Resonance Raman Spectra of Myoglobins Reconstituted with Spirographis and Isospirographis Hemes and Iron 2,4-Diformylprotoporphyrin IX. Effect of Formyl Substitution at the Heme Periphery†

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ABSTRACT: Spirographis and isospirographis hemes and iron 2,4-diformylprotoporphyrin IX were synthesized and their resonance Raman spectra were observed by incorporating them into sperm whale apomyoglobin (Mb) to protect them from photodegradation. The reconstituted Mb's exhibited the formyl C=O stretching band around 1650–1670 cm^{-1} both in reduced and in oxidized states and even in the ferric low-spin state, although the band is reported to be unobservable for cytochrome *a* of cytochrome oxidase. The C=O stretching frequencies clearly reflected nonequivalence of positions 2 and 4 of the porphyrin ring in the heme cavity of apoprotein. The Raman lines of native deoxy-Mb at 342 and 242 cm^{-1} showed a frequency shift upon formyl substitution and were assigned to the porphyrin ν_8 and ν_{17} modes, respectively. The porphyrin $C_\alpha C_m$ stretching (ν_{10}) and, though less sensitively, $C_\alpha N$ stretching frequencies (ν_4) differed with the substituted positions of formyl group. However, the $Fe–O_2$ stretching frequencies of the reconstituted oxy-Mb's depended little upon the substituted positions of formyl group despite large differences in oxygen affinities. The $Fe–N_\epsilon$(His-F8) stretching frequencies of three reconstituted deoxy-Mb's and native deoxy-Mb were alike (221–224 cm^{-1}). Therefore, it was concluded that the large difference in oxygen affinity among those Mb's has no correlation with the bond energy of the $Fe–O_2$ bond of oxy-Mb and the $Fe–N_\epsilon$(His-F8) bond of deoxy-Mb. The CO stretching frequency of the reconstituted COMb's was highest (1954 cm^{-1}) for the 2,4-diformyl heme as anticipated from the strong electron-withdrawing power of the formyl group. The present results appeared consistent with the classification that the 215- and 270-cm^{-1} lines of reduced cytochrome oxidase in mitochondria arise from high-spin cytochrome a_3 and low-spin cytochrome *a*, respectively.

Resonance Raman spectroscopy has revealed the vibrational spectra of chromophoric groups of biological molecules (Spiro & Gaber, 1977) and has been extensively used to study structural details of heme group for various hemoproteins (Spiro, 1975; Felton & Yu, 1978; Kitagawa et al., 1978b). In its application to whole mitochondria, the resonance Raman spectra (RRS)[1] of cytochromes *b* and *c* were selectively observed by appropriate choice of the excitation wavelengths (Adar & Erecińska, 1978), and, moreover, a photoreductive titration experiment was performed by Adar & Erecińska (1979). The latter study found three redox states for cytochrome oxidase in mitochondria which were characterized by intensities of a few Raman lines.

Cytochrome oxidase, the terminal oxidase in mitochondrial respiration, contains heme *a* prosthetic groups. The importance of unusual peripheral substituents of heme *a* porphyrin ring, which are a formyl group at position 8 and a hydroxyfarnesylethyl group at position 2, was pointed out by the reconstitution experiments (Hill & Wharton, 1978). The RRS of isolated cytochrome oxidase (Salmeen et al., 1973, 1978; Adar & Yonetani, 1978; Kitagawa & Orii, 1978) as well as heme *a* derivatives (Kitagawa et al., 1977; Salmeen et al., 1978; Babcock & Salmeen, 1979) were extensively studied. The Raman lines associated with the heme *a* formyl group are detectable in the high-spin state or in CH_2Cl_2 but not in the low-spin state or in H_2O (Babcock & Salmeen, 1979) and are found to serve as marker bands distinguishing the intermediate redox states of cytochrome oxidase in mitochondria (Adar & Erecińska, 1979). The presence or absence of the formyl band was interpreted in terms of coplanarity of the formyl and porphyrin groups or a change of the electron-withdrawing power of the formyl group by formation of a hydrogen bond (Babcock & Salmeen, 1979). Thus, a study of the effect of formyl substitution at the heme periphery upon RRS is of biological as well as spectroscopic interest. Accordingly, in the present study, we investigated the RRS of formyl-substituted iron protoporphyrins IX, namely, iron 2-formyl-4-vinyl- (2F-4V), iron 2-vinyl-4-formyl- (2V-4F), and iron 2,4-diformylprotoporphyrin IX (2F-4F) (see Figure 1).

The 2F-4V and 2V-4F hemes are called spirographis and isospirographis hemes, respectively. Especially the former is a prosthetic group of chlorocruorin, functioning as an oxygen carrier in some marine annelida, and, accordingly, analysis of its RRS implicates biological importance. Since these porphyrins are photoreactive, they were reconstituted into sperm whale apomyoglobin to protect them from photodegradation upon laser illumination.

