Hemes a and a_3 Environments of Plant Cytochrome c Oxidase[†,‡]

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Received April 13, 1990; Revised Manuscript Received June 12, 1990

ABSTRACT: The structures of hemes a and a_3 of maize and wheat germ cytochrome c oxidase were investigated by resonance Raman spectroscopy. Comparison between the plant and mammalian cytochrome oxidases revealed that (i) the vinyl groups associated with hemes a and a_3 vibrate at higher frequencies in the plant enzyme than in the mammalian enzyme, suggesting different degrees of interaction between the heme cores and their periphery; (ii) aside from the geometry of the vinyl group, the structure of heme a_3 in plant cytochrome oxidase is essentially unchanged from that of its mammalian counterpart; (iii) the vibrational band associated with the formyl group of reduced heme a shows relatively weak enhancement in the Soret-excited resonance Raman spectra of maize and wheat germ cytochrome oxidase, suggesting that the formyl group of ferrous heme a in the plant enzymes is conjugated only slightly to the porphyrin ring; and (iv) for oxidized heme a, the formyl vibration is strongly enhanced, but its frequency indicates a weaker interaction with the protein milieu relative to the mammalian enzyme. These observations suggest that the local environment around the formyl position of the heme a chromophore differs in the plant and mammalian cytochrome oxidases. The implication of the latter feature in the mechanism of proton pumping by cytochrome oxidase is discussed.

Cytochrome c oxidase (EC 1.9.3.1), the terminal enzyme in the respiratory electron transport chain, catalyzes the four-electron reduction of O_2 to water and also functions as a redox-linked proton pump (Wikström et al., 1981; Scott et al., 1989). The enzyme has four catalytically active metals: two heme iron ions and two copper ions. Heme a_3 and Cu_B form a binuclear site where O_2 binding occurs, while heme a and Cu_A play a role in electron transfer from cytochrome c to the O_2 binding site. Other metals (a copper, a magnesium, and a zinc ion) also bind to mammalian cytochrome oxidase (Einarsdottir & Caughey, 1984, 1985; Bombelka et al., 1986), but there is no evidence for their involvement in electron or proton transport.

Substantial literature on mammalian and bacterial cytochrome oxidases has accumulated over the years, but in spite of these efforts, many questions remain unanswered. This is particularly true of the mechanism of proton pumping (Copeland & Chan, 1989). Two recent models are based on the premise that proton pumping activity is related to specific structural features of Cu_A or heme a. Gelles et al. (1986) have described a mechanism whereby shuttling of protons is promoted by redox-linked changes in the coordination of Cu_A. In support of this model, Nilsson et al. (1988) have observed that perturbation of the Cu_A site leads to a marked decrease in proton pumping activity of cytochrome oxidase. On the basis of spectroscopic observations, Babcock and Callahan (1983) postulated that the formyl C=O group of heme a is hydrogen-bonded to a protein residue. They further suggested that the energy differential between hydrogen bonds to the formyl group in the oxidized and reduced forms of heme a is used to pump protons across the membrane.

Yet another picture may be emerging, however. Wikström (1989a,b) has proposed that the heme a_3–Cu_B site provides the mechanistic coupling between electron transfer and proton translocation in cytochrome oxidase. This view is supported indirectly by the recent report (Puustinen et al., 1989) that cytochrome *bo*, a bacterial oxidase, acts as a proton pump. This enzyme contains Cu and heme *b*. The Cu ion appears to be coupled to a heme *b* molecule, forming a site similar in structure to the Cu_B–heme a_3 site of cytochrome oxidase (Salerno et al., 1989). There is no evidence for a site analogous to Cu_A in cytochrome *bo* (Salerno et al., 1989). Also, heme *b* does not have the peripheral formyl group that is thought to be the critical feature of a heme a mediated proton pump.

We have used a comparative approach to link structure to function in cytochrome oxidase. We have isolated cytochrome oxidase from maize and wheat germ and determined the spectroscopic properties of the Cu_A and heme a sites. By comparing our results with those for the beef heart enzyme, it is possible to pinpoint those structural characteristics that are common to the Cu_A and heme a sites of mammalian and plant cytochrome oxidases. These comparisons, in turn, will help us determine if the presence of proton pumping activity is linked to specific structural features of the Cu_A or heme a sites.

