Resonance Raman Studies on the Cu_A Site of Cytochrome *c* Oxidase Using a Multichannel Scanning Raman Spectrometer with a CCD Detector†

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ABSTRACT: A new Raman measurement system with a CCD (charge coupled device) detector was constructed and applied successfully to observe far-red excited Raman spectra of bovine cytochrome *c* oxidase. In resonance with the 830-nm absorption band, Raman bands were observed at around 330 cm^{-1} and assigned to the Cu_A–S(Cys) and Cu_A–N(His) stretching vibrations. Although the Cu_A center has been believed to be coupled with the other redox center and to serve as the electron acceptor from cytochrome *c*, the frequency and intensity of these bands were influenced by neither the redox and ligation changes in cytochrome a_3–Cu_B center nor the binding of cytochrome *c*, indicating the highly independent nature of the Cu_A center. In the higher frequency region of the far-red excited Raman spectra, some protein vibrations which could not be observed in resonance Raman spectra of heme proteins upon visible excitation were observed together with some preresonant heme modes. The amide I and III frequencies indicated a predominance of α-helix in the enzyme, and the amino hydrogens were scarcely exchanged with deuterons in D_2O, while some Trp side chain bands clearly displayed the deuteration shift.

Mitochondrial cytochrome *c* oxidase is a metalloenzyme that catalyzes the reduction of molecular oxygen to water with electrons donated by cytochrome *c* (Wikstrom et al., 1981), and there is now general agreement that the intramolecular electron transfer is coupled to an active transmembrane transport of protons contributing to the formation of a membrane electrochemical potential for ATP synthesis (Wikstrom 1977). The structure and conformation of this enzyme is best known for beef heart cytochrome *c* oxidase with M_w = 202 787 and 13 different subunits (Buse et al., 1985), but its metal content is controversial between two propositions, namely, 3Cu/2Fe/1Mg/1Zn and 2.5Cu/2Fe/1Mg/1Zn per monomer [see recent article by Pan et al. (1991a) and references sited therein]. It is established that two heme irons, designated as cytochromes *a* and a_3, and one Cu ion, designated as Cu_B, are contained in subunit I and that cytochrome a_3 and Cu_B, which are EPR silent in the resting state on account of antiferromagnetic coupling, constitute the catalytic site for O_2 reduction.

The other Cu center, designated as Cu_A, is contained in subunit II and displays unusual spectroscopic properties in the resting state. Both the hyperfine coupling and the *g* values are quite small (Beinart et al., 1962; Beinart & Palmer, 1964), which are distinct from the type I (blue) copper proteins having usual *g* values (Peisach & Blumberg, 1974). Upon reduction, the X-ray absorption edge remains unchanged for the Cu_A while it changes from the cupric to cuprous positions for blue coppers (Hu et al., 1977; Powers et al., 1979; Scott et al., 1986). The cupric Cu_A moiety gives rise to an absorption band at a significantly longer wavelength (around 830 nm; Griffiths & Wharton, 1961, Beinart et al., 1980) than blue copper proteins (around 600 nm). To explain the peculiarities mentioned above, several model structures have been proposed, including a model of a single copper coordinated by two cysteine and two histidine residues (Stevens et al., 1982) and a Cu(II)–Cu(I) mixed valence binuclear copper center (Kroneck et al., 1988, 1990). Although general agreement about the selection of the alternatives has not been obtained, the extended studies on the Cu_A site revealed the following properties: EPR and ENDOR studies with [^{15}N]His and [^{2}H]Cys (Stevens et al., 1982) and [^{13}C]Cys-substituted (Martin et al., 1988) enzymes confirmed the coordination of, at least, one cysteine and one histidine. A recent high-field ENDOR study (Fee et al., 1992) confirmed the coordination of two histidine residues. The EXAFS experiments (Powers et al., 1981; Scott et al., 1986; Li et al., 1987) indicated the coordination of two N (or O) and two S (or Cl) atoms.

A physiological role of Cu_A has not been fully established. It is apparent that Cu_A is involved in the electron transfer reaction. Chan and co-workers (Gelles et al., 1986; Chan & Li, 1990) proposed that Cu_A serves as a coupling apparatus between the electron and proton transfers by replacing the Cys ligand with Tyr upon reduction of Cu_A. Saraste and co-workers (Holm et al., 1987; Saraste, 1990) proposed that the Cu_A moiety of subunit II is located outside of the membrane and acts as a cytochrome *c* binding site. The lack of experimental techniques accessible to Cu_A has prohibited us from clarifying these arguments.

