiro, T. G. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 4146–4149.

Dutta, P. K., Nestor, J. R., & Spiro, T. G. (1978) *Biochem. Biophys. Res. Commun. 83*, 209–216.

Felton, R. H., & Yu, N.-T. (1978) *Porphyrins 3*, Chapter 8.

Fukumori, Y. (1980) Ph.D. Thesis, Osaka University.

Fukumori, Y., & Yamanaka, T. (1979) *J. Biochem. (Tokyo) 85*, 1405–1414.

Hendra, P. J., & Loader, E. J. (1968) *Chem. Ind. (London)* 718–719.

Hendriks, R., & Cronin, J. R. (1971) *Biochem. Biophys. Res. Commun. 44*, 313–318.

Kenney, W. C., & Singer, T. P. (1977) *J. Biol. Chem. 252*, 4767–4772.

Kihara, H., Hon-nami, K., & Kitagawa, T. (1978) *Biochim. Biophys. Acta 532*, 337–346.

Kitagawa, T., & Orii, Y. (1978) *J. Biochem. (Tokyo) 84*, 1245–1252.

Kitagawa, T., & Nagai, K. (1979) *Nature (London) 281*, 503–504.

Kitagawa, T., Kyogoku, Y., Iizuka, T., Ikeda-Saito, M., & Yamanaka, T. (1975) *J. Biochem. (Tokyo) 78*, 719–728.

Kitagawa, T., Kyogoku, Y., Iizuka, T., & Ikeda-Saito, M. (1976) *J. Am. Chem. Soc. 98*, 5169–5173.

Kitagawa, T., Ozaki, Y., Teraoka, J., Kyogoku, Y., & Yamanaka, T. (1977) *Biochim. Biophys. Acta 494*, 100–114.

Kitagawa, T., Abe, M., & Ogoshi, H. (1978a) *J. Chem. Phys. 69*, 4516–4526.

Kitagawa, T., Ozaki, Y., & Kyogoku, Y. (1978b) *Adv. Biophys. 11*, 153–192.

Kitagawa, T., Nishina, Y., Kyogoku, Y., Yamano, T., Ohishi, N., Takai-Suzuki, A. & Yagi, K. (1979a) *Biochemistry 18*, 1804–1808.

Kitagawa, T., Nishina, Y., Shiga, K., Watari, H., Matsumura, Y., & Yamano, T. (1979b) *J. Am. Chem. Soc. 101*, 3376–3378.

Kusai, A., & Yamanaka, T. (1973) *Biochim. Biophys. Acta 325*, 304–314.

Lanir, A., Yu, N. T., & Felton, R. H. (1979) *Biochemistry 18*, 1656–1660.

Meyer, T. E., & Bartsch, R. G. (1976) in *Flavin and Flavoproteins* (Singer, T. P., Ed.) pp 312–317, Elsevier, Amsterdam.

Moss, T. H., Bearden, A. J., Bartsch, R. G., & Cusanovich, M. A. (1968) *Biochemistry 7*, 1583–1590.

Müller, F., & Massey, V. (1971) *Methods Enzymol. 18b*, 468–473.

Nafie, L. A., Pezolet, M., & Peticolas, W. L. (1973) *Chem. Phys. Lett. 20*, 563–568.

Nishimura, Y., Hirakawa, A. Y., & Tsuboi, M. (1977) *J. Mol. Spectrosc. 68*, 335–358.

Nishina, Y., Kitagawa, T., Shiga, K., Horiike, K., Matsumura, Y., Watari, H., & Yamano, T. (1978) *J. Biochem. (Tokyo) 84*, 925–932.

Nishina, Y., Kitagawa, T., Shiga, K., Watari, H., & Yamano, T. (1980a) *J. Biochem. (Tokyo) 87*, 831–839.
Nishina, Y., Shiga, K., Horiike, K., Tojo, H., Kasai, S., Matsui, K., Watari, H., & Yamano, T. (1980b) *J. Biochem. (Tokyo) 88*, 411–416.
Ondrias, M. R., Findsen, E. W., Leroi, G. E., & Babcock, G. T. (1980) *Biochemistry 19*, 1723–1780.
Placzek, G. (1934) in *Rayleigh-Streuung und Raman Effekt, Handbuch der Radiologie*, Heft VI, Teil 2, Akademische Verlagsgesellschaft, Leipzig.
Salmeen, I., Rimai, L., & Babcock, G. T. (1978) *Biochemistry 17*, 800–806.
Spiro, T. G. (1975) *Biochim. Biophys. Acta 416*, 169–187.
Spiro, T. G., & Strekas, T. C. (1972) *Proc. Natl. Acad. Sci. U.S.A. 69*, 2622–2626.
Spiro, T. G., & Strekas, T. C. (1974) *J. Am. Chem. Soc. 96*, 338–345.
Spiro, T. G., & Gaber, B. P. (1977) *Annu. Rev. Biochem. 46*, 553–572.
Strekas, T. C. (1976) *Biochim. Biophys. Acta 446*, 179–191.
Vorkink, W. P., & Cusanovich, M. A. (1974) *Photochem. Photobiol. 19*, 205–215.
Yamamoto, T., Palmer, G., Gill, D., Salmeen, I. T., & Rimai, L. (1973) *J. Biol. Chem. 248*, 5211–5213.
Yong, F. C., & King, T. E. (1970) *J. Biol. Chem. 245*, 1331–1335.

