Resonance Raman Study of the aa_3-Type Cytochrome Oxidase of Thermophilic Bacterium PS3†

Takashi Ogura, Nobuhito Sone, Kunio Tagawa, and Teizo Kitagawa*

ABSTRACT: Resonance Raman spectra of the aa_3-type cytochrome oxidase of thermophilic bacterium PS3, which has a simpler subunit composition than the mitochondrial enzymes but very similar enzymatic properties, are investigated under various conditions and compared with those of mitochondrial enzymes. The intensities of the two marker lines of reduced cytochrome a_3 at 1667 and 213 cm^{-1} had different dependences on the incubation temperatures and pH. With regard to the incubation temperature dependence, the intensity of the 1667-cm^{-1} line, the peripheral CH=O stretching mode of the a_3 heme, behaved in nearly the same way as that of the oxidase activity whereas the intensity of the 213-cm^{-1} line, the Fe–histidine stretching mode of the a_3 heme, exhibited a similar dependence to that of the proton pumping activity. The 213-cm^{-1} line disappeared upon binding of carbon monoxide, upon raising the pH above 9.2, or after incubating above 55 °C. The Raman line at 1611 cm^{-1}, which was recently suggested to probe the proton pump activity [Babcock, G. T., & Callahan, P. M. (1983) *Biochemistry 22*, 2314–2319], remained unaltered after incubation at 60 °C for 20 min despite a reduction of proton pumping activity to one-third. This argues against the proposed mechanism. The frequencies of the Raman lines were the same for the intact membrane and the isolated enzyme in the reduced state. The Raman spectra of cytochrome oxidase isolated from bacterium, yeast, and bovine heart were different in the lower frequency region below 600 cm^{-1} but closely alike in the higher frequency region above 1200 cm^{-1}. The oxidase of the present preparation was apparently autoreduced in the presence of 100 mM, but not 2 mM, cyanide. The Raman spectra of the resting enzyme in the presence or absence of 2 mM cyanide were almost the same, although the cytochrome *c* oxidase activity was completely lost in the presence of 2 mM cyanide.

The molecular structure of the metal center of cytochrome oxidase (EC 1.9.3.1) and its relevance to the reaction mechanism have been extensively investigated with various spectroscopic techniques but still are a matter of debate [for review, see Malmström (1979), Wikström et al. (1981), and Babcock (1982)]. The metal center of the aa_3-type cytochrome oxidase is composed of two heme *a*'s and two copper atoms. The portion containing the low-spin heme *a* and EPR-active copper and another portion containing the high-spin heme *a* and the EPR-silent copper are designated as cytochrome *a* and cytochrome a_3, respectively. Recently cytochrome oxidases have been isolated from several bacteria (Yamanaka et al., 1979; Sone et al., 1979; Yamanaka & Fujii, 1980; Hon-nami & Oshima, 1980; Ludwig & Schatz, 1980; Fee et al., 1980). These enzymes possess a similar metal center (aa_3 type), but their subunit structures are much simpler than those of the mitochondrial enzymes; two to three subunits are found in bacterial enzymes, compared to seven or more in mitochondrial (Cabral & Schatz, 1978). The enzyme isolated from thermophilic bacterium PS3 has three peptides with M_r 56 000 (I), 38 000 (II), and 22 000 (III) and is relatively thermostable (Sone & Yanagita, 1981). This enzyme also served as a proton pump when it was reconstituted into phospholipid vesicles (Sone & Hinkle, 1982).

Resonance Raman (RR)[1] scattering from hemoproteins provides information about structural details of the heme vicinity (Rousseau & Ondrias, 1982; Asher, 1981; Kitagawa & Teraoka, 1982; Spiro, 1975). There are several reports on the RR spectra of mitochondrial cytochrome oxidase (Adar & Yonetani, 1978; Salmeen et al., 1978; Kitagawa & Orii, 1978; Bocian et al., 1979; Babcock & Salmeen, 1979) as well as heme *a* derivatives (Kitagawa et al., 1977; Callahan & Babcock, 1981), and the assignments of their Raman lines have been discussed (Choi et al., 1983). The postulated difference of the Soret absorption maximum between cytochrome *a* and cytochrome a_3 was considered to yield a differentiated RR effect of the two cytochromes (Babcock et al., 1981), and their difference in the formyl CH=O stretching frequencies was pointed out (Callahan & Babcock, 1983). In our previous study the Fe–histidine stretching Raman line of reduced cytochrome a_3 was assigned with the ^{54}Fe-incorporated cytochrome oxidase isolated from thermophilic bacterium HB8 (Ogura et al., 1983). Here we describe the RR spectra of the cytochrome oxidase isolated from thermophilic bacterium PS3 and their dependence on incubation temperature and pH in relation to the oxidase activity and the proton pumping activity.