Resonance Raman (RR)[1] spectroscopy has made a significant contribution to reveal the reaction mechanism of cytochrome *c* oxidase at room temperature (Babcock, 1988; Han et al., 1990a,b, 1991; Varotsis et al., 1989; Varotsis & Babcock, 1990; Ogura et al., 1990a,b, 1991; Babcock & Wikstrom, 1992), but none of them has brought about structural information on Cu_A. The Cu_A–ligand stretching modes, if observed, are expected to work as an acute probe of the Cu_A site. On the other hand, RR spectroscopy has been extensively used to characterize blue copper proteins

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[1] Abbreviations: RR, resonance Raman; CCD, charge coupled device.

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[see Woodruff et al. (1988) for a review]. Excitation of Raman scattering from blue copper proteins by red light around 590–610 nm yielded a number of RR bands below 500 cm^{-1} assignable to the Cu–Cys and Cu–His stretching and related modes (Han et al., 1991), but red excitations of cytochrome *c* oxidase brought no Cu_A-associated Raman bands (Bocian et al., 1979; Callahan & Babcock, 1983; Centeno & Babcock, 1991). This failure has been probably caused by the low sensitivity of a photomultiplier in the far-red region.

Recently, a new array detector called CCD (charge coupled device), which is sensitive particularly in the far-red region, became available to Raman experiments. Accordingly, we constructed a new Raman observation system using a CCD detector attached to a bright single monochromator and applied it successfully to observe the Cu_A–ligand RR bands of cytochrome *c* oxidase (Takahashi et al., 1991). Here we report effects of the oxidation and ligation changes of another redox center and also of binding of cytochrome *c* on the Cu_A-associated RR bands in addition to the detailed description of a new procedure adopted for Raman measurements. The observed results indicate that the Cu_A–ligand stretching bands are affected neither by the binding of a ligand to cytochrome a_3 and of cytochrome *c* to subunit II nor by the redox changes of cytochrome a_3 and Cu_B. These results were unexpected on the basis of current ideas about the Cu_A center but should be significantly taken into consideration upon modeling of the Cu_A site.

EXPERIMENTAL PROCEDURES

Sample Preparations. Cytochrome *c* oxidase was purified through the reported method (Yoshikawa et al., 1977), but the final stage of purification adopted was crystallization (Yoshikawa et al., 1988), which was essential to prepare the fluorescence-free enzyme. The concentration of the enzyme, which was determined spectrophotometrically with the value of ϵ (reduced form, 603–630 nm) = 8.25 mM^{-1} cm^{-1}, was adjusted to 500 μM in terms of Cu_A in 10 mM sodium phosphate buffer, pH 7.4, unless otherwise stated.

The CN^- bound oxidized form was prepared by adding a fresh solution of 1 M NaCN (pH 8.5) to the resting enzyme so that the final cyanide concentration could be 20 mM and leaving it at room temperature for 1 h at least. The CO-bound half-reduced form was prepared by flushing with CO gas on a resting enzyme and leaving it at room temperature over an hour. The fully reduced form was obtained by addition of a small amount of sodium dithionite or sodium ascorbate to the resting form in an airtight cell whose inside atmosphere was replaced by N_2. The final concentration of reductant was made 20 mM at least. The CN-bound half-reduced form was obtained by reducing the CN-bound oxidized form by dithionite or ascorbate. Although the final pH was not examined, the formation of the individual compounds mentioned above and the sample integrity before and after the Raman measurement were ascertained by visible absorption spectra. The D_2O solution of the enzyme was obtained through repetition of dissolving the enzyme into 10-fold (v/v) D_2O buffer followed by filtration through membrane filter (Amicon YM-5), twice at least. Horse cytochrome *c* (Sigma, type IV) was purified by cation-exchange column chromatography (Whatman CM-52) and then dialyzed against 5 mM sodium phosphate buffer pH 7.4.