The first target of our investigation was Cu_A [see Peiffer et al. (1990)]. Our electron paramagnetic resonance (EPR)[1] results show that the spectroscopic properties and, therefore, the structures of the Cu_A^{2+} sites are similar in the plant and mammalian systems. We can infer that their functions are also similar, but our comparative study cannot specify a role for Cu_A in the mechanism of proton pumping.

[†] This research was funded by grants from the National Institutes of Health to G.T.B. (GM25480) and to S.F.-M. (GM26916).

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[1] Abbreviations: EPR, electron paramagnetic resonance; RR, resonance Raman; TMPD, *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine.

0006-2960/90/0429-8702$02.50/0

In this paper, we continue our investigation of plant cytochrome oxidases with the resonance Raman spectroscopic characterization of the heme sites from the maize and wheat germ enzymes. The similarities and differences between the plant and mammalian systems are discussed in light of the possible involvement of heme a in proton pumping.

Experimental Procedures

Beef heart cytochrome oxidase was isolated by using a modified Hartzell–Beinert protocol (Centeno, 1987). Maize and wheat germ cytochrome oxidases were prepared according to the procedure outlined in the preceding paper (Peiffer et al., 1990).

Absorption spectra were obtained at room temperature with a Perkin-Elmer Lambda 4B spectrophotometer. Unless otherwise noted, samples for resonance Raman spectroscopy were typcically 25–70 μM enzyme in a buffer system that consists of 100 mM KH_2PO_4 (pH 7.5), 0.5 M KCl, 5% sucrose, and 4 mM lauryl maltoside. Reduction of cytochrome oxidase was achieved by addition of excess sodium dithionite under anaerobic conditions. CN^- treatment entailed the addition of 1 mM KCN to aerated 25 μM enzyme, which was previously reduced with 1 μM *N,N,N′,N′*-tetramethyl *p*-phenylenediamine (TMPD) and 1 mM ascorbate.

Excitation into the Soret bands of hemes a and a_3 was achieved by using either a Coherent INNOVA-90K Kr^+ laser (406.7 nm) or a Liconix 4240N He–Cd laser (441.6 nm). Visible band excitation at 602 nm was derived from a Spectra Physics Model 375 dye laser, with Rhodamine R6G as the dye, pumped by a Spectra Physics Model 164 Ar^+ laser. The fully oxidized samples were contained in a quartz spinning cell that was open to the atmosphere. These conditions, combined with low laser powers (5–10 mW), prevented the photoreduction of the enzyme. The fully reduced and mixed-valence samples were contained in 0.2-cm quartz cuvettes adapted for anaerobic work. Raman scattering was collected in a 90° geometry and dispersed in a Spex 1401 spectrometer. The detector was a thermoelectrically cooled photomultiplier tube (RCA 34031A) operated in the photon counting mode. Data acquisition and signal averaging were accomplished with a DEC LSI-11 computer. Spectral resolution was 5–6 cm^{-1}. The temperature of the samples was kept between −5 and 4 °C by flowing cold nitrogen gas around the sample cell. O_2 consumption measurements showed that prolonged exposure to the laser beam decreased the activity of wheat germ cytochrome oxidase only by 10–25%. No changes in the absorption spectrum of the enzyme was detected after the Raman experiment.

Results

Absorption Spectra. The absorption spectra of maize cytochrome oxidase are shown in Figure 1; the preceding paper (Peiffer et al., 1990) reports the absorption spectra of the wheat germ enzyme. The spectra show that the Soret and α bands in the reduced enzymes, which occur at 440–441 and 602 nm, respectively, are blue-shifted relative to the equivalent bands in the beef heart enzyme, where they occur at 443 and 604 nm, respectively. The visible absorption bands in the reduced plant enzymes are still, however, considerably red-shifted relative to the absorption bands of six-coordinate heme a^{2+} model compounds in protic solvents (Babcock & Callahan, 1983).

