Tanford, C., Nozaki, Y., Reynolds, J. A., and Makino, S. (1974), *Biochemistry 13,* 2369.
Tsugita, A., Kobayashi, M., Tani, S., Kyo, S., Rashid, M. A., Yoshida, Y., Kajihara, T., and Hagihara, B. (1970), *Proc. Nat. Acad. Sci. U.S. 67,* 442.
Weber, K., and Osborn, M. (1969), *J. Biol. Chem. 244,* 4406.
Wetlaufer, D. B. (1962), *Advan. Protein Chem. 17,* 303.

Comparison of the Resonance Raman Spectra of Carbon Monoxy and Oxy Hemoglobin and Myoglobin: Similarities and Differences in Heme Electron Distribution†

Lajos Rimai,* Irving Salmeen; and David H. Petering

ABSTRACT: With 441.6-nm excitation, which is near the Soret band, we observe that the resonance Raman spectra of hemoproteins contain not only the bands between 650 and 1700 cm^{-1} which arise from vibrations of the conjugated macrocycle, but also bands below 650 cm^{-1}, some of which involve vibrations of the iron pyrrole–nitrogen bonds. The spectra of the oxygen and carbon monoxide complexes of both myoglobin and hemoglobin are sufficiently similar to those of low spin met derivatives, that the electronic distribution on the heme for both ligands can be interpreted as that of a low spin ferriheme. This agrees with an earlier interpretation, by others, of comparative optical absorption spectra and, as pointed out previously, would imply that in the complex the ligands are bound as O_2^- and CO^-. However, band frequencies and relative intensities differ somewhat between the carbon monoxide and oxygen complexes of the same protein, which indicates differences between the details of the π-electron distributions in the corresponding complexes.

In a previous paper (Yamamoto *et al.,* 1973) we presented a comparative study of resonance Raman spectra, with excitation near the Soret band, of a number of hemoproteins including oxyhemoglobin (HbO_2)[1] and deoxyhemoglobin (Hb). These vibrational spectra showed that the electronic charge distribution on the iron in HbO_2 is essentially the same as that in low spin ferric hemoproteins. Although these spectra do not reflect directly the electron distribution on the ligand, the diamagnetism of HbO_2, as first proposed by Weiss (1964), implies that the oxygen is bound as O_2^- with the unpaired electron ($S = \frac{1}{2}$) on the O_2^- antiferromagnetically spin paired with the low spin ferric ($S = \frac{1}{2}$) ion. Similar correlations among the resonance Raman spectra of some of the same hemoglobin derivatives have also been noted more recently by Spiro and Strekas (1974) using excitation near the α and β bands, at which excitation frequency the intensity distribution among the vibrational lines differs markedly from that observed with Soret excitation.

In this paper we show that, by the same Raman criteria, the electron distribution on the iron in HbCO also is similar to that of the low-spin ferrihemoproteins and, thus, from the diamagnetism of HbCO, the carbon monoxide should be bound formally as CO^-. Whereas our previous paper (Yamamoto *et al.,* 1973) was limited to the spectral range 1300–1700 cm^{-1}, this paper includes spectra in the frequency range 100–900 cm^{-1} which contains bands with contributions from iron–pyrrole nitrogens. In both spectral ranges differences occur between the oxygen and carbon monoxide complexes. We also observe that the spectra of MbO_2 and MbCO are similar to those of the respective hemoglobin complexes, but spectral differences exist between corresponding myoglobin and hemoglobin derivatives which must reflect the influence of the different proteins on the heme. Spiro and Strekas (1974) have already suggested that the iron in HbCO is low spin ferric; however, their suggestion is based on the observation of only a single line in the high frequency region and they fail to report any differences between carbon monoxide and oxygen derivatives.

Experimental Procedure

Raman spectra were recorded with the 441.6-nm line of a Spectra Physics He–Cd laser, Model 185, using a spectrometer described elsewhere (Rimai *et al.,* 1973). Samples were contained in standard 5 mm × 10 mm rectangular cuvets and temperatures were maintained at 4° by blowing cold gas through a jacketed cuvet holder.