Materials and Methods

Preparation of Reconstituted Myoglobins. Myoglobin (Mb) was prepared from sperm whale muscle by the method of Yamazaki et al. (1964) with slight modifications. The minced muscle was squeezed through a thick cloth, and the solution was 55% saturated with $(NH_4)_2SO_4$ after adjustment of its pH to 7.8 with 1 M Tris. The precipitate was removed by centrifugation, and the supernatant was 95% saturated with $(NH_4)_2SO_4$. The solution was spun and the precipitate was

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[1] Abbreviations used: RRS, resonance Raman spectra; 2F-4V, iron 2-formyl-4-vinylprotoporphyrin IX; 2V-4F, iron 2-vinyl-4-formylprotoporphyrin IX; 2F-4F, iron 2,4-diformylprotoporphyrin IX; 2V-4V, iron 2,4-divinylprotoporphyrin IX; Mb, myoglobin; Hb, hemoglobin; Tris, tris(hydroxymethyl)aminomethane; Ni(OEP), (octaethylporphyrinato)-nickel(II).

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dissolved in a minimum amount of distilled water and dialyzed several times against 0.005 M Tris-HCl buffer, pH 8.4. The dialyzed solution was applied to a column of DE-23 (Whatman) equilibrated with the same buffer, and the first main peak was collected. The high purity of Mb was confirmed by isoelectric focusing on polyacrylamide gel containing Ampholine, pH 6–8 (LKB) (Righetti & Drysdale, 1971).

2F-4V, 2V-4F, and 2F-4F hemes were obtained according to Sono & Asakura (1974). Incorporation of the iron porphyrins into apo-Mb was carried out by the method of Asakura & Sono (1974). The reconstituted Mb was reduced by sodium dithionite under N_2 atmosphere and then was equilibrated with CO gas. The (carbon monoxy)-Mb (COMb) thus obtained was passed through a column of Sephadex G-25 (fine) (Pharmacia) equilibrated with deoxygenated 0.01 M sodium phosphate buffer, pH 6.0 ($NaH_2PO_4 \cdot 2H_2O$, 2.26 g; Na_2HPO_4, 2.52 g/5 L), and then was applied to a column of CM-52 (Whatman) equilibrated with the same buffer. After washing, we eluted the adsorbed COMb by 0.02 M sodium phosphate buffer, pH 7.2 ($NaH_2PO_4 \cdot 2H_2O$, 0.80 g; Na_2HPO_4, 1.06 g/1 L). By this procedure met-Mb was completely removed. The COMb solution was concentrated by ultrafiltration in a collodion bag (Sartorius membrane filter, SM 13200). Then carbon monoxide was removed from COMb according to Kilmartin & Rossi-Bernardi (1971). The Mb solution was gel-filtered against 0.02 M sodium phosphate buffer, pH 7.2. The absorption spectra of deoxy-, oxy-, and (carbon monoxy)-Mb's thus reconstituted were in good agreement with those reported by Sono & Asakura (1975), and the concentration of oxy-Mb's was determined spectrophotometrically according to Sono & Asakura (1975).

Measurements of Resonance Raman Spectra. For measurements of RRS of oxy-Mb, 0.3 mL of oxy-Mb solution (0.3 mM) was put into a screw-cap septum cell after filtration through a membrane filter (Sartorius, 0.8 μm), and the cell was saturated with O_2 gas. Deoxy-Mb was prepared from oxy-Mb in the Raman cell by repeated evacuation and flushing with N_2 gas and finally by adding a minimum amount of sodium dithionite solution anaerobically.

The Raman scattering was excited by the 488.0- and 457.9-nm lines of an Ar–Kr laser (Spectra Physics, Model 164) and the 441.6-nm line of a He–Cd laser (Kinmon Electric Corp.) and was recorded on a JEOL-400D Raman spectrometer. The spectrometer was calibrated with indene (Hendra & Loader, 1968) for each experiment. The cell was placed in a thermostated metal cell holder kept at 10 ± 2 °C. After measurements of Raman spectra, nondegradation of the formyl heme was confirmed by the absorption spectrum of its diluted solution (Hitachi 124).

Measurements of Infrared Spectra. Infrared (IR) spectra were measured for COMb with a Hitachi 225 IR spectrophotometer. For measurement ~1 mM solution of the reconstituted COMb was put in the 0.05-mm thick fixed cell equipped with CaF_2 windows. The native met-Mb solution of identical concentration was placed in the reference beam. The spectra were recorded in a transmittance mode, and calibration of the spectrometer was performed with the 1942.6-cm^{-1} band of H_2O vapor.

Results

Absorption spectra of deoxy- and oxy-Mb's reconstituted with 2F-4V, 2V-4F, and 2F-4F hemes are shown in Figure 1, where the excitation wavelengths of Raman scattering are designated with arrows. Atomic numbering of the porphyrin ring is also represented in Figure 1. Due to the presence of the electron-withdrawing formyl group, both Soret and Q