Babcock and Callahan (1983) have shown that the absorption spectra of bisimidazole heme a^{2+} model compounds shift to the red as an aprotic solvent is replaced with a protic, hydrogen-bonding solvent. The extent of the red-shift correlated with the strength of the hydrogen bond between the

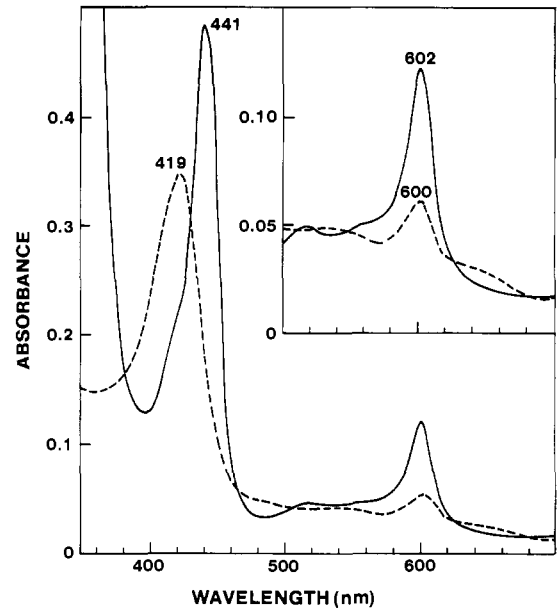

FIGURE 1: Visible absorption spectrum of maize cytochrome c oxidase after purification by anion-exchange FPLC as described in Peiffer et al. (1990). Oxidase was 4.3 μM in 5% sucrose, 20 mM KH_2PO_4, and 4 mM lauryl maltoside, pH 7.5. Air-oxidized (---); dithionite-reduced (—).

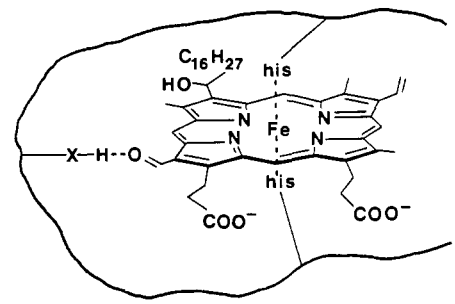

FIGURE 2: Postulated heme a structure in cytochrome oxidase. The heme a iron is six-coordinate with histidines occupying the two axial sites. The peripheral formyl group is involved in a hydrogen bond with a proton donor designated as X–H, which is associated with the polypeptide backbone [adapted from Babcock and Callahan (1983)].

solvent and the formyl group of the heme. The large red-shift in the absorption spectrum of heme a^{2+} in beef heart cytochrome oxidase thus suggested the presence of a relatively strong hydrogen bond between the protein and the heme formyl groups (Figure 2). In agreement with this interpretation of the optical data, resonance Raman spectroscopy provided evidence that the formyl group of heme a forms an energetically significant hydrogen bond to a protein group in its environment and that an analogous interaction does not occur for heme a_3 [Babcock & Callahan, 1983; see also Sassaroli et al. (1989)]. When viewed in this light, the absorption spectrum of plant cytochrome oxidase indicates that its heme a environment is different from that found in mammalian cytochrome oxidase. The extent of these differences and their possible physiological importance are discussed below.

Resonance Raman Spectroscopy. Hemes a and a_3 of maize and wheat germ cytochrome c oxidase were investigated by resonance Raman spectroscopy (Figures 3a,b, 4, and 5). By comparing our results to those from the well-characterized beef heart enzyme [Figure 3c,d; see Babcock (1988) for a comprehensive review of resonance Raman studies of cytochrome oxidase], we were able to infer structural features of the chromophores in the plant enzymes.

It is clear from Figure 3 that almost all vibrational bands of the beef heart cytochrome oxidase (c, d) have direct ana-