In the 441.6-nm laser beam, both oxygen and carbon monoxide readily photodissociate from hemoglobin and myoglobin. In order to obtain spectra of the fully liganded proteins we utilized the highest possible sample concentrations consistent with preparative convenience and the need to monitor the α–β bands of the optical absorption spectra. In addition we controlled the gas phase over the sample utilizing a cuvet to which was attached a configuration of stopcocks whereby: (1) the sample could be isolated from the ambient atmosphere, (2) the gas over the sample could be exchanged, and (3) the gaseous ligands (*i.e.,* CO or O_2) could be maintained at 1 atm of pressure. Further the laser

† From the Scientific Research Staff, Ford Motor Company, Dearborn, Michigan, 48121 (L.R. and I.S.), and the Department of Chemistry, University of Wisconsin-Milwaukee, Milwaukee, Wisconsin, 53201 (D.H.P.).

[1] Abbreviations used are: Hb, hemoglobin; Mb, myoglobin; HbO_2, MbO_2, HbCO, MbCO, corresponding oxygen and carbon monoxide liganded derivatives.

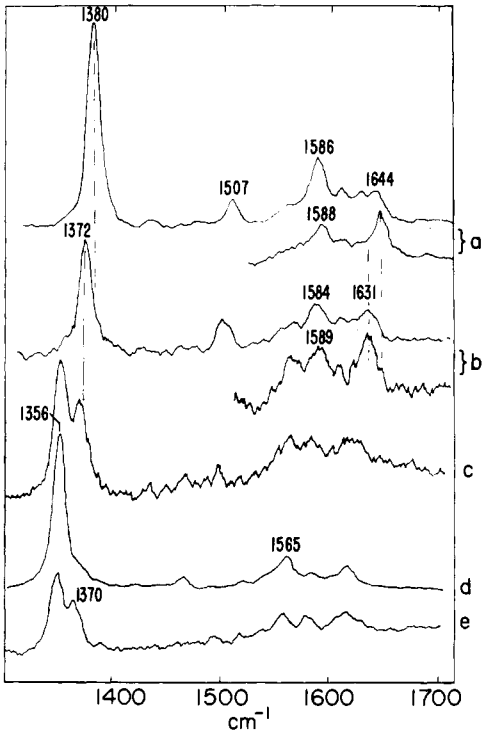

FIGURE 1: Raman spectra in the range 1300–1700 cm^{-1}. Excitation at 441.6 nm polarized perpendicular to scattering plane. Spectral slit width, 5 cm^{-1}; scan speed, 1 cm^{-1}/sec; sample temperature, 4°. All full traces taken with polarization analyzer in scattered beam oriented such that the polarization of the scattered beam was parallel to that of the incident beam. The partial traces below a and b were taken with the analyzer rotated by 90° so that the polarization of the scattered beam was perpendicular to that of the incident beam. The gains for the partial traces are twice the gains of the corresponding full traces; however, the gains of traces a–e are not directly comparable because of different optical absorbances and because the alignment of the system optics is not precisely reproducible upon changing samples. (a) Oxyhemoglobin 70 μM, transmission geometry, 25 mW laser power, 1 scan; (b) carbonmonoxyhemoglobin 1.2 mM, reflection geometry, 1.5 mW laser power, 12 scans accumulated; (c) carbonmonoxyhemoglobin 70 μM, transmission geometry, 1.5 mW laser power, 6 scans accumulated; (d) carbonmonoxyhemoglobin 70 μM, transmission geometry, 25 mW laser power, 1 scan (spectrum identical with deoxyhemoglobin due to full photodissociation; (e) carbonmonoxymyoglobin, approximately 70 μM, transmission geometry, 1.5 mW laser power, 12 scans accumulated.