Materials and Methods

Thermophilic bacterium PS3 was cultivated at about 70 °C in the medium containing 0.3% NaCl, 0.8% polypepton, and 0.4% yeast extract under continuous air bubbling and stirring. Cytochrome oxidase was purified from the cytoplasmic membrane as described previously (Sone & Yanagita, 1982). One of the enzyme was chromatographed on octyl-Sepharose (CL-4B) according to Rosén (1978). The purified enzyme was dissolved in 50 mM phosphate buffer, pH 7.1, with 0.5% Tween 20 and was dialyzed thoroughly against the same buffer. Yeast cytochrome oxidase was purified from pressed yeast (*Saccaromyces cerevisiae*) according to Sekuzu et al. (1967). The purified enzyme was dissolved in 10 mM phosphate buffer, pH 7.4, containing 0.25 M sucrose and 1 mM

† From the Institute for Molecular Science, Okazaki National Research Institutes, Myodaiji, Okazaki, Aichi, 444 Japan (T.O. and T.K.), Department of Biochemistry, Jichi Medical School, Minamikawachi-machi, Tochigi, 329-04 Japan (N.S.), and Department of Molecular Physiological Chemistry, Osaka University Medical School, Kitaku, Osaka, 530 Japan (K.T.). *Received August 15, 1983.* This study was partly supported by the Naito Foundation Research Grant for 1983 and Grant-in-Aid for Scientific Research of the Ministry of Education, Science and Culture (No. 58480458) to T.K.

[1] Abbreviations: Hb, hemoglobin; Mb, myoglobin; HRP, horseradish peroxidase; EDTA, ethylenediaminetetraacetate; RR, resonance Raman.

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EDTA. Beef heart cytochrome oxidase was kindly given by Prof. S. Yoshikawa, Konan University.

Upon pH titration the Raman measurements were started at 10 min after the pH change with a NaOH solution. The pH values were determined with a Beckman pH meter (Model ϕ71) after the Raman measurements for the sample in the Raman cell. For the experiments on temperature variations, the resting unreduced enzyme in the Raman cell was incubated at the designated temperatures for 20 min, and then the cell was put in an ice bath for 5 min to quench the reaction. The Raman spectra were measured at 5 °C after reduction of the enzyme by dithionite. Oxidase activity was evaluated with oxygen electrode by measuring oxygen consumption and cytochrome *c* oxidase activity by monitoring the absorbance at 550 nm of reduced cytochrome *c*. The proton pumping activity was measured after incorporating the enzyme used for the Raman measurements into phospholipid vesicles and was evaluated in terms of the initial velocity of H^+ extrusion when yeast ferrocytochrome *c* was added (Sone & Hinkle, 1982). EPR spectra were measured at liquid helium temperature with an X band spectrometer (JEOL ME-2X).

Raman scattering was excited with the 441.6-nm line of a He/Cd laser (Kinmon Electrics, Model CDR80MGE) or the 514.5-nm line of an Ar/Kr laser (Spectra Physics, Model 165) and recorded with a JEOL-400D Raman spectrometer equipped with a cooled Hamamatsu HTV-R649 photomultiplier. Calibration of the frequencies of Raman lines was performed with indene (1200–1700 cm^{-1}) and CCl_4 (150–650 cm^{-1}) as standards. The spectra of the resting and carbon monoxide bound and cyanide-bound forms were measured with a spinning cell (1800 rpm, 2-cm diameter). Cold nitrogen gas was flushed against the cell during the measurements to keep the sample around 5 °C. For the measurements of the reduced form, ca. 50 μL of the enzyme was put in a cylindrical cell, which was evacuated to 0.01 torr and then was placed in a thermostatic water-jacket at 5 °C. Intensity of a Raman line was estimated as peak height from the assumed base line represented by a broken line in individual spectra and accordingly may involve some systematic errors. Since a small amount of the enzyme was precipitated during pH variation or incubation at elevated temperatures, the effective concentration of the enzyme in each Raman measurement was altered a little. Therefore, we monitored the intensity of the 1667- and 213-cm^{-1} lines with other heme modes as intensity standards instead of referring an internal standard such as sulfate anion.