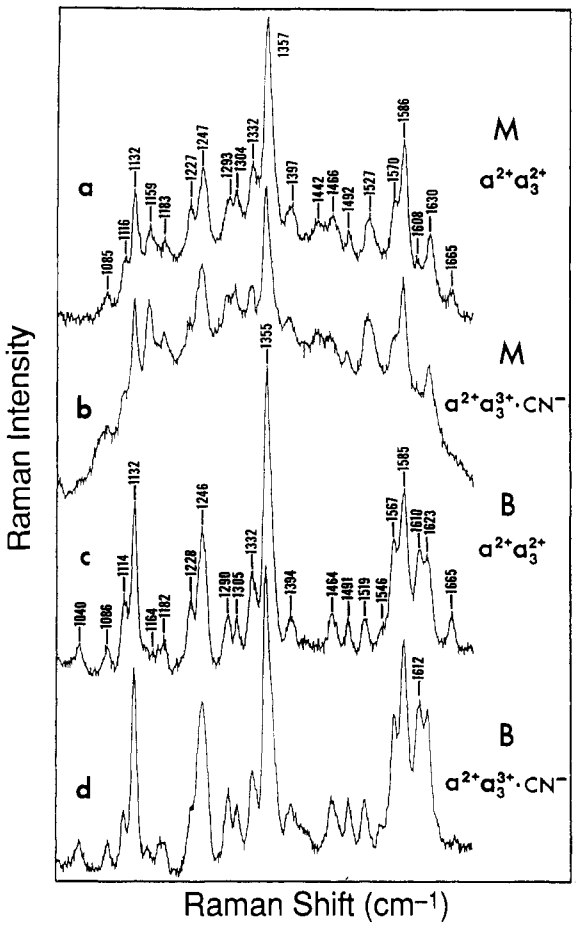

FIGURE 3: Resonance Raman spectra of beef heart and maize cytochrome oxidase. Laser excitation was at 441.6 nm (20 mW). (a) Maize cytochrome oxidase fully reduced with sodium dithionite at 8.3 μM enzyme, pH 7.9; (b) maize cytochrome oxidase in the mixed-valence state reduced with TMPD–ascorbate in the presence of cyanide at 8.3 μM enzyme, pH 7.9; (c) beef heart cytochrome oxidase fully reduced with TMPD–ascorbate at 30 μM enzyme, pH 7.4; (d) beef heart cytochrome oxidase in the mixed-valence state reduced with TMPD–ascorbate in the presence of cyanide at 40 μM enzyme, pH 7.4.

logues in the maize system (a, b). Further comparison between Figures 3a and 4c (reduced form, 441.6-nm excitation), and between Figures 3b and 5a (mixed-valence form, 441.6-nm excitation) illustrates that the hemes in the maize and wheat germ enzymes have similar structures. On the strength of the latter observation, we decided to study only one of the plant enzymes in detail—the wheat germ system—and compare its properties to those of the mammalian system.

Our strategy for the assignment of the resonance Raman spectra of wheat germ cytochrome oxidase takes advantage of the selective enhancement of heme *a* and heme a_3 modes as a function of redox state and excitation wavelengths [reviewed by Babcock (1988)]. Thus, fully oxidized samples excited at 406.7 nm show vibrations due to hemes a^{3+} and a_3^{3+}. Likewise, fully reduced samples probed at 406.7 and 441.6 nm bring out vibrations from hemes a^{2+} and a_3^{2+}, although the core vibrational mode ν_{10} is more strongly enhanced at 406.7 nm than at 441.6 nm. The CN^--treated, mixed-valence samples exist as $a^{2+}(a_3^{3+}CN^-)$ and, consequently, when probed at 441.6 and 602 nm, allow for the observation of heme a^{2+} modes only.

The principles outlined above proved useful in the assignment of the 1665 cm^{-1} mode to a heme a_3 vibration because it is enhanced by Soret excitation of the fully reduced enzyme but not by Soret or visible excitation of the mixed-valence