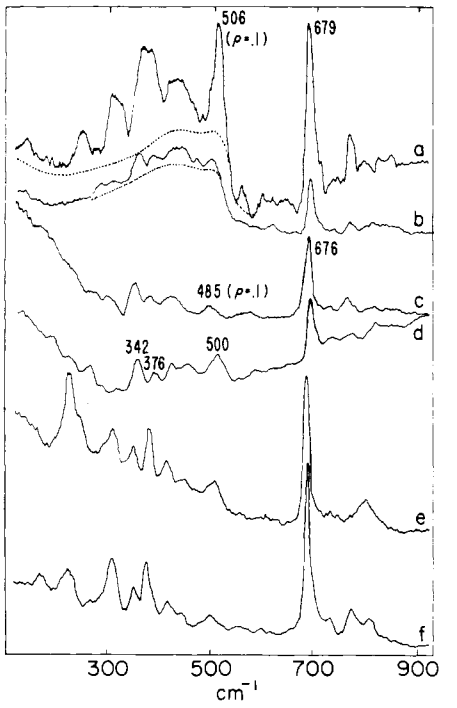

FIGURE 2: Raman spectra in the range 100–900 cm^{-1}. General conditions same as for data in Figure 1 except no polarization analyzer in the scattered beam. As determined independently, the depolarization ratio, ρ, is ≤ 0.2 for all bands between 200 and 700 cm^{-1}. (a) Carbonmonoxyhemoglobin 1.2 mM, reflection geometry, 1.5 mW laser power, 12 scans accumulated; (b) oxyhemoglobin 70 μM, reflection geometry, 25 mW laser power, single scan; (c) oxyhemoglobin 70 μM, transmission geometry, 25 mW laser power, single scan; (d) oxymyoglobin 70 μM, transmission geometry, 25 mW laser power, single scan; (e) deoxymyoglobin 70 μM, transmission geometry, 25 mW laser power, single scan; (f) deoxyhemoglobin 70 μM, transmission geometry, 25 mW laser power, single scan.

beam was attenuated with calibrated neutral density filters until contributions from photodissociated protein could not be detected in the Raman spectra (see Figure 1).

At high sample concentrations, the incident and scattered laser beams are strongly absorbed. Thus in order to record the Raman spectra, surface reflection geometry was required in which the cuvet was tilted so that the laser beam was incident at an angle of about 30° with respect to the face of the cuvet and the scattered light was collected at 90° with respect to the incident beam. With this geometry, the Raman spectrum of the cuvet glass often appeared as a broad background in the 100–900-cm^{-1} spectral range (see Figure 2, traces a–c). Generally, the protein concentration and the focusing of the system optics were such that the glass spectrum did not interfere with the observation of the sample spectrum.

Spectra were recorded at scan rates of 1 cm^{-1}/sec and spectral slit width of 5 cm^{-1}. The frequency scale was calibrated using benzene and carbon tetrachloride. In this manner absolute frequencies could be determined to ± 1 cm^{-1}. Sample concentrations, laser powers, and number of accumulated scans are noted in the figure legends.

Human oxyhemoglobin was prepared according to the method of Perutz (1968) from whole blood obtained from the Milwaukee Blood Center. Sperm whale metmyoglobin, batch 10, was purchased from Miles-Servac. It was reduced with a small excess of sodium dithionite followed quickly by Sephadex G-25 chromatography to isolate the reduced protein. Spectra of all samples were taken within several days after preparation. The buffer in all cases was 0.01 M phosphate at pH 7.0.

For recording Raman spectra of oxygenated derivatives, the cuvet was flushed extensively with oxygen and then the stopcocks were closed, trapping oxygen at a pressure of 1 atm. The carbon monoxide derivatives were prepared after deoxygenating the proteins by alternately flushing with nitrogen and then pumping off the nitrogen with a water aspirator. The optical absorption spectrum was monitored to check the state of deoxygenation. After oxygen removal the sample was flushed thoroughly with carbon monoxide and the cuvet finally closed at 1 atm of carbon monoxide pressure. This procedure was adopted because we found that, due to the more efficient photodissociation of carbon monoxide with respect to oxygen, residual oxygen in the sample could replace the carbon monoxide when the laser power was sufficient to photodissociate carbon monoxide, but not oxygen.