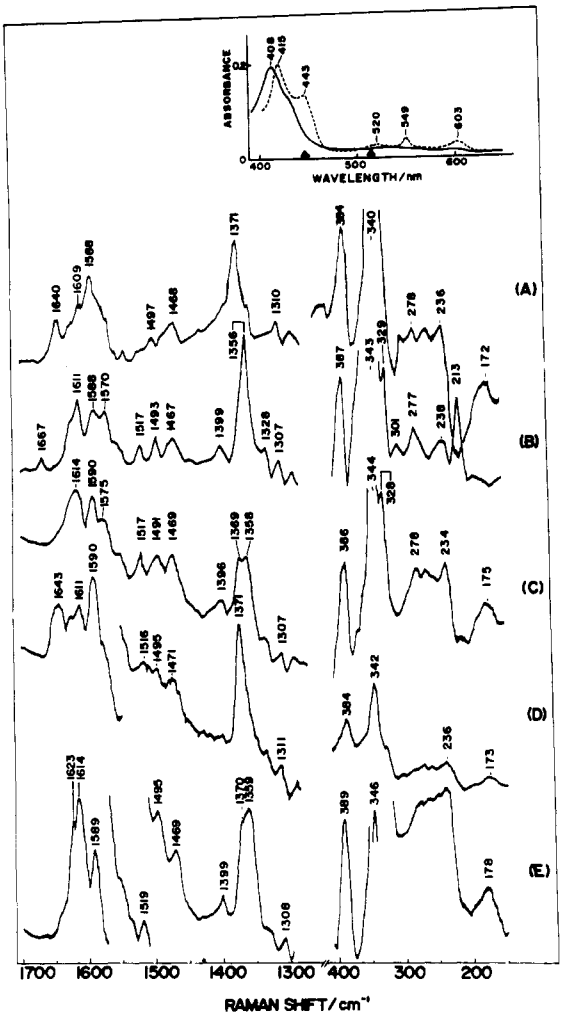

FIGURE 1: Resonance Raman spectra of resting (A), dithionite-reduced (B), reduced and carbon monoxide bound (C), resting and 2 mM cyanide bound (D), and resting and 100 mM cyanide-bound forms (E) of PS3 cytochrome oxidase excited at 441.6 nm. Samples with the heme *a* concentration of ca. 0.1 mM were kept at 5 °C during the measurements. Spectra A and C–E were obtained with a spinning cell. The carbon monoxide bound form was prepared by bubbling CO gas through the sample solution for 2 min after dithionite reduction. The cyanide-bound form was obtained by mixing a KCN solution with the enzyme solution to give a final CN^- concentration of 2 mM, pH 7.4, or of 100 mM, pH 10.0. Instrumental conditions: laser 40 mW; slit width 5 cm^{-1}; sensitivity 1000 counts/s for (A) and (E) and 2500 counts/s for (B–D); scan speed 10 cm^{-1}/min; time constant 8 s. The inset displays the visible absorption spectra of the resting (solid line) and reduced PS3 enzyme (broken line).

Results

The RR spectra of the resting (A), dithionite reduced (B), reduced and carbon monoxide bound (C), resting and 2 mM cyanide bound (D), and resting and 100 mM cyanide bound forms (E) of the PS3 cytochrome oxidase are shown in Figure 1. The visible absorption spectra of the resting and reduced forms are depicted in the inset of Figure 1, where arrows denote the excitation wavelengths. Since this enzyme contains firmly bound heme *c* in a 1:2 ratio with heme *a*, absorptions due to cytochrome *c* are identified at 415, 520, and 549 nm for the reduced form. Nonetheless, all the Raman lines observed in Figure 1 are considered to arise from the cytochrome aa_3 moiety of the enzyme, since horse cytochrome *c* at the same concentration as the cytochrome oxidase gave no Raman line under the same instrumental conditions upon excitation at 441.6 nm. Upon excitation at 514.5 nm, however, the reduced form of this enzyme had almost the same spectrum as that of horse cytochrome *c*. This indicates that the heme *c* of this enzyme is not greatly distorted by interactions with the cytochrome aa_3 chromophores.

The Raman lines of the reduced form at 1667 and 213 cm^{-1} (spectrum B) were previously assigned to the peripheral CH=O stretching (Salmeen et al., 1978) and Fe–histidine stretching modes (Ogura et al., 1983) of cytochrome a_3, respectively. In accord, these lines disappeared when CO was bound to the a_3 heme (spectrum C), presumably due to the change of spin state from high to low spin. The additional Raman line of the CO-bound form at 1369 cm^{-1} (spectrumC) is assigned to the ν_4 mode of the a_3·CO complex and the 1358-cm^{-1} component to the ν_4 mode of the low-spin *a* heme [the mode number is based on Abe et al. (1978)]. The Raman line of the a_3·CO complex diminished when the spinning of cell was abolished, and the resultant spectrum resembled that of the reduced enzyme (spectrum B), indicating that spinning the cell apparently protected the a_3·CO complex from photodissociation. The relative intensities of Raman lines around 1570–1620 cm^{-1} were altered by CO binding. This suggests

appreciable contribution of the a_3·CO complex to the spectrum there.

The resting enzyme in the spinning cell under aerobic conditions gave spectrum A in Figure 1. When spinning of the cell was stopped, a spectrum similar to spectrum B except for the absence of the 213- and 1667-cm^{-1} lines and the presence of a weak shoulder around 1370 cm^{-1} was observed. On the other hand, when the resting enzyme was measured under anaerobic conditions without spinning the cell, immediately appeared a Raman spectrum identical with spectrum B. Evidently, photoreduction took place, as in the case of mitochondrial enzymes (Adar & Yonetani, 1978; Babcock & Salmeen, 1979; Kitagawa & Orii, 1978). Note that the a_3 heme was also photoreduced for the PS3 enzyme under anaerobic conditions since it exhibited the Raman lines at 1667 and 213 cm^{-1} and no shoulder at 1371 cm^{-1}, although Salmeen et al. (1978) stressed no photoreduction of the a_3 heme for mammalian cytochrome oxidase. The a_3 heme of the photoreduced PS3 enzyme is not reduced under aerobic conditions as evidenced by the absence of the 213- and 1667-cm^{-1} lines and the presence of the 1370-cm^{-1} shoulder. This is presumably due to the fact that dioxygen reduction by a_3 heme takes place much faster than electron transfer from cytochrome a to cytochrome a_3. It may imply that cytochrome a_3 receives electrons solely from cytochrome a even in the photoreduction.