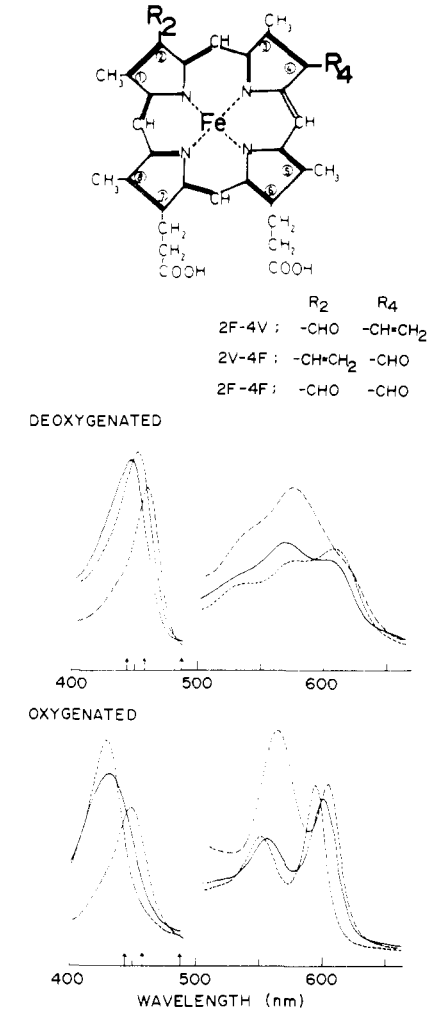

FIGURE 1: Atomic numbering of porphyrin ring atoms and absorption spectra of reconstituted deoxy-Mb's (upper) and oxy-Mb's (lower). The substituents at positions 2 and 4 are designated as R_2 and R_4, respectively, and other peripheral substituents are identical with those of protoporphyrin IX. (—) 2F-4V (spirographis heme); (---) 2V-4F (isospirographis heme); (–·–) 2F-4F (2,4-diformyl heme). Arrows indicate the wavelengths of excitation lines used.

bands are shifted to red as in cytochrome oxidase. The Soret peak of deoxy-Mb can clearly distinguish 2F-4V (448.5 nm) from 2V-4F (441 nm) heme, although the spectra of free hemes are almost identical. As Sono & Asakura (1975) pointed out, formyl groups of the isomers interact differently with the surrounding amino acid residues and, as a result, give rise to the absorption spectra specific to the substituted positions.

The RRS of deoxy-Mb's excited at 457.9 nm in the higher and lower frequency regions are shown in parts a and b of Figure 2, respectively, where the RRS of 2F-4V-Mb (A), 2V-4F-Mb (B), and 2F-4F-Mb (C) are compared with the RRS of native 2V-4V-Mb (D). The Raman spectral characteristics were almost unaltered by excitation at 441.6 nm. These RRS of native sperm whale Mb agreed with those of horse heart Mb (Desbois et al., 1979; Kincaid et al., 1979).

The most prominent feature of the RRS in Figure 2a lies in the appearance of a new Raman line around 1650–1670 cm^{-1} upon formyl substitution. This Raman line is assignable to the formyl C=O stretching mode and corresponds to the characteristic line of reduced cytochrome oxidase at 1670 cm^{-1} (Salmeen et al., 1978). It is noted that the frequency of the C=O stretching mode depends upon whether the formyl group is located at position 2 (1648 cm^{-1}) or at position 4 (1660 cm^{-1})

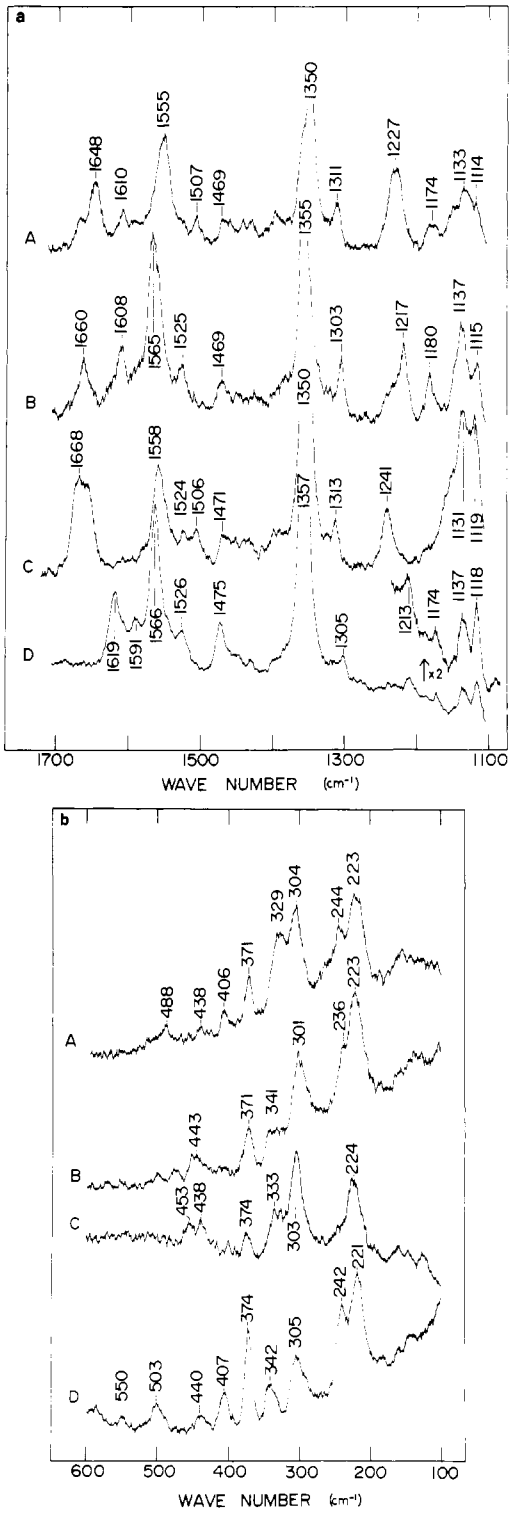

FIGURE 2: Resonance Raman spectra of reconstituted deoxy-Mb's in the higher (a) and lower (b) frequency regions. Laser: 457.9 nm, 30 mW at sample. (A) 2F-4V-Mb; (B) 2V-4F-Mb; (C) 2F-4F-Mb; (D) 2V-4V-Mb (native). All were in 0.02 M sodium phosphate buffer, pH 7.2; heme concentration, 0.3 mM.