Results

Typical results are presented in Figures 1 and 2. Figure 1 shows the spectral range 1300–1700 cm^{-1} which is domi-

nated by conjugated double bond stretching vibrations. Figure 1a is the spectrum of HbO_2 taken with the polarization analyzer in the scattered beam set parallel to the polarization of the incident beam. The partial spectra shown below Figure 1a and b were taken with the polarization analyzer perpendicular to the polarization of the incident beam and show a strongly depolarized line at *ca.* 1640 cm^{-1} which appears in the spectra of all low spin ferrihemoproteins. Traces b–d show spectra of HbCO under three different experimental conditions. The spectrum in Figure 1d was obtained on a *low* concentration sample in the *transmission* mode with the highest available laser power (25 mW); it is identical with that of deoxyhemoglobin and is the result of complete photodissociation of the carbon monoxide. The spectrum in Figure 1c is of the same sample but with the laser power attenuated to about 1.5 mW which results in only partial photodissociation of the carbon monoxide. The spectrum in Figure 1b was obtained on a *high* concentration sample in the *reflection* geometry with laser power attenuated to 1.5 mW. Under these conditions, photodissociation of carbon monoxide was minimized and we attribute the spectrum in Figure 1b to HbCO. The spectral changes as a function of laser power are reversible. The spectrum in Figure 1b (HbCO) is similar to the spectrum in Figure 1a (HbO_2), but as noted in the figures the frequencies of the corresponding lines are different. These differences are discussed below; at this point they help to establish that the spectrum in Figure 1b is indeed that of HbCO and not HbO_2 which may have formed accidentally as a result of the photodissociation of carbon monoxide in the presence of oxygen.

The trace in Figure 1e shows the spectrum of a sample of MbCO at an intermediate degree of photodissociation. As for HbCO the photodissociation is reversible. In this region the spectrum of MbO_2 is very similar to that of HbO_2 and therefore is not shown. The differences between MbO_2 and MbCO parallel those between HbO_2 and HbCO, for the bands in this region.

Figure 2 shows spectra of HbCO (a), HbO_2 (b, c), MbO_2 (d), Mb (e), and Hb (f) in the low-frequency region. Of particular interest are the bands below 650 cm^{-1} since, as discussed later, some of these are contributed by vibrations of bonds directly involving the iron. The background indicated in dashed lines in the spectra in Figure 2a and b is due to Raman scattering from the cuvet glass (as described under Experimental Procedures). The spectra which do not show this background were recorded in transmission from low concentration samples (compare traces b, c, both from HbO_2). These low-frequency spectra clearly distinguish among the three liganded states of hemoglobin and are additional experimental verification of the particular liganded state of the protein. The spectra in Figure 2d and e are from MbO_2 and Mb. The spectrum of MbCO in the low-frequency region is not shown because, at the lowest power for which a reasonable signal-to-noise ratio could be obtained in the high-frequency region (see Figure 1, trace e), the MbCO still photodissociated significantly. Since spectra in the low-frequency range are generally weaker than those in the high-frequency region, the spectrum in the 100–900-cm^{-1} range was too noisy to be useful.

Discussion

In a previous paper (Yamamoto *et al.*, 1973) we demonstrated that, with 441.6-cm excitation, the resonance Raman spectra of all unambiguously ferrihemoproteins have a very strong polarized line between 1367 and 1380 cm^{-1}. Upon reduction, this band shifts to lower frequency by at least 10 cm^{-1}. We also observed that the spectra of low-spin complexes have a prominent polarized band at *ca.* 1584 cm^{-1}, while those of high-spin complexes have one at *ca.* 1566 cm^{-1}. In addition to these two lines, we note here the presence of a strongly depolarized line between 1628 and 1645 cm^{-1}, which occurs in the spectra of all *low-spin ferric* complexes.