The RR spectrum obtained in the presence of 2 mM cyanide at pH 7.4 (spectrum D) was essentially the same as that of the resting enzyme, although the cytochrome c oxidase activity was lost completely under this condition. Since cyanide has been considered to bind to the oxidized a_3 heme, the spectral invariability may indicate that most of the Raman lines of the resting enzyme arise from the cytochrome a component upon excitation at 441.6 nm, as previously suggested for bovine cytochrome oxidase (Babcock & Salmeen, 1979). The ν_{10} frequency (1640 cm^{-1} for resting form), which has been utilized as an indicator of the core expansion (Spaulding et al., 1973; Spiro et al., 1979) and thus of a coordination number of the heme iron (Teraoka & Kitagawa, 1980), is consistent with the six-coordinate ferric low-spin heme postulated for cytochrome a. Nevertheless, we cannot rule out the possibility that cyanide is not bound to the axial position of the a_3 heme at the concentration of 2 mM, since a more concentrated cyanide solution (100 mM) caused a distinct change of the Raman spectrum, as shown by spectrum E; there appeared another ν_4 line at 1358 cm^{-1} in addition to the 1370-cm^{-1} line, and the 1640-cm^{-1} line was completely replaced by a doublet at 1623 and 1614 cm^{-1}. The absorption spectrum of this solution displayed maxima at 415, 443, 520, 549, and 603 nm, indicating reduction of the enzyme, and this occurred even in the dark after a day. The EPR spectrum of the original resting enzyme at liquid helium temperature revealed absorptions at g = 2.98, 2.23, and 2.02, but that of the CN^--bound form gave only absorption at g = 2.00 (spectrum not shown). Since the oxidized cytochrome a is known to give EPR signals at g = 3.03 and 2.25 (Ohnishi et al., 1982), it is clear that cytochrome a, at least, was reduced in the presence of 100 mM CN^- at pH 10.

Figure 2 shows the RR spectra of reduced enzyme at two different pH values. The Fe–histidine stretching Raman line at 213 cm^{-1} exhibited a pH-dependent intensity decreasing to zero at pH 9.2. This is illustrated in the inset of Figure 2, where the relative intensities of the 213- and 238-cm^{-1} lines, I_{213}/I_{238}, are plotted vs. pH (squares). On the other hand, the relative intensities of the 278- and 238-cm^{-1} Raman lines (triangles in the inset) remain unaltered with pH. The other

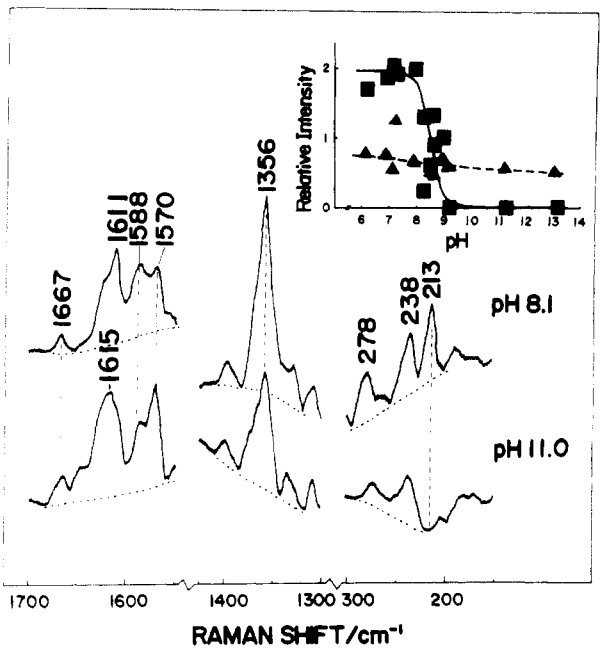

FIGURE 2: Resonance Raman spectra of reduced PS3 cytochrome oxidase at pH 8.1 (upper) and pH 11.0 (lower). Broken lines denote the assumed base lines from which the peak heights were measured to estimate the intensities of the Raman lines. Instrumental conditions are the same as in Figure 1. Inset figure plots the pH dependence of relative intensities, I_{213}/I_{238} (■) and I_{278}/I_{238} (▲).

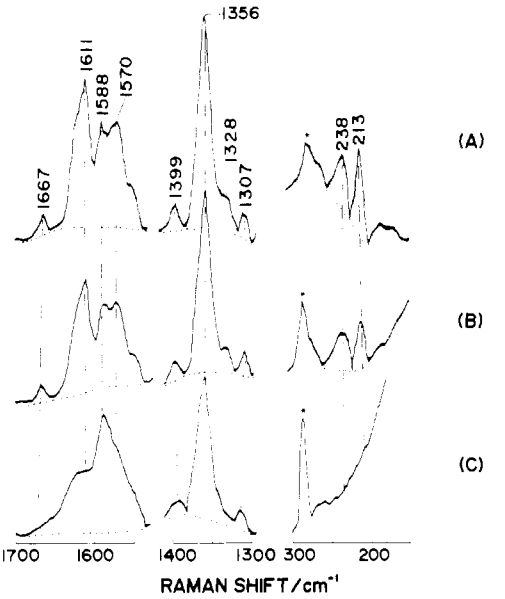

FIGURE 3: Resonance Raman spectra of reduced PS3 cytochrome oxidase after incubation for 20 min at 50 (A), 60 (B), and 70 °C (C). Enzymes were reduced just before the Raman measurements at 5 °C (see Materials and Methods). Instrumental conditions are the same as in Figure 1. An asterisk denotes the plasma line of a He/Cd laser. Broken lines denote the base lines from which peak height was measured.

marker line of cytochrome a_3, at 1667 cm^{-1}, was also hardly altered between pH 8.1 and pH 11.0, although the relative intensities of Raman lines around 1620–1570 cm^{-1} were appreciably changed.