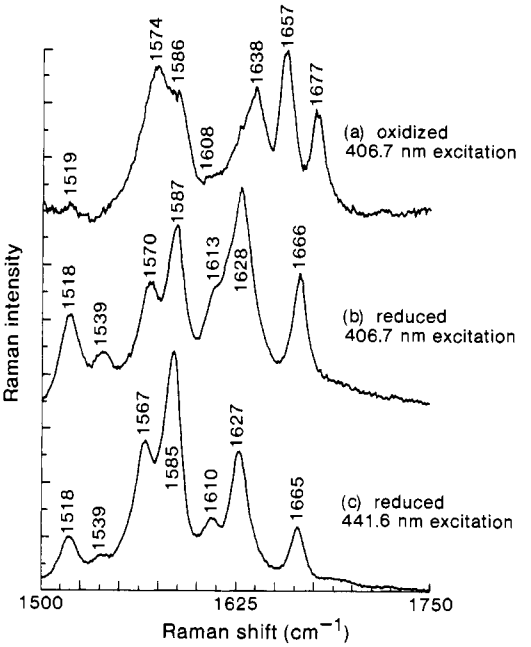

FIGURE 4: Resonance Raman spectra of wheat germ cytochrome oxidase: (a) oxidized enzyme, excited at 406.7 nm (10 mW); (b) dithionite-reduced enzyme, excited at 406.7 nm (5 mW); (c) dithionite-reduced enzyme, excited at 441.6 nm (10 mW). Further details are described under Experimental Procedures.

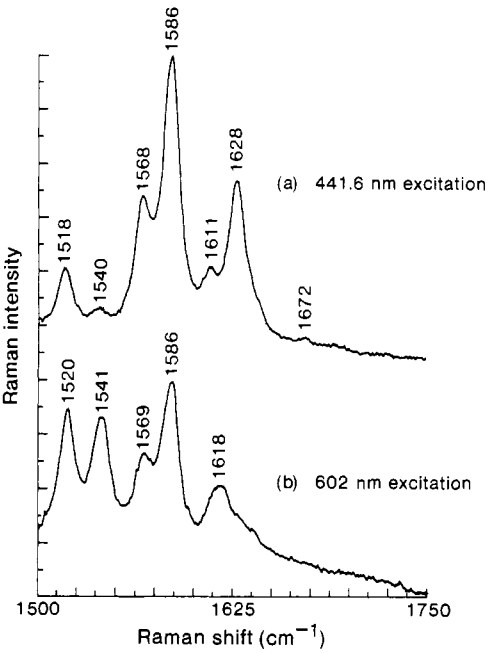

FIGURE 5: Resonance Raman spectra of mixed-valence, CN^--treated wheat germ cytochrome oxidase excited at (a) 441.6 nm (10 mW) and (b) 602 nm (100 mW). Further details are described under Experimental Procedures.

enzyme. A comparison with the resonance Raman spectra of mammalian cytochrome oxidase further indicates that the 1665 cm^{-1} mode is comprised mostly of a C=O stretching vibration of the formyl group of heme a_3^{2+}.

Our approach has also led to the deconvolution of the 1610–1630 cm^{-1} region of the Soret-excited RR spectrum of wheat germ cytochrome oxidase. Figure 4b shows that this spectral region is composed of at least three bands when the reduced enzyme is excited at 406.7 nm. The RR spectra of the mixed-valence enzyme (Figure 5) show that these three bands arise from heme a^{2+}, not a_3^{2+}, and occur at 1610, 1618, and 1628 cm^{-1}. The fact that the 1618 cm^{-1} band is enhanced

Table I: Assignment of High-Frequency Vibrations (cm^{-1}) in Plant Cytochrome Oxidase and Comparisons with the Mammalian Enzyme

	reduced				oxidized			
	wheat germ		beef heart		wheat germ		beef heart	
mode	a^{2+}	a_3^{2+}	a^{2+}	a_3^{2+}	a^{3+}	a_3^{3+}	a^{3+}	a_3^{3+}
$\nu_{C=O}$	1610	1665	1610	1665	1657	1677	1650	1676
$\nu_{C=C}$	1627	1627	1623	1623				
ν_{10}	1618	NO[a]	1614	1607	1638	NO	1641	1615
ν_2	1587	NO	1585	1579	1586	1576	1590	1572
ν_{19}	1586		1586					
$\nu_{38}(1)$	1567		1567					
$\nu_{38}(2)$	1541		1546					
ν_{11}	1518		1519					
ν_3	1492	NO	1491	1464	1503	1477	1506	1478
ν_4	1356	1361	1354	1356	1371	1371	1373	1373

[a] NO = not observed due to overlapping bands.

with visible (602 nm) excitation only indicates that it is composed of a C–C stretching vibration of the porphyrin core [the so-called ν_{10} mode; see Babcock (1988) for a summary of the assignments]. The other two vibrations can be assigned to the RR spectra of mammalian cytochrome oxidase as arising from a vinyl C=C stretch (1628 cm^{-1}) and a formyl C=O stretch (1610 cm^{-1}). The latter vibrates at the same frequency as in beef heart, but shows weaker enhancement.