In the spectrum of HbCO (trace in Figure 1b) the line at 1372 cm^{-1} lies well within the range corresponding to ferrihemoproteins, and the lines at 1586 and 1631 cm^{-1} are within the range characteristic of low-spin ferric complexes. Thus, the resonance Raman data imply that the electronic configuration of the heme in HbCO is essentially the same as that of low-spin ferrihemoproteins. The net diamagnetism of HbCO then implies that, upon binding, the CO acquires an electron, and the unpaired electron ($S = \frac{1}{2}$) associated with the carbon monoxide is antiferromagnetically coupled to the low-spin ferric ($S = \frac{1}{2}$) ion (Weiss, 1964; Peisach *et al.*, 1968) resulting in a diamagnetic complex.

Although these spectra show that the gross electronic structure of the heme is the same in HbCO and HbO_2, the absolute frequencies and relative intensities of corresponding bands are somewhat different. This is illustrated, for example, by the lines at 1372, 1631, and 506 cm^{-1} in HbCO and 1380, 1642, and 485 cm^{-1} in HbO_2. A detailed interpretation of the differences requires a normal coordinate analysis, but the following considerations offer a plausible explanation.

The frequency range below 650 cm^{-1} contains bands which have significant contributions from modes associated with the iron–pyrrole nitrogen stretching vibrations. This is shown by the resonance Raman (Brunner and Sussner, 1973) and infrared absorption (Alben *et al.*, 1973) spectra of metal-free porphyrins, which have very few vibrational modes below 550 cm^{-1}, and by a normal coordinate analysis of in-plane infrared active modes (Ogoshi *et al.*, 1972). Thus changes in electron distribution due to ligand replacement (*e.g.*, O_2 for CO) should be reflected by changes in these low-frequency vibrational bands. In addition to the spectral differences between HbCO and HbO_2, described in this paper, Yamamoto (1973) has recorded differences among various met derivatives of the same spin state, *e.g.*, between high-spin fluoro- and aquometmyoglobin (pH 7) and between low-spin azo- and cyanomethemoglobin.

All the bands between 650 and 1700 cm^{-1} are contributed by the conjugated macrocycle. As inferred from previous work (Rimai *et al.*, 1971) on the linear conjugated chains, the bands in the range 675–900 cm^{-1} correspond to skeletal vibrations. The region between 900 and 1300 cm^{-1} (when excited near the Soret band) contains a number of relatively weak lines, presumably due to single bond C–C stretching and methine carbon bending motions, which serve neither as oxidation state, spin state, nor as specific structural markers—this spectral region therefore is excluded from further discussion.

The bands in the range 1500–1700 cm^{-1} correspond to the ethylenic modes of the linear conjugated chains and contain major contributions from conjugated double bond stretching motions (Rimai *et al.*, 1973; Alben *et al.*, 1973; Ogoshi *et al.*, 1972). In general, for these vibrations, the higher the π-electron density in the bonds, the stiffer the spring, and the higher the corresponding frequency (Wilson *et al.*, 1955). Since carbon monoxide is a better π-electron

acceptor than oxygen, some of the bands in the conjugated double bond region of the HbCO spectrum should exhibit frequencies lower than the corresponding ones in the HbO_2 spectrum. The bands at 1372 and 1631 cm^{-1} of HbCO and 1380 and 1642 cm^{-1} of HbO_2 seem to illustrate this effect. This lower π-electron density in the conjugated bond system of HbCO should correspond to a higher π-electron density on the iron–ligand system, which would shift some of the modes below 650 cm^{-1} to higher frequency. Indeed, we apparently observe this when we compare the line at 506 cm^{-1} in HbCO with the corresponding one at 485 cm^{-1} in HbO_2.