In spite of the fact that the PS3 bacterium survives around 70 °C, the isolated cytochrome oxidase loses its enzymatic activity above 65 °C (Sone et al., 1979). The RR spectra of the isolated enzyme incubated at 50, 60, and 70 °C for 20 min are shown in Figure 3. A large spectral change occurred at 65–70 °C, but the 213-cm^{-1} line lost intensity at lower temperatures (~60 °C).

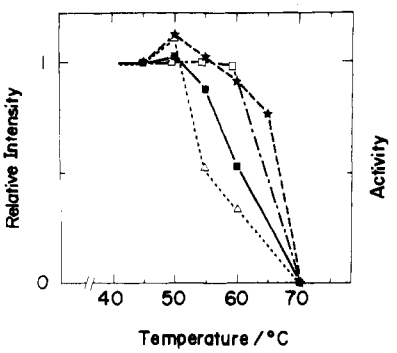

FIGURE 4: Incubation temperature-dependent changes of the Raman intensities at 213 (■) and 1667 cm^{-1} (★) and of the proton pumping (△) and oxidase activities (□) of the samples used for the Raman measurements. The values are normalized to unity at 45 °C. The abscissa represents the temperature at which the sample solution was incubated for 20 min before being put into an ice bath and reduced. The Raman and activity measurements were carried out at 5 and 25 °C, respectively. The Raman intensities are represented in terms of the peak height, relative to that of the 1356-cm^{-1} line.

Figure 4 plots the proton pumping and cytochrome *c* oxidase activities as well as the relative intensities, I_{213}/I_{1356} and I_{1667}/I_{1356}, of the enzyme which was incubated at the indicated temperatures for 20 min. The incubation temperature dependence of the oxidase activity qualitatively agrees with the behavior of the 1667-cm^{-1} line. On the other hand, the proton pumping activity, which was measured at 25 °C after the incubated enzymes were incorporated into phospholipid liposomes, seems to run parallel with the intensity of the 213-cm^{-1} line.

Figure 5 compares the RR spectra of PS3 cytoplasmic membrane and isolated cytochrome oxidase with those of mitochondrial cytochrome oxidases purified from yeast, and beef heart, all in the reduced state. The RR spectrum of the PS3 cytoplasmic membrane (spectrum A) is exceedingly close to that of the isolated enzyme (spectrum B) upon excitation at 441.6 nm, despite the presence of other cytochromes in the membrane. The formyl CH=O stretching mode (1667 cm^{-1}), the Fe–histidine stretching mode of the ferrous high-spin heme (213 cm^{-1}), and other lines of ferrous low-spin heme are shifted little between the membrane and isolated enzyme. This similarity was anticipated from the previous observation for mitochondria (Adar & Erecińska, 1978).

The spectra of isolated cytochrome oxidase from bacteria (spectrum B), yeast (spectrum C), and mammalian sources (spectrum D) differ distinctly with each other in the low-frequency region (200–600 cm^{-1}). This probably means that the heme environments in these cytochrome oxidases are somewhat different from each other, since the low-frequency porphyrin vibrations involve considerably the bending modes of the peripheral substituents (Abe et al., 1978) which are most easily affected by the protein–heme interactions. In this regard it is noteworthy that the RR spectrum of cytochrome oxidase of an extreme thermophile *Thermus thermophilus* HB8 (Ogura et al., 1983) is markedly close to spectrum B of Figure 5 even in the low-frequency region, indicating close resemblance of the heme environments.

In the higher frequency region, the relative intensities of Raman lines around 1570–1620 cm^{-1} vary with the source of the enzyme, although the frequencies of the Raman lines remain almost unaltered. The variation of the relative intensity of two lines around 1250 and 1230 cm^{-1} seems consistent with the sensitive solvent dependence of the corresponding two lines of the ferrous high-spin heme *a* compounds (Steelandt-Frentrup et al., 1981). This may imply that the geometry of

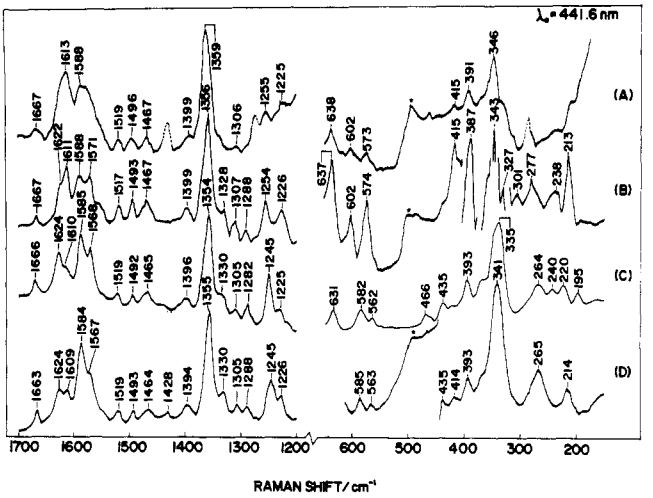

FIGURE 5: Resonance Raman spectra of PS3 cytoplasmic membrane (A), isolated PS3 (B), yeast (C), and bovine heart cytochrome oxidase (D), all in the reduced state. The part represented by a broken line indicates the plasma lines of a He/Cd laser, and the Raman lines marked by a star are due to the Raman cell. Instrumental conditions are the same as in Figure 1. Heme *a* concentration is about 0.1 mM.

the formyl group is susceptible to environmental effects, because the latter line arises from the C_b–CHO stretching mode (Choi et al., 1983).