or at both (1668 cm^{-1}). Increased delocalization of the formyl π electrons to the C_β–CHO bond decreases the bond order of the C=O bond and thus lowers its stretching frequency. Therefore, the difference in the C=O stretching frequency implies that π electrons are more donated from the –CHO group to the C_β–CHO bond when it is located at position 2 compared with when it is at position 4. Alternatively, the formyl group at position 2 forms a stronger hydrogen bond with the surrounding amino acid residues compared with that at position 4, because the stronger CO···H interaction generally gives the lower C=O stretching frequency. If the C=O stretching frequencies differed between positions 2 and 4 due to different strength of hydrogen bonding, 2F-4F-Mb would display two Raman lines at almost the same frequencies as those of 2F-4V- and 2V-4F-Mb's, but this contradicts the results of spectrum C of Figure 2a. Thus the latter alternative seems less likely.

When both positions are occupied by formyl groups, donation of electrons from the individual –CHO groups becomes less than in the case of monosubstitution probably due to mutual repulsion of electrons. Then the C=O groups may possibly have a similar stretching frequency, giving rise to apparently one broad band. In this regard, substitution at position 8 in heme *a* (1670 cm^{-1}) is close to the case of 2F-4F-Mb. Frequencies of other Raman lines above 1200 cm^{-1} were appreciably altered by the 2- or 4-formyl substitution and will be discussed later.

In the lower frequency region (Figure 2b), the RRS of the formyl-substituted Mb's differed less from the RRS of native Mb compared with the higher frequency region. Salmeen et al. (1978) previously pointed out that the appearance of Raman lines of reduced cytochrome oxidase around 215 and 364 cm^{-1} was owed to protein–heme interaction and also to a cytochrome a_3 component. The former line was observed at 221 cm^{-1} for native deoxy-Mb. This line was recently shown to be sensitive to ^{54}Fe substitution and was assigned to the Fe–N_ϵ (His-F8) stretching mode (Kitagawa et al., 1979). The frequencies of this mode for the reconstituted Mb's (223, 223, and 224 cm^{-1} for 2F-4V-, 2V-4F-, and 2F-4F-Mb's, respectively) were reasonably unaltered by formyl substitution. Thus, the suggestion by Salmeen et al. (1978) that the 215-cm^{-1} line of reduced cytochrome oxidase might arise from the Fe–axial ligand stretching mode is supported by the present study. Since these lines shifted toward higher frequency upon conversion to ferrous low-spin state, the classification of the 215-cm^{-1} line of reduced cytochrome oxidase to the high-spin cytochrome a_3 component is also consistent with the present observation.

The shoulder band at the higher frequency side of the 221-cm^{-1} line of native deoxy-Mb is noted to be sensitive to formyl substitution: 244 cm^{-1} for 2F-4V-Mb, 236 cm^{-1} for 2V-4F-Mb, nothing for 2F-4F-Mb, and 242 cm^{-1} for 2V-4V-Mb. Therefore, the shoulder band is presumably associated with the vibration of vinyl bending modes. It has been wondered why the shoulder band is present for deoxy-Mb and absent for deoxy-Hb (Nagai et al., 1980). The present observation suggested that the difference was caused by the dissimilar situation of vinyl groups between deoxy-Hb and deoxy-Mb.

The other important feature in the lower frequency region is a frequency change of the Raman line of native Mb at 342 cm^{-1} upon formyl substitution: 329 cm^{-1} for 2F-4V-Mb, 341 cm^{-1} for 2V-4F-Mb, and 333 cm^{-1} for 2F-4F-Mb. The Raman line was previously assigned to the mode involving primarily the C_β–C(peripheral) bending motion [ν_8, Abe et al. (1978)]. The sensitive change of its frequency upon formyl substitution supports the assignment. Other prominent Raman lines of native Mb at 305, 374, 407, and 440 cm^{-1} were found to be relatively insensitive to formyl substitution.

The RRS of oxy-Mb's excited at 457.9 nm in the higher and lower frequency regions are shown in parts a and b of Figure 3, respectively, where the RRS of 2F-4V-Mb (A), 2V-4F-Mb (B), and 2F-4F-Mb (C) are compared with the RRS of native 2V-4V-Mb (D). The RRS of all oxy-Mb's