The remaining assignments of our RR spectra are summarized in Table I, where data from wheat germ and beef heart cytochrome oxidases are compared. In the table, ν denotes a stretching vibration; C=O and C=C refer to the formyl and vinyl groups, respectively. The numbered vibrations are composed mostly of C–C stretches of the porphyrin ring [see Kitagawa and Osaki (1987) for a review]. Modes ν_2, ν_3, ν_{10}, and ν_{19} vary with the size of the porphyrin central core. Mode ν_4 reflects the extent of oxidation of the Fe ion. The numbering system adopted here is that of Abe (1986). Of particular note, as discussed below, are the differences between the plant and mammalian enzymes with respect to the frequency of the oxidized heme a formyl vibration (1657 versus 1650 cm^{-1}, respectively) and the reduced heme a vinyl vibration (1627 versus 1623 cm^{-1}, respectively).

Discussion

Vinyl Modes. In general, the resonance Raman spectra show that the structure of heme a_3 is essentially the same in the mammalian and plant cytochrome oxidases. There is one exception to this observation: the vibrational bands arising from the C=C stretch of the vinyl groups of both heme a and a_3 occur at higher frequency in the plant enzymes than in the mammalian enzyme. In model porphyrin compounds with vinyl substituents, the vinyl group and the porphyrin are not coplanar, a geometry that does not favor extensive conjugation between the two π systems and leads to an increase in the frequency of the C=C vibration. Gersonde et al. (1989) have proposed that slight variations in the vinyl–porphyrin angle can explain shifts in the frequency of the C=C stretching vibration. Therefore, we conclude that the angle between the heme core and the vinyl group is different in plant and beef heart cytochrome oxidases. Namely, the vinyl group appears to be rotated more out of the porphyrin plane in the ground state of plant heme a than in mammalian heme a. Our data also indicate that the vinyl group rotates back into the plane of the porphyrin in the excited state, because the vinyl mode is enhanced strongly in the Soret-excited RR spectrum of heme a^{2+}.

The differences in the conformation of the vinyl group described above are not major. They may be the result of different steric interactions between the periphery of the prosthetic group and the protein in the two systems.

Formyl Modes. More noticeable differences in the heme environments of plant and mammalian cytochrome oxidases are revealed by the formyl C=O stretches. The frequency of the C=O vibration reflects interactions between the heme a formyl group and its environment. The interaction may be a hydrogen bond to the protein (Babcock & Callahan, 1983) or resonance energy transfer with a nearby water molecule (Sassaroli et al., 1989). In the reduced enzymes, the heme a–protein interactions are of similar strength in the plant and the mammalian systems, since the formyl C=O vibrations both occur at 1610 cm^{-1}. In the fully oxidized form, however, the interaction is weaker in plant heme a ($\nu_{C=O}$ = 1657 cm^{-1}) than in mammalian heme a ($\nu_{C=O}$ = 1650 cm^{-1}).

If we assume that the formyl C=O group is hydrogen-bonded to a protein group, then we can use the Badger–Bauer rule to estimate the strength of the interaction. Babcock and Callahan (1983) have used this method to calculate the enthalpy of the hydrogen bond (ΔH_{hb}) between heme a^{3+} and the protein for beef heart cytochrome oxidase, arriving at a value of 3.0 kcal/mol. The same formalism, when applied to wheat germ heme a^{3+}, yields a value of ΔH_{hb} = 1.9 kcal/mol. The hydrogen bond is, thus, weaker by 1.1 kcal/mol in plant heme a^{3+} relative to mammalian heme a^{3+}. Since the hydrogen bonds are of equal strength in plant and mammalian heme a^{2+}, where the ΔH_{hb} = 5.3 kcal/mol, the change in bond energy between the oxidized and reduced states will be greater for plant heme a (3.4 kcal/mol) than mammalian heme a (2.3 kcal/mol).