Discussion

Fe–Histidine and Formyl Stretching Modes. Stevens & Chan (1981) demonstrated in an EPR study of [^{15}N]-histidine-substituted yeast cytochrome oxidase that the axial ligand of the ferrous high-spin cytochrome a_3 is a histidine residue, as in Mb, Hb, and HRP. The Fe–histidine stretching Raman lines of ferrous high-spin hemoproteins usually are intensity enhanced upon excitation at 441.6 nm; they have been observed at 220 cm^{-1} for deoxy-Mb (Kitagawa et al., 1979; Kincaid et al., 1979), at 216 cm^{-1} for deoxy-Hb (Nagai et al., 1980), and at 244 cm^{-1} for reduced HRP (Teraoka & Kitagawa, 1981). The Fe–imidazole stretching Raman line of ferrous high-spin iron porphyrin was also observed around 210 cm^{-1} (Hori & Kitagawa, 1980), although the frequency undergoes solvent effects (Teraoka & Kitagawa, 1981). The Fe–histidine as well as the Fe–imidazole stretching Raman lines apparently disappear upon binding of a ligand such as CO, O_2, CN^-, or imidazole to the sixth-coordination site and also upon oxidation of the heme iron. Furthermore, for all the plant peroxidases, only the Fe–histidine stretching Raman line exhibited a pH-dependent intensity change (Teraoka et al., 1983). Such properties are in agreement with those observed for the 213-cm^{-1} line of the PS3 cytochrome oxidase in the reduced state. Accordingly, the 213-cm^{-1} line had been inferred to be associated with the Fe–histidine stretching mode.

Recently we succeeded in obtaining ^{54}Fe-substituted cytochrome oxidase from thermophilic bacterium HB8 and examined its RR spectrum. Only the 210-cm^{-1} line of the HB8 enzyme exhibited an upward frequency shift (by 2 cm^{-1}) upon ^{54}Fe substitution (Ogura et al., 1983). Consequently, it seems clear that the 210-cm^{-1} line arises mainly from the Fe–histidine stretching mode of the ferrous high-spin a_3 heme.

The intensity of the 213-cm^{-1} line of the PS3 enzyme diminished significantly around pH 9.2 and was not restored upon pH reduction to 7.4, while the ν_4 line was observed at the same frequency as the ferrous state (1356 cm^{-1}; Figure 2). This implies conversion of the a_3 heme to the low-spin state at alkaline pH which is presumably caused by coordination of an amino acid residue to the sixth-coordination site of the

a_3 heme, in agreement with the suggestion from the MCD spectra (Callahan & Babcock, 1983). Note that the 1667-cm^{-1} line is still observable at pH 11 after the spin-state change. Since in the RR spectra of bovine cytochrome oxidase [Figure 8 of Callahan & Babcock (1983)], the 1665-cm^{-1} line is completely deleted at pH 10, we examined a possible spectral change when the enzyme was kept at pH 11 for longer time (7 h). However, the spectrum remained unaltered. Therefore, this spectral difference is probably caused by different pH resistances of the two enzymes. We stress the existence of the state in which the 1667-cm^{-1} line is definitely observable but the 213-cm^{-1} line is absent.

This observation may indicate that the appearance of the peripheral CH=O stretching Raman line is not always correlated with the spin state of the heme. When the electronic conjugation between the CH=O group and the porphyrin skeleton decreases in a certain geometry, the intensity of the CH=O stretching RR line is expected to diminish. Accordingly, one of the plausible interpretations assumes that a geometry of the CH=O group relative to the porphyrin skeleton is determined by specific interactions of the CH=O group with the protein moiety and changes upon binding of an external ligand such as CO or CN^- but not upon binding of an internal ligand at higher pH. If the protein–heme interaction were weak, the CH=O group would be expected to adopt the geometry determined solely by the spin state of the heme iron (Steelandt-Frentrup et al., 1981).

The intensity of the 1667-cm^{-1} line was altered when the enzyme was incubated at higher temperatures. With regard to the incubation temperature dependence, the intensity of the 1667-cm^{-1} line bore close resemblance to the oxidase activity. Therefore, a specific geometry of the formyl group relative to the heme plane may possibly be necessary for oxidase activity. However, the intensity change of the 1667-cm^{-1} line ran parallel with the overall spectral change and accordingly seemed to be more likely to reflect a conformational change of the whole protein.

Cyanide Binding and Respiratory Inhibition. Cytochrome *c* oxidase activity was lost completely in the presence of 2 mM cyanide, pH 7.4, when neither the autoreduction nor Raman spectral change was recognized. In the presence of 100 mM cyanide at pH 10, cyanide was bound to cytochrome a_3 with concomitant reduction of cytochrome *c* and cytochrome *a* (Figure 1, spectrum E). Cyanide is unlikely to be the reductant, since the resting enzyme under anaerobic conditions (10^{-3} torr) also was reduced without cyanide after a day. The following procedures were tried, in attempts to remove a possible "intrinsic" reductant: (1) dialysis against phosphate buffer, (2) hydrophobic chromatography with octyl-Sepharose (CL-4B) to remove impurities and phospholipids, and (3) stirring under aerobic conditions to exhaust the reductant. These attempts were unsuccessful.