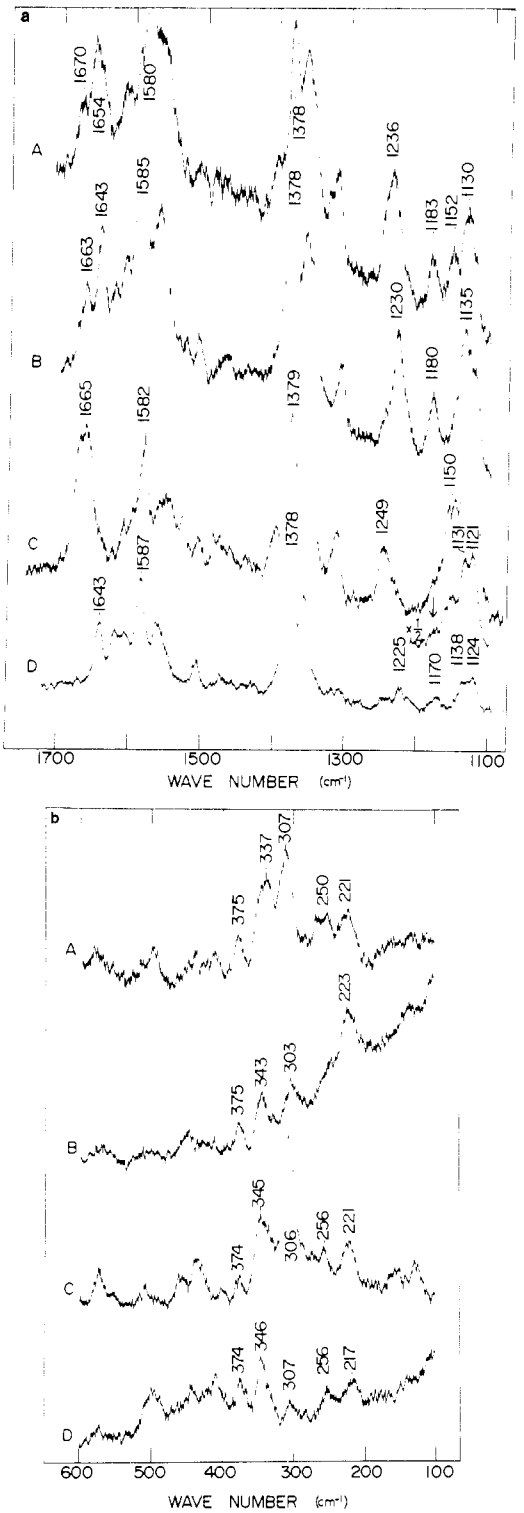

FIGURE 3: Resonance Raman spectra of reconstituted oxy-Mb's in the higher (a) and lower (b) frequency regions. (A) 2F-4V-Mb; (B) 2V-4F-Mb; (C) 2F-4F-Mb; (D) 2V-4V-Mb (native Mb). Experimental conditions are the same as those described in Figure 2.

around 1350–1380 cm^{-1} consisted of two lines and the lower frequency counterpart is due to photodissociated deoxy-Mb. Even with a spinning cell the photodissociation could not be eliminated. The Raman line around 1670 cm^{-1} may possibly be derived from deoxy-Mb, and therefore the C=O stretching frequency of the reconstituted oxy-Mb's could not be confidently determined from the present experiment.

Despite the incomplete spectra, we emphasize that a new Raman line is clearly recognized for native oxy-Mb at 256

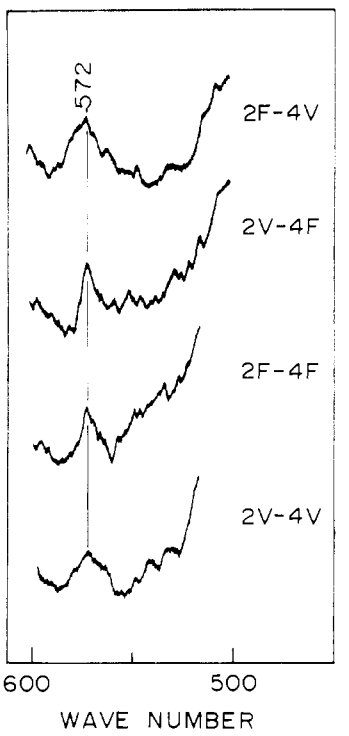

FIGURE 4: Resonance Raman spectra of reconstituted oxy-Mb's in the Fe–O_2 stretching region Laser, 488.0 nm. The types of hemes are specified at the right side of the individual spectra.

cm^{-1}. In the recent report by Desbois et al. (1979), the RRS of oxy-Mb and deoxy-Mb excited at 441.6 nm were closely alike, and there was no trace of a Raman line around 256 cm^{-1} for oxy-Mb. However, existence of a Raman line around 250 cm^{-1} was also confirmed for 2F-4V-Mb, 2V-4F-Mb, and 2F-4F-Mb. Furthermore, Raman lines of deoxy-Hb were more strongly resonance-enhanced than those of oxy-Hb by this excitation line. Therefore, we speculate that the RRS of oxy-Mb of Desbois et al. (1979) might be derived from the photodissociated deoxy-Mb component.

The extent of photodissociation could be deduced from the relative intensity of the two lines, 1375/1355. This, shown in Figure 3a, indicates that 2F-4F- and 2V-4V-oxy-Mb's are most and least photodissociable, respectively. Consideration on wavelength difference between the excitation line (457.9 nm) and λ_{max} of the Soret band of oxy-Mb's (447 nm for 2F-4F-oxy-Mb, 435 nm for 2F-4V-oxy-Mb, 429 nm for 2V-4F-oxy-Mb, and 418 nm for 2V-4V-oxy-Mb) may allow us to infer that the closer the Soret band is to the excitation wavelength, the more the photodissociation takes place. Alternative interpretation may attribute the photoliability to lower oxygen affinity. Indeed, the oxygen affinity of 2F-4V-Mb (p_{50} = 2.7 mmHg) and 2F-4F-Mb (p_{50} = 2.8 mmHg) is about one-third of those for 2V-4F-Mb (p_{50} = 1.0 mmHg) and Mb (p_{50} = 0.5 mmHg) (Sono & Asakura, 1975). Such nonequivalence of the formyl groups at positions 2 and 4 in the reconstituted Mb's was previously noticed from a distinct difference in optical and oxygen binding properties between 2V-4F-Mb and 2F-4V-Mb (Asakura & Sono, 1974; Sono & Asakura, 1975).