Inspection of the data in Figure 2 also shows that the spectra of corresponding derivatives of hemoglobin and myoglobin are different. Such effects must reflect perturbations of the heme electronic structure by protein interactions and should be taken into consideration when comparisons are made between spectra of hemes differing simultaneously in axial ligand and general heme environment.

Similarities between the heme electronic structures of HbO_2 and HbCO and those of typical low-spin ferrihemoproteins also have been pointed out by Peisach *et al.* (1968) on the basis of comparative optical absorption spectra. Since both optical and resonance Raman spectra depend on the π-electron distribution over the entire heme, similar conclusions based on the two methods may have been expected. However, other spectroscopic studies have been interpreted differently. Mössbauer (Lang and Marshall, 1966) and X-ray fluorescence (Koster, 1972) spectra, both of which are sensitive to the electronic distribution over the metal, indicated that the iron is ferric in HbO_2 but ferrous in HbCO. Infrared absorption spectra of hemoglobin bound oxygen shows an intramolecular stretching frequency of 1107 cm^{-1} (Barlow *et al.*, 1973) which is intermediate between that of free oxygen (1555 cm^{-1}) and peroxide (750–900 cm^{-1}) (Loehr *et al.*, 1974). This intermediate frequency is consistent with the O_2^- structure for bound oxygen. The observed stretching frequency for hemoglobin bound CO is 1952 cm^{-1} (Barlow *et al.*, 1973) whereas that of CO gas is 2143 cm^{-1}. By analogy with oxygen, one would like to attribute this lowering of the CO frequency to a CO^- structure for the bound form, but a frequency shift of 200 cm^{-1} in going from the gas to the bound CO has been observed in many iron carbonyls (Cotton and Wilkinson, 1972; Adams, 1967), for which there seems to be only a small polarity in the Fe–CO bond (Cotton and Wilkinson, 1972). However, in such compounds, the CO is formally neutral and the formal oxidation state of the iron is low (*i.e.*, zero or less), whereas in hemoglobin the iron is in a higher oxidation state and is bound to pyrrole and imidazole nitrogens which have much more σ-bonding character than the Fe–CO bonds of the iron carbonyls. Thus comparisons between hemoglobin and iron carbonyls may not be reliable in the resolution of this question.

As noted above, differences do exist even between the resonance Raman spectra of HbCO and HbO_2. Either these are a result of just quantitative differences which infrared, Mössbauer, and X-ray fluorescence spectra reflect more markedly than optical and resonance Raman spectra, or these are a result of fundamental differences and the general similarities between the optical spectra and between the resonance Raman spectra are fortuitous.

If it is accepted that oxygen and CO acquire a negative charge upon binding to hemoglobin and myoglobin, then the stability of *all* liganded derivatives of hemoglobin and myoglobin may be attributed to the same basic mechanism; *viz.*, the electrostatic interaction between a negatively charged ligand and the positively charged ferriheme. This is most obvious for the derivatives of methemoglobin and metmyoglobin where the ferriheme (positive charge of +1) and the ligands such as F^-, CN^-, N_3^-, and OH^- are charged even before binding. However, the nonpolar ligands, oxygen and carbon monoxide, bind only to the ferroheme of deoxyhemoglobin. As noted in the literature (Wang *et al.*, 1958, Collman *et al.*, 1973) the hydrophobic environment of the binding site at the electrically neutral ferroheme provides a medium favoring access of nonpolar ligands, while simultaneously hindering competition from polar water. Upon binding, charge transfer could occur between the iron and the ligand, which would result in a stable complex with essentially the same ground state charge distribution as that of the met derivatives. This similarity in charge distribution at the ligand binding sites, for *all* ligands, is consistent with the conclusions from X-ray crystallography and other spectroscopic methods, that the quaternary structure of the methemoglobin derivatives is that of the relaxed (R) state (Perutz, 1972; Perutz *et al.*, 1974a–c).