The spectral changes caused by 100 mM cyanide were reversed by removal of the cyanide through dialysis. Therefore, irreversible denaturation did not take place when the autoreduction was observed. The pH dependence of autoreduction suggested that the molecular species to bind is CN^-. The amounts of reduced cytochrome *c* and cytochrome *a* (evaluated by the absorbance at 549 and 603 nm, respectively) increased at higher pH, and their pH dependences were alike and close to the ionization curve of HCN (pK_a = 9.3). Consequently, it appears most likely that there is always a little transport of electrons from an unknown reductant to oxygen through the enzyme and that the transported electrons accumulate in cytochrome *c* and cytochrome *a* when CN^- binds to the axial position of the a_3 heme and thus prevents oxygen from binding. An important suggestion from our observations is that the respiratory inhibition by cyanide, at low concentrations in particular, does not always indicate the binding of cyanide to the axial position of the a_3 heme.

Hydrogen Bonding between Heme and Protein. To keep the stoichiometry of the H^+:e^- ratio (=0.8) (Sone & Hinkle, 1982) during the oxidase reaction, some communication between the proton pump site and the catalytic site of dioxygen reduction seems necessary. Since intensity changes in the formyl CH=O and Fe–histidine stretching Raman lines, which should involve an appreciable structural change of the a_3 heme, were observed upon binding of CO or CN^-, these two groups were anticipated to serve as communication points between the heme group and polypeptide chain. However, it became clear that the proton pump activity was not correlated with the CH=O stretching mode (Figure 4). There still remains the possibility that the fifth ligand of the a_3 heme communicates the oxygen binding to the gate site of the proton pump through a conformation change.

Recently Callahan & Babcock (1983) assigned the 1609-cm^{-1} line of the reduced enzyme to the formyl stretching mode of cytochrome *a*. This Raman line in their spectrum (Figure 8) disappears around pH 10 and instead appears a new Raman line at 1633 cm^{-1}. From this observation they assigned the presence of very strong hydrogen bond between the protein and the heme CH=O group, which is assumed to result in the characteristic red shift of the visible absorption. Furthermore, Babcock & Callahan (1983) proposed that a change of the formyl hydrogen bonding of cytochrome *a* upon the redox change served as a driving force for proton translocation. On the other hand, in the RR spectrum of the PS3 enzyme at pH 11.0 (Figure 2), it seems as if the 1611-cm^{-1} line at pH 7.4 were shifted to 1615 cm^{-1} at pH 11.0, but actually, it was the relative intensity of the two lines at 1611 and 1622 cm^{-1} which changed with the pH variation. The spectral feature remained unaltered, and there was no Raman line between 1630 and 1650 cm^{-1} even after prolonged incubation at pH 11.0 (7 h). Therefore, the 1633-cm^{-1} line of Callahan and Babcock's seems to be characteristic of the 406.7-nm excitation, although the absorption maxima of cytochrome a_3 and cytochrome *a* in the reduced state are considered to be alike (442.5 and 444 nm, respectively, at pH 8.0) (Vanneste, 1966).

In the RR spectra of heme *a* derivatives excited at 441.6 nm (Steelandt et al., 1981), a Raman line was observed at 1608 cm^{-1} for both the CH_2Cl_2 and aqueous solutions, in which the formyl CH=O mode was observed at 1660 and 1640 cm^{-1}, respectively. Therefore, the 1608-cm^{-1} line of the model compound and the 1611-cm^{-1} line of the PS3 enzyme as well probably are assignable to the porphyrin ν_{10} mode (Abe et al., 1978), and the frequency seems insensitive to the hydrogen bonding of the CH=O group.

If the CH=O stretching Raman line of the low-spin heme *a* were down shifted due to strong hydrogen bonding and overlapped with the ν_{10} mode at 1611 cm^{-1}, its band shape would be deformed with a shoulder on the higher frequency side upon weakening of the hydrogen bond. It is generally expected that such hydrogen bonds are weakened or destroyed upon releasing of the tertiary structure of the protein due to an increase of temperature or pH. However, such a large spectral change was not detected except the relative intensity change of the two lines at 1611 and 1622 cm^{-1}. Furthermore, the proton pumping activity of the enzyme incubated at 60 °C was about one-third of that at room temperature (Figure 4) while the 1611-cm^{-1} feature in spectrum B in Figure 3

(incubated at 60 °C) remained almost unaltered. These observations are unfavorable to the proposal by Babcock and Callahan, although there remains the possibility that the proton channel is damaged at 60 °C while the driving force at the heme is still unaffected.

There are large differences between the RR spectra of the PS3, yeast, and beef heart enzymes, particularly in the low-frequency region (Figure 3). The Raman line of the PS3 enzyme at 277 cm^{-1} is assignable to the low-spin cytochrome *a*, because many ferrous low-spin hemoproteins give a Raman line around 270 cm^{-1}. The corresponding Raman line of the mammalian enzyme is seen at 265 cm^{-1}. The Fe–histidine stretching mode of the ferrous high-spin a_3 heme is seen at 213, 220, and 214 cm^{-1} for the PS3, yeast, and bovine enzymes, respectively. These Raman lines may be useful as probes of the heme environments of cytochrome *a* and cytochrome a_3. Nonetheless, the frequencies of Raman lines around 1610 and 1622 cm^{-1} differ little among the three kinds of enzymes. Therefore, we are attempting to assign the 1611- and 1622-cm^{-1} lines to the porphyrin ν_{10} mode of cytochrome *a* and cytochrome a_3, respectively, although the latter could involve significant contribution from the peripheral vinyl stretching mode (Choi et al., 1983).