The photodissociation was more serious upon excitation at 441.6 nm but much less at 488.0 nm. The RRS of the reconstituted oxy-Mb's between 500 and 600 cm^{-1} are shown in Figure 4. Unexpectedly, the Fe–O_2 stretching band was observed in all at 572 ± 1 cm^{-1}, though somewhat higher than the 567 cm^{-1} of oxy-Hb (Brunner, 1974). This clearly indicates that the Fe–O_2 bond strength is almost identical between

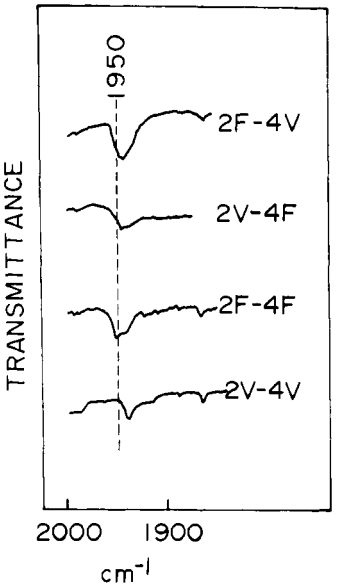

FIGURE 5: Infrared absorption spectra of the reconstituted COMb's in the CO stretching region. The spectra are represented in a transmittance mode. The types of hemes are specified at the right side of individual spectra. A broken line is drawn at 1950 cm^{-1} for easier comparison.

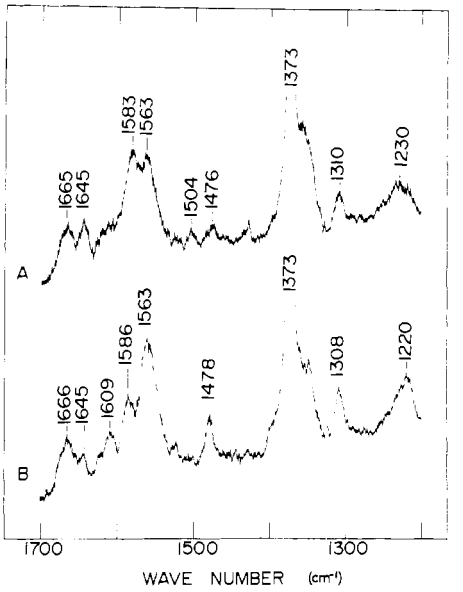

FIGURE 6: Comparison of the resonance Raman spectra of low-spin and high-spin met-Mb's reconstituted with spirographis heme. (A) Imidazole complex (low spin); (B) fluoride complex (high spin). Both are in 0.1 M Tris-HCl buffer, pH 8.0. Other experimental conditions are the same as those described in Figure 2.

2F-4V- and 2V-4F-oxy-Mb's, and, accordingly, the oxygen affinity does not depend upon the bond energy of the Fe–O_2 bond.

Figure 5 shows the IR spectra of the reconstituted COMb's in the CO stretching region. 2F-4F-COMb exhibited the CO stretching band at the highest frequency (1954 cm^{-1}) and 2V-4V-COMb (native) did at the lowest (1944 cm^{-1}). The magnitude of frequency difference is almost the same as that between COHb [1951 cm^{-1}, Alben & Caughey (1968)] and COMb [1944 cm^{-1}, Maxwell et al. (1974)]. Here again we note that the state of the CO molecule differs little between 2F-4V- and 2V-4F-COMb's, and thus their distinct dissimilarity in the electronic spectra is not transmitted to the bound CO molecule in the ground electronic state.

In the study of RRS of oxidized cytochrome oxidase, Babcock & Salmeen (1979) pointed out that all the observed Raman lines were assignable to low-spin cytochrome *a* but not to high-spin cytochrome a_3 and also that cytochrome *a* did not exhibit the Raman line of the formyl group at 1670 cm^{-1} at all. Thus, we examined the RRS of the formyl-substituted met-Mb in the high- and low-spin states as shown in Figure 6, which compares the RRS of the imidazole complex of 2F-4V-met-Mb (low spin) (A) with the RRS of 2F-4V-fluoro-met-Mb (high spin) (B). The intensity ratio of two Raman lines, 1583/1563, changed upon the conversion of spin states consistently with the empirical rule pointed out by Yamamoto et al. (1973). Nevertheless, the Raman line of the formyl C=O stretching mode was observed at an identical frequency (1666 cm^{-1}). A small shoulder present around 1350 cm^{-1} may be due to photoreduced Mb. But the portion of photoreduced molecule, if any, is extremely small, and the Raman line at 1666 cm^{-1} cannot be attributed to the photoreduced component in consideration of its intensity.

Babcock & Salmeen (1979) suggested that the Raman line of the formyl group is observed only when the formyl group is placed in a hydrophobic environment without forming a hydrogen bond. Normally, positions 2 and 4 of the porphyrin ring are located in the depth of the heme cavity of the protein for Mb (Takano, 1977a,b) and solvent water may not reach there. If the structure is retained for the reconstituted Mb's, the formyl group would be placed in the hydrophobic environment. In accord, the formyl group in the reconstituted Mb's did not react with $NaHSO_3$ and HCN, although both reacted with the formyl group of isolated heme *a*, yielding the addition compound (Kitagawa et al., 1977). Therefore, the appearance of the Raman line of formyl group for the reconstituted Mb is consistent with Babcock and Salmeen's suggestion.