The RR spectrum of plant heme a^{2+} reveals another unique structural feature. The 1610 cm^{-1} formyl C=O mode is strongly enhanced in the beef heart enzyme (Figure 2c,d) but only weakly enhanced in the reduced forms of the maize and wheat germ enzymes (Figures 3a,b, 4c, and 5a). Enhancement of the C=O vibration is determined by the degree of conjugation of the formyl group with the porphyrin π system either in the ground or in the excited electronic states. The C=O formyl mode of heme a can be strongly enhanced in the RR spectrum even if it is rotated out of the plane of the porphyrin in the ground state, provided that electronic excitation increases the extent of conjugation with the porphyrin π system. If the C=O formyl and porphyrin π systems are not strongly conjugated in either the ground or the excited electronic states, then the C=O mode is expected to show low activity in the RR spectrum. When viewed in this light, our results indicate that the formyl C=O groups have different conformations in the reduced forms of plant and mammalian hemes a. The lower intenisty of the C=O mode in the reduced plant enzyme suggests that the formyl group conjugates weakly with the porphyrin plane both in the ground and in the excited elec-

tronic states. A corresponding situation does not appear to occur for oxidized heme *a* in the plant enzymes since the enhancement of the formyl mode at 1657 cm^{-1} is comparable to that observed for the a^{3+} formyl mode in the mammalian enzyme.

Reconciling the Absorption and RR Data. We saw [Figure 1 and Peiffer et al. (1990)] that the heme *a* absorption bands of reduced plant oxidases are blue-shifted with respect to reduced mammalian cytochrome oxidases. According to the model put forth by Babcock and Callahan (1983), this means that the hydrogen bond between the protein and the formyl group of heme *a* is weaker in the plant system. The strengths of these putative hydrogen bonds, as revealed by the frequencies of the formyl C=O stretching vibrations, are similar, however, in plant and mammalian oxidases (vide supra). We can explain this paradox by recalling that the formyl group appears to conjugate only weakly with the porphyrin group in plant oxidases. The resultant decrease in delocalization of π electron density from the porphyrin to the formyl group is most likely the origin of the blue-shift in the absorption spectrum of plant heme *a* relative to mammalian heme *a*.

Implications for Proton Pumping Activity. It is generally recognized that the vibrational frequency of the formyl C=O group of heme *a* bound to cytochrome oxidase is indicative of a strong interaction of heme *a* with its environment. There are currently two views on the nature of this interaction. Babcock and Callahan (1983) proposed that the formyl group of heme *a* is hydrogen-bonded to the protein. The energy difference between the hydrogen bonds to heme *a* in its oxidized and reduced states was proposed to provide the driving force for a redox-linked proton translocation event. More recently, Sassaroli et al. (1989) gave an alternative interpretation of the resonance Raman spectrum of heme *a* in beef heart cytochrome oxidase. They suggest that water molecules in close proximity to the heme affect the vibrational frequencies of the vinyl C=C and formyl C=O groups. Although specific functions for these water molecules were not given, Sassaroli et al. (1989) speculated that they could play a role in proton pumping by being part of a hydrogen-bonding network linking heme *a* to the outside of the membrane.

Our results indicate that the extent of interaction between the formyl C=O group of heme *a* and its environment is different in the plant and mammalian cytochrome oxidases. Our data indicate that the formyl–protein interactions are of similar strength in the reduced forms of the plant and mammalian enzymes; in the oxidized form, however, the C=O group vibrates at a higher frequency in wheat germ cytochrome oxidase than in beef heart enzyme. According to the model put forth by Sassaroli et al. (1989), this effect can be explained by a spatial arrangement of the water molecule relative to the heme plane that is not conducive to extensive resonance vibrational energy transfer. This feature may affect the function of the site in either electron transfer or proton translocation.

If the interaction of heme *a* with its protein environment is a determinant of proton pumping activity (Babcock & Callahan, 1983), then our results suggest that wheat germ cytochrome oxidase should be a very effective proton pump. In any case, the fact that this structural feature of cytochrome oxidase is conserved in the plant enzyme emphasizes its likely importance in oxidase function, whether or not it is directly involved in the mechanism of proton translocation.

Registry No. H^+, 12408-02-5; cytochrome *c* oxidase, 9001-16-5; heme *a*, 57560-10-8; heme a_3, 58916-42-0.

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