A New Raman Measurement System with a CCD. While the use of a CCD detector in Raman spectroscopy is increasing (Pemberton & Sobocinski, 1989; Wang & McCreery, 1989; Harris et al., 1989), to date its biological applications taking full advantages of the characteristics of CCD are rare. In this study, a CCD with 578 × 384 channels (Astromed CCD3200) was attached to a 50-cm single polychromator (Jasco CT-50) equipped with a 750-nm blazed ruled grating (1200 grooves/mm) or a 100-cm single polychromator (Ritsu MC-100DG) with a 900-nm blazed holographic grating (1200 grooves/mm). Reflected light from some electronic elements around the CCD chip was found to become stray light, yielding serious disturbance to Raman spectra, particularly in the low-frequency region. This was circumvented by placing an appropriate mask in front of the detector head. When the longer axis of the rectangular chip, along which the stored electrons are added up on the chip (binning) before reading out, was made parallel to the direction of wavelength dispersion, spectral distortions due to a charge trap (Pemberton et al., 1990) were noticed in the test measurement of cytochrome *c*. Therefore, the longer axis of the chip was made parallel to the entrance slit. With this geometry, the charge trap problems were eliminated, but the spectral range measured in one exposure became narrow (140 cm^{-1} in one exposure). In order to widen the wavenumber range of a spectrum examined, we adopted an idea of the multichannel scanning method (Knoll et al., 1990; Deckert & Kiefer, 1992).

Multichannel Scanning Method. This technique involves repetition of the multichannel measurement of a certain range of a spectrum followed by polychromator scanning. Practical procedures adopted in this study for obtaining a spectrum are explained below and illustrated in Figure 1.

(*Step 1*) *Flat Field Correction.* Sensitivity differences among individual channels are corrected by dividing the raw spectra by the spectrum of white light (not shown in Figure 1) passed through the spectrometer ($B_i = A_i$/white spectrum). The white spectra were measured with a tungsten lamp as a source separately from the Raman measurements.

(*Step 2*) *Standardization.* The center wavelength of the polychromator is shifted by $\Delta\lambda$ between two successive measurements (B_i and B_{i+1}). The integrated intensity of the spectra in the overlapped region in two successive multichannel measurements should be coincident as long as the laser power is constant. If not, it is due to laser power fluctuation, and a whole spectrum of the second measurement was multiplied by an appropriate factor deduced from the intensity ratio of the overlapped region (see Figure 1 caption for the practical procedures).

(*Step 3*) *Removal of the Cosmic Rays Effects.* The CCD detector cannot be protected from being hit by cosmic rays, which make spike noises in an otherwise smooth spectrum. Since the cosmic rays rarely hit exactly the same position of the CCD chip, the noise due to cosmic rays was removed by comparing the successive spectra. In comparison of the first spectrum (C_1) with the second one (C_2) in the overlapped region, a smaller value in counting of light intensity is always adopted at each channel, giving rise to a new combined spectrum (D_1). Next, the second (C_2) and third spectra (C_3) are compared, and the same procedure yields the second combined spectrum (D_2). In this way $N - 1$ spectra (D_1 – D_{N-1}) are generated from N spectra (C_1 – C_N).

(*Step 4*) *Averaging.* The $N - 1$ spectra (D_1 – D_{N-1}) thus obtained are averaged to yield a single spectrum (E). In this process the corresponding $N - 1$ spectra (D_1 – D_{N-1}) of the standard sample for calibration were added under the assumption that D_i and D_{i+1} are shifted by $\Delta\lambda+\delta$, where $\Delta\lambda$ is a predetermined value for the measurements of the sample. This calculations were carried out for several different values of δ near zero, and its practical value was determined so that