Recent studies of cobalt-substituted hemoglobin (Hoffman *et al.*, 1972; Little and Ibers, 1974), and the observed cooperativity of the binding of azide to aquomethemoglobin (Banerjee *et al.*, 1973), suggest that the stereochemical mechanism of Perutz (1970, 1972) may not account for all of the experimental data on cooperative ligand binding. Since the available experimental data, discussed above, suggest the existence of a dipole associated with the oxygen–heme bond, the possibility should not be neglected that electrostatic interactions in the hydrophobic pocket, involving this dipole, contribute to the mechanisms controlling the cooperativity.

References

Adams, D. (1967), Metal Ligand and Related Vibrations, London, Edward Arnold.

Alben, J. O., Choi, S. S., Adler, A. D., and Caughey, W. S. (1973), *Ann. N.Y. Acad. Sci. 206,* 278–295.

Banerjee, R., Henry, Y., and Cassoly, R. (1973), *Eur. J. Biochem. 32,* 173–177.

Barlow, C. H., Maxwell, J. C., Wallace, W. J., and Caughey, W. S. (1973), *Biochem. Biophys. Res. Commun. 55,* 91–95.

Brunner, H., and Sussner, H. (1973), *Biochim. Biophys. Acta 310,* 20–31.

Collman, J. P., Gague, R. R., Halbert, T. R., Marchon, J. C., and Reed, C. A. (1973), *J. Amer. Chem. Soc. 95,* 7868–7870.

Cotton, F. A., and Wilkinson, G. (1972), Advanced Inorganic Chemistry, 3rd Ed., New York, N.Y., Interscience.

Hoffman, B. M., Spillburg, C. A., and Petering, D. H. (1972), *Cold Spring Harbor Symp. Quant. Biol. 36,* 343–348.

Koster, A. S. (1972), *J. Chem. Phys. 56,* 3161–3164.

Lang, G., and Marshall, W. (1966), *Proc. Roy. Soc. London 87,* 3–34.

Little, R. G., and Ibers, J. A. (1974), *J. Amer. Chem. Soc. 96,* 4452–4463.

Loehr, J. S., Freedman, T. B., and Loehr, T. M. (1974), *Biochem. Biophys. Res. Commun. 56,* 510–515.

Ogoshi, H., Saito, Y., and Nakamoto, K. (1972), *J. Chem. Phys. 57,* 4194–4201.

Peisach, J., Blumberg, W. E., Wittenberg, B. A., and Wittenberg, J. B. (1968), *J. Biol. Chem. 243,* 1871-1880.
Perutz, M. F. (1968), *J. Cryst. Growth 2,* 54-56.
Perutz, M. (1970), *Nature (London) 228,* 726-739.
Perutz, M. (1972), *Nature (London) 237,* 495-499.
Perutz, M., Fersht, A. R., Simon, S. R., and Roberts, G. C. K. (1974b), *Biochemistry 13,* 2174-2186.
Perutz, M., Heidner, E. J., Ladner, J. E., Beetlestone, J. G., and Ho, C. (1974c), *Biochemistry 13,* 2187-2200.
Perutz, M., Ladner, J. E., Simon, S. R., and Ho, C. (1974a), *Biochemistry 13,* 2163-2173.
Rimai, L., Gill, D., and Parsons, J. L. (1971), *J. Amer. Chem. Soc. 93,* 1353-1357.
Rimai, L., Heyde, M. E., and Gill, D. (1973), *J. Amer. Chem. Soc. 95,* 4493-4501.
Spiro, T. G., and Strekas, T. C. (1974), *J. Amer. Chem. Soc. 96,* 338-345.
Wang, J. H., Nakahara, A., and Fleischer, E. B. (1958), *J. Amer. Chem. Soc. 80,* 1109-1113.
Weiss, J. J. (1964), *Nature (London) 202,* 83-84.
Wilson, E. B., Jr., Decius, J. C., and Cross, P. C. (1955), Molecular Vibrations, New York, N.Y., McGraw-Hill.
Yamamoto, T. (1973), Resonance Raman Spectroscopy of Heme and Iron Sulfur Proteins, Ph.D. dissertation, University of Michigan, Ann Arbor, Mich.
Yamamoto, T., Palmer, G., Gill, D., Salmeen, I. T., and Rimai, L. (1973), *J. Biol. Chem. 248,* 5211-5213.