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References

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

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

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

Asher, S. A. (1981) *Methods Enzymol. 76*, 371–413.

Babcock, G. T. (1982) in *Inorganic Reactions and Methods*, Verlag Chemie (in press).

Babcock, G. T., & Salmeen, I. (1979) *Biochemistry 18*, 2493–2498.

Babcock, G. T., & Callahan, P. M. (1983) *Biochemistry 22*, 2314–2319.

Babcock, G. T., Callahan, P. M., Ondrias, M. R., & Salmeen, I. (1981) *Biochemistry 20*, 959–966.

Bocian, D. F., Lemley, A. T., Peterson, N. O., Brudvig, G. W., & Chan, S. I. (1979) *Biochemistry 18*, 4396–4402.

Cabral, F., & Schatz, G. (1978) *J. Biol. Chem. 253*, 4396–4401.

Callahan, P. M., & Babcock, G. T. (1981) *Biochemistry 20*, 952–958.

Callahan, P. M., & Babcock, G. T. (1983) *Biochemistry 22*, 452–462.

Choi, S., Lee, J. J., Wei, Y. H., & Spiro, T. G. (1983) *J. Am. Chem. Soc. 105*, 3692–3707.

Fee, J. A., Choc, M. G., Findling, K. L., Lorence, R., & Yoshida, T. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 147–151.

Hon-nami, K., & Oshima, T. (1980) *Biochem. Biophys. Res. Commun. 92*, 1023–1029.

Hori, H., & Kitagawa, T. (1980) *J. Am. Chem. Soc. 102*, 3608–3613.

Kincaid, J., Stein, P., & Spiro, T. G. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 549–552, 4156.

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

Kitagawa, T., & Teraoka, J. (1982) in *The Biological Chemistry of Iron* (Dunford, H. B., Dolphin, D., Raymond, K. N., & Sieker, L., Eds.) pp 375–389, D. Reidel Publishing Co., Boston, MA.

Kitagawa, T., Kyogoku, Y., & Orii, Y. (1977) *Arch. Biochem. Biophys. 181*, 228–235.

Kitagawa, T., Nagai, K., & Tsubaki, M. (1979) *FEBS Lett. 104*, 376–378.

Ludwig, B., & Schatz, G. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 196–200.

Malmström, B. G. (1979) *Biochim. Biophys. Acta 549*, 281–303.

Nagai, K., Kitagawa, T., & Morimoto, H. (1980) *J. Mol. Biol. 136*, 271–289.

Ogura, T., Hon-nami, K., Oshima, T., Yoshikawa, S., & Kitagawa, T. (1983) *J. Am. Chem. Soc. 105*, 7781–7783.

Ohnishi, T., LoBrutto, R., Salerno, J. C., Bruckner, R. C., & Frey, T. G. (1982) *J. Biol. Chem. 257*, 14821–14825.

Rosén, S. (1978) *Biochim. Biophys. Acta 523*, 314–320.

Rousseau, D. L., & Ondrias, M. R. (1983) *Annu. Rev. Biophys. Bioeng. 12*, 357–380.

Salmeen, I., Rimai, L., & Babcock, G. T. (1978) *Biochemistry 17*, 800–806.

Sekuzu, I., Mizushima, H., Hirota, S., Yubisui, T., & Okunuki, K. (1967) *J. Biochem. (Tokyo) 62*, 710–718.

Sone, N., & Hinkle, P. C. (1982) *J. Biol. Chem. 257*, 12600–12604.

Sone, N., & Yanagita, Y. (1982) *Biochim. Biophys. Acta 682*, 216–226.

Sone, N., Ohyama, T., & Kagawa, Y. (1979) *FEBS Lett. 106*, 39–42.

Spaulding, L. D., Chang, C. C., Yu, N. T., & Felton, R. H. (1975) *J. Am. Chem. Soc. 97*, 2517–2524.

Spiro, T. G. (1975) *Biochim. Biophys. Acta 416*, 169–187.

Spiro, T. G., Stong, J. D., & Stein, P. (1979) *J. Am. Chem. Soc. 101*, 2648–2655.

Steelandt-Frentrup, J. V., Salmeen, I., & Babcock, G. T. (1981) *J. Am. Chem. Soc. 103*, 5981–5982.

Stevens, T. H., & Chan, S. I. (1981) *J. Biol. Chem. 256*, 1069–1071.

Teraoka, J., & Kitagawa, T. (1980) *J. Phys. Chem. 84*, 1928–1935.

Teraoka, J., & Kitagawa, T. (1981) *J. Biol. Chem. 256*, 3969–3977.

Teraoka, J., Job, D., Morita, Y., & Kitagawa, T. (1983) *Biochim. Biophys. Acta 747*, 10–15.

Vanneste, W. H. (1966) *Biochemistry 5*, 838–848.

Wikström, M., Krab, K., & Saraste, M. (1981) *Cytochrome Oxidase: A Synthesis*, Academic Press, London.

Yamanaka, T., & Fujii, K. (1980) *Biochim. Biophys. Acta 591*, 53–62.

Yamanaka, T., Fujii, K., & Kamita, Y. (1979) *J. Biochem. (Tokyo) 86*, 821–824.