Amino Acid Sequence of Ragweed Allergen Ra3†

David G. Klapper,* L. Goodfriend, and J. D. Capra

ABSTRACT: The complete amino acid sequence of ragweed pollen allergen Ra3 has been determined. The molecule consists of 101 amino acid residues and to date is the only allergen isolated from *Ambrosia elatior* (short ragweed) which contains carbohydrate. This particular preparation of allergen has a single, unique amino acid sequence, but there is evidence suggesting that, like Ra5, Ra3 isolated from pollen collected in diverse geographical areas shows amino acid sequence variation. The complete amino acid sequence was derived by utilizing only 10–12 mg of material (approximately 1 μM) as a result of recent technical innovations such as DEAE–glass ion exchangers and Polybrene as a useful sequencing aid. This is the second pollen allergen active in man which has been sequenced, and information resulting from these data should be useful in dissecting the molecular mechanisms involved in atopic allergy.

Sexual reproduction in plants depends in large part upon airborne distribution of pollen from one plant to another. Inhalation of these pollens leads to sensitization of certain individuals such that they develop symptoms of allergic disease (Wodehouse, 1971). Probably the best studied common weed pollen is that from short ragweed (*Ambrosia elatior*). Early studies by King et al. (1964) provided evidence that antigen E was the major allergen of ragweed pollen, but more recently other active allergens have been isolated [King et al., 1967; Underdown & Goodfriend, 1969; Griffiths & Brunet, 1971; Lichtenstein et al., 1973; Lapkoff & Goodfriend, 1974; reviewed by King (1976)]. Although some of these other ragweed allergens are considerably smaller than antigen E and present in only minute quantities, they are quite active and do not appear to cross-react antigenically or allergenically with antigen E (Lichtenstein et al., 1973).

Since ragweed allergens have been implicated in sensitizing certain individuals and eliciting an IgE immune response, it seems important to attempt to understand if ragweed allergens themselves are unique proteins, whether the genetic endowment of a sensitized individual is responsible, or perhaps some combination of these and other factors leads to an allergic state.

The first ragweed allergen to have its complete amino acid sequence determined was Ra5, a 5000 molecular weight protein containing no detectable carbohydrate (Mole et al., 1975). The structure of Ra5 is unusual, having eight cysteines among its 45 amino acids and, at the COOH terminus, three of the last five residues are lysine. The functional relationship of the high cysteine content and very basic "tail" is presently under study.

In addition, hypersensitivity to Ra5 is associated with HLA-B7 histocompatibility antigens (Marsh et al., 1973, 1975). This might suggest that hypersensitivity is simply a "responder" phenomenon and is not a property of the specific allergen involved.

To further investigate this problem, we have determined the primary amino acid sequence of another small ragweed allergen, Ra3 (Underdown & Goodfriend, 1969). This molecule consists of a single polypeptide chain of approximately 100 amino acids and contains some 8% carbohydrate (Goodfriend et al., 1980). An association has been described between sensitivity to Ra3 and the HLA-A2 phenotype (Marsh et al., 1977, 1979).

Materials and Methods

Allergen Ra3. Purified Ra3 was obtained from the Research Resources Branch of the National Institutes of Health. This allergen preparation was extracted from pollen collected by Greer Laboratories (Lanier, NC) and was purified essentially by following the procedures described by Underdown & Goodfriend (1969). For convenience, this preparation will be referred to as NIH Ra3.

Enzyme Digests. Tryptic and chymotryptic digests were performed on extensively reduced (7 M guanidine hydrochloride) and ^{3}H-alkylated Ra3. Dithiothreitol (0.01 M) was added to the protein solution buffered at pH 8.6 with 0.1 M Tris-HCl, and reduction of disulfide bonds proceeded at 37 °C for 30 min. This solution was returned to room temperature and trace labeled in the dark for 15 min with 5×10^{-7}

† From the Department of Bacteriology and Immunology, University of North Carolina, School of Medicine, Chapel Hill, North Carolina 27514 (D.G.K.), the Department of Experimental Medicine, McGill University, Montreal, Canada (L.G.), and the Department of Microbiology, University of Texas Health Science Center at Dallas, Dallas, Texas 75235. *Received February 21, 1980.* This work was supported by National Institutes of Health Grants 1 R01 AE 12796, 1 F32 AI 05018, and 1 R23 AI 14908 and MRC Grant (Canada) MT-2010.

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