Conformational and Functional Studies of Chemically Modified Cytochromes: *N*-Bromosuccinimide- and Formyl-Cytochromes *c*†

Daniel J. O'Hern, Pranab K. Pal,‡ and Yash P. Myer*

ABSTRACT: *N*-Bromosuccinimide-cytochromes *c* (Myer, Y. P. (1972), *Biochemistry 11,* 4195) and formyl-cytochrome *c* (Aviram, I. and Schejter, A. (1971), *Biochim. Biophys. Acta 229,* 113) have been chromatographically purified, and the resulting components have been characterized in terms of their structure, conformation, and function. The activity measurements are considered in terms of the oxidizability, as the transference of an electron to solubilized cytochrome *c* oxidase, and reducibility, as the tendency to accept an electron from NADH-cytochrome *c* reductase. Conformational characterization has been carried out by absorption measurements, pH-spectroscopic behavior, circular dichroism, thermal denaturation, ionization of phenolic hydroxyls, the tendency to form the CO complex, and autoxidation with molecular oxygen. NBS-cytochrome *c* yields two major components, the relative proportions of which, with increasing modification of the protein, exhibit a pattern typical of the formation of the two in a consecutive manner. The first product contains the modification of the Trp-59 and Met-65 side chains, and the second contains the added modification of Met-80. The former in both valence states of iron is more or less like the native protein, except for an apparently slightly loosened heme crevice; the latter, as in other modifications involving modification of centrally coordinated Met-80, was found to be in a conformational state characteristic of the native protein with a disrupted central coordination complex, a loosened heme crevice, and small, but finite derangement of the polypeptide conformation. Functionally, the first component reflected 55% of the reducibility property and an unimpaired oxidizability property, while the latter exhibited derangement of both aspects of cytochrome *c* activity. Formyl-cytochrome *c* yielded a single component with modification of Trp-59. Conformationally, in both valence states, it is a molecular form with a disrupted central coordination complex, a loosened heme crevice, and gross derangement of the overall protein conformation. It exhibits a minimal reducibility property, 12%, whereas it retains a native-like tendency to transfer an electron to cytochrome *c* oxidase. The data from the NBS-cytochrome *c* components are analyzed with reference to the two forms in the earlier studies of the unpurified preparations. The results are found to be in agreement with one another. The selectivity between the reducibility and the oxidizability exhibited by the first NBS component and formyl-cytochrome *c*, irrespective of significant differences in the conformational and coordinational configurations of the two, has been viewed in light of a two-path, two-function model for oxidoreduction, as well as with reference to conformational and structural requirements for the oxidizability and reducibility properties of the molecule.

Chemical modification of specific functional groups has been one of the principal avenues of approach to the elucidation of structure-biological function relationships and the functioning of enzymes on a molecular level. In the case of cytochrome *c*, the role of the invariant tryptophanyl residue in the oxidoreduction function of the molecule has been investigated by the use of two specific modifications of the functional group, the formylation (Aviram and Schejter, 1971) and the *N*-bromosuccinylation (Stellwagen and Van Rooyan, 1967; Yonetani, 1968). In general, these investigations showed that the modification of the tryptophan resi-

† From the Department of Chemistry, State University of New York at Albany, Albany, New York 12222. *Received June 28, 1974.* This work was supported by research Grants BO-39855X and B17S73-01090 from the National Science Foundation. This article constitutes paper X of the series entitled Conformation of Cytochromes.

‡ Present address: Department of Chemistry, University of Delaware, Newark, Delaware.