It is well-known that oxidized cytochrome oxidase is quickly photoreduced by illumination of laser light (Adar & Yonetani, 1978; Salmeen et al., 1978; Kitagawa & Orii, 1978). However, the reconstituted Mb's were merely partially photoreduced, and the extent of photoreduction did not depend upon the spin state. On the other hand, met-Hb in the T structure was partially photoreduced by laser illumination at 441.6 nm, but met-Hb in the R structure was not (Kitagawa & Nagai, 1979). These facts imply that occurrence of photoreduction depends upon protein–heme interactions but not upon the physical property of heme provided by the peripheral substituents.

Discussion

The vibrations of porphyrin ring were analyzed based on the RRS data of isotope-substituted (octaethylporphyrinato)nickel(II) [Ni(OEP)] (Kitagawa et al., 1978a) and subsequent normal coordinate calculations (Abe et al., 1978). The totally symmetric $C_\alpha N$ stretching mode (ν_4) which has been practically used as an oxidation state marker of hemoproteins is usually less influenced by the peripheral substituents. The low-frequency shift of this mode in the reduced state was ascribed to more delocalization of π electrons to the porphyrin Π^* orbital (Spiro & Strekas, 1974; Kitagawa et al., 1976). The corresponding line of the reconstituted Mb's was observed at 1350 cm^{-1} for 2F-4V-Mb and 2F-4F-Mb, 1355 cm^{-1} for 2V-4F-Mb, 1357 cm^{-1} for 2V-4V-Mb, and 1360 cm^{-1} for reduced cytochrome oxidase (Adar & Yonetani, 1978; Salmeen et al., 1978; Kitagawa & Orii, 1978). This may indicate that even the central $C_\alpha N$ bond can appreciably sense the substituted position of formyl group. Conversely, the Fe–O_2 bond strength of reconstituted oxy-Mb's was much less

affected by the number and the substituted positions of formyl group. It is an extremely important finding in this study that the Fe–O_2 bond energy has no correlation with the oxygen affinity.

The CO stretching frequency of COMb is usually shifted to higher frequency, with an increase of the electron-withdrawing ability of the substituents at positions 2 and 4 (Alben & Caughey, 1968). The frequency, 1954 cm^{-1}, for 2F-4F-COMb is reasonably higher than 1944 cm^{-1} of native COMb. Between the two frequencies, the CO stretching bands of the monoformyl-COMb's were located as shown in Figure 5, but the difference between 2V-4F-COMb and 2F-4V-COMb was quite small. Therefore, the π donor property of ferrous heme decreases with the number of formyl groups at the heme periphery, but this property depends little upon the substituted positions for monoformyl hemes. The dissimilar appearance of the effects of formyl substitution on the Fe–O_2 stretching frequency of oxy-Mb and the CO stretching frequency of COMb is noted.

The highest frequency porphyrin mode (ν_{10}, B_{1g}), mainly due to the $C_\alpha C_m$ stretching vibration, was observed at 1610 cm^{-1} for 2F-4V-deoxy-Mb, at 1608 cm^{-1} for 2V-4F-deoxy-Mb, and at 1619 cm^{-1} for 2V-4V-deoxy-Mb. However, for 2F-4F-deoxy-Mb, the corresponding line was not seen (spectrum C, Figure 2a). Therefore, the electronic effect of formyl substitution is complicatedly transmitted to bonds at the methine bridge. This difference among the reconstituted Mb's is also preserved after oxygenation, in which the Raman line is seen around 1643 cm^{-1} for 2F-4V-Mb, 2V-4F-Mb, and 2V-4V-Mb but not for 2F-4F-Mb. In reduced mitochondria, two Raman lines were observed in this frequency region [1623 and 1609 cm^{-1}, Adar & Erecińska (1979)]. The two lines might indeed belong to two different heme *a* groups of cytochrome oxidase.

Salmeen et al. (1978) attributed the Raman line at 1230 cm^{-1} of reduced cytochrome oxidase to the cytochrome a_3 component. The frequency of this line of the reconstituted deoxy-Mb's is particularly sensitive to the formyl substitution: 1227 cm^{-1} for 2F-4V-Mb, 1217 cm^{-1} for 2V-4F-Mb, 1241 cm^{-1} for 2F-4F-Mb, and 1213 cm^{-1} for 2V-4V-Mb. This line corresponds to the 1220-cm^{-1} line of Ni(OEP) which was assigned to ν_{13} (B_{1g}), the methine bridge C_mH bending mode (Abe et al., 1978). The 1305-cm^{-1} line of native deoxy-Mb is also assignable to the C_mH bending (ν_{21}, A_{2g}). In fact, the frequency change of this mode upon formyl substitution is parallel to that of ν_{13}. This feature is also preserved for oxy-Mb's and met-Mb's (Figures 3a and 6).

The C=O stretching mode was observed around 1660 cm^{-1} irrespective of the oxidation and spin states of the heme iron. However, it is likely that the C=O stretching fundamental is in Fermi resonance with the combination mode of ~1355-cm^{-1} (ν_4) and 303-cm^{-1} modes, particularly for 2V-4F-Mb and 2F-4F-Mb, resulting in a complicated intensity pattern in the 1650–1670-cm^{-1} region. Therefore, whether the 1650-cm^{-1} line of oxidized cytochrome oxidase (Babcock & Salmeen, 1979) is associated with the formyl group or ν_{10} of the porphyrin ring would require more study.

It is especially important to analyze the RRS in the lower frequency region because the difference in the RRS between the T and R quaternary structures of deoxy-Hb was found only in this frequency region (Nagai et al., 1980). As described above, the Raman lines of native deoxy-Mb at 342 and 242 cm^{-1} showed a frequency shift upon the formyl substitution and are presumably due to ν_8 and ν_{17} of porphyrin ring modes. They involve predominantly the C_β–C(peripheral) bending (Abe et al., 1978). The assignment of the 221-cm^{-1} line of native deoxy-Mb is somewhat controversial; Desbois et al. (1979) and Kincaid et al. (1979) attributed it to the porphyrin ring mode whereas Kitagawa et al. (1979) claimed it to be associated with the Fe–N_ϵ(His-F8) stretching mode. The experimental evidence for the latter proposal lies in the observation of the ^{54}Fe isotopic frequency shift of that Raman line for deoxy-Mb as well as for the iron protoporphyrin–2-methylimidazole complex. Furthermore, for the picket fence porphyrin–2-methylimidazole complex, the frequency shifts upon ^{54}Fe substitution and upon deuteration of 2-methylimidazole were also observed recently (Hori & Kitagawa, 1980). Accordingly, we believe that the Raman lines of reconstituted deoxy-Mb's at 223–224 cm^{-1} arose from the Fe–N_ϵ(His-F8) stretching mode, although it may be vibrationally coupled with the lowest frequency totally symmetric mode of the porphyrin ring (ν_9) around 300 cm^{-1}. For a ferrous low-spin hemoprotein, a Raman line was observed at 277 cm^{-1} instead of the 223-cm^{-1} line (Kitagawa et al., 1979). On the basis of this distinct feature between the ferrous low-spin and high-spin hemoproteins, the 216- and 270-cm^{-1} lines of reduced cytochrome oxidase in mitochondria (Adar & Erecińska, 1979) may belong to the vibrations of high-spin cytochrome a_3 and low-spin cytochrome *a* components, respectively.

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Interaction of Opioid Peptides with Model Membranes. A Carbon-13 Nuclear Magnetic Study of Enkephalin Binding to Phosphatidylserine†

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ABSTRACT: The binding of enkephalin to phosphatidylserine has been studied, by using ^{13}C NMR, as a model for interactions with components of biological receptors. Chemical shifts, line widths and spin–lattice relaxation times were measured for peptides enriched to 90% in ^{13}C. The pK_a values of the terminal amino and carboxyl groups were determined from the pH dependence of the ^{13}C chemical shifts. Interaction of (2-[2-^{13}C]glycine)methionine-enkephalin, (3-[2-^{13}C]-glycine)methionine-enkephalin, and (3-[2-^{13}C]glycine)methionine-enkephalinamide with phosphatidylserine (PS) was studied as a function of pH. Salt and morphine antagonism to binding was manifest. Binding was shown to be pH dependent, exhibiting a maximum under slightly acidic conditions. Whereas the $-NH_3^+$ group of enkephalin is essential for binding, the data suggest that neither the tyrosyl hydroxyl group nor the COO^- group is involved. Binding affects the ^{13}C spin–lattice relaxation times most strongly; the chemical shifts and line widths of the ^{13}C-enriched material show little perturbation in the presence of PS. The internal flexibility of the peptides is decreased, on binding to model membranes, by 1 order of magnitude. Dissociation constants have been measured as 4×10^{-1} M and 2.6×10^{-3} M for enkephalin and enkephalinamide, at pH 6.3 and 6.4, respectively.

Structure–activity studies on the opioid peptides, in particular the enkephalins, have attempted to relate not only structural but also conformational features of these peptides to the geometry of morphine. Although X-ray (Smith & Griffin, 1978) and solution (Bleich et al., 1977; Jones et al., 1977; Roques et al., 1976; Garbay-Jaurequiberry et al., 1976) studies, as well as theoretical calculations (De Coen et al., 1977; Isogai et al., 1977), may indicate a preferred conformer or set of preferred conformations (Combrisson et al., 1976; Deslauriers et al., 1978; Tancrède et al., 1978; Fischman et al., 1978; Higashijima et al., 1979), such studies do not take into account the nature of substrate–receptor interactions and therefore can provide little insight into the conformation(s) of the receptor-bound peptide. The flexibility of these compounds raises the possibility that the conformation of the bound peptide is completely different from that in the absence of receptor. Structure–activity studies (Gorin et al., 1978) have been used to infer possible peptide conformations at the receptor, but such studies are indirect and therefore less satisfactory than direct observation for the elucidation of receptor-bound peptide conformation. We felt that, by monitoring the bound form of the opioid peptides via NMR spectroscopy, direct indications of peptide conformations on the receptor might be obtained. Thus, we undertook studies of the binding properties of these peptides (Tancrède et al., 1978; Deslauriers et al., 1978; Jarrell et al., 1979).

Phospholipids have been implicated as essential components of the opiate receptor (Abood & Takeda, 1976; Abood et al., 1977a,b), while treatment of brain tissue with lipolytic enzymes greatly inhibits opiate binding (Abood et al., 1978). Lipids have also been shown to bind morphine stereospecifically (Abood & Hoss, 1975). Although the binding of the enkephalins to lipid is generally weaker than to complex biological extracts, it is quite conceivable that lipid is an essential component of the binding site. A systematic study of binding to possible components of the biological receptor is therefore a

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