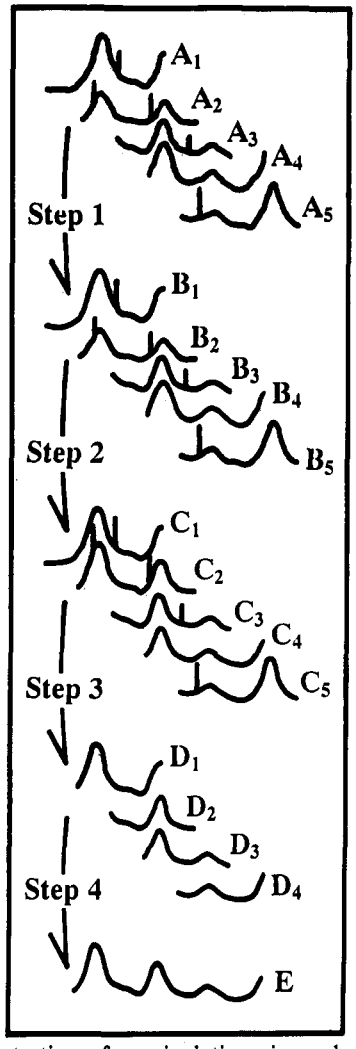

FIGURE 1: Illustration of manipulations in each step of data acquisition involved in the multichannel scanning spectrometry. (Step 1) Correction by white spectra. A_i is divided by a white spectrum in each spectral region, yielding B_i. (Step 2) Standardization of spectral intensity. The band intensity of B_{i+1} is standardized to B_i on the basis of their intensities in their overlapped region by multiplying an appropriate factor to spectrum B_{i+1}, and the resultant spectrum, C_{i+1} = constant × B_{i+1}, is stored in place of old B_{i+1}. Next the procedure is repeated with B_{i+2} and is repeated until B_N. (Step 3) Removal of cosmic rays effects. Spectra C_i and C_{i+1} are compared, and smaller values are adopted to give rise to spectrum D_i. (Step 4) Averaging. $N - 1$ spectra are added and averaged.

the synthesized spectrum could yield the highest peak height for well-known standard peaks. This δ value was used also for a sample. Finally, the wavelength scale was calibrated by measuring spectra of standard samples.

Raman Measurements. The sample was contained in a spinning cell (1800 rpm; diameter = 5 mm), and its temperature was maintained at ~5 °C by flushing with cold N_2 gas. In order to obtain the difference spectra very accurately without introducing systematic errors upon the exchange of samples, a light collecting system using optical fibers (Kamogawa & Kitagawa, 1990) was used. The scattered light from the sample was focused into one end of the linearly arranged optical fibers (diameter = 200 μm) and led to the entrance slit of the polychromator at the other end of the fibers. Although the energy throughput from the optical fibers is nearly half of what is attained by the direct measurement without the device, the difference spectrum obtained with this device does not suffer from artifacts caused by movements of the laser or the sample cell. This was applied

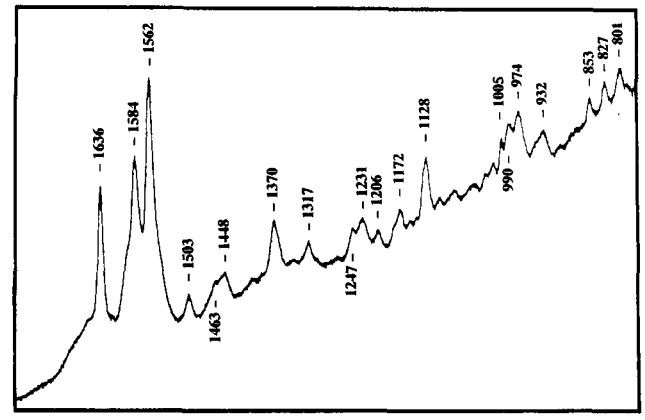

FIGURE 2: 695-nm excited Raman spectrum of ferric cytochrome *c* obtained with the multichannel scanning technique and a CCD detector. The 300 spectra which were obtained through the exposure of detector for 15 s at each wavelength upon scanning of the spectrometer stepwise by 0.3 nm were combined to a single spectrum with a method described under Experimental Procedures.

to the resting vs CN^--bound resting forms and the resting vs CO-bound half-reduced forms. However, for the cytochrome *c* bound form, the low throughput seriously affected the spectral quality, and, therefore, the two samples to be compared were alternatively measured several times without the optical fiber device. The invariance of the spectra of the same species upon the sample exchange was confirmed by difference calculations.

The short cut interference filter with the cut wavelength at 830 nm was placed in front of the entrance slit. In addition, a spatial filter (Ogura et al., 1991) was necessary to measure the spectrum in a low-frequency region in the case of the CT-50 spectrometer. In the practical multichannel scanning measurements, data accumulation for 30 s followed by scanning of the spectrometer by $\Delta\lambda$ (=0.4 nm) was repeated 100 times (N = 100) unless otherwise stated. The averaged spectra were calibrated with an accuracy of 1 cm^{-1} using CCl_4 and indene for the frequency regions of 200–500 and 500–1650 cm^{-1}, respectively. The spectral slit width was typically 6 cm^{-1}.

RESULTS

In order to examine the validity of the present method of synthesizing a whole spectrum from the component multichannel spectra, ferric cytochrome *c*, which has a weak band around 695 nm, was examined first. Figure 2 displays the RR spectrum of ferric cytochrome *c* excited at 695 nm. This is a composite of 300 multichannel spectra of 15-s exposure (i = 1 ~ 300) measured with $\Delta\lambda$ = 0.3 nm for the wavelength range from 710 to 800 nm. This spectrum is in qualitative agreement with the reported spectrum obtained with a Fourier transform method in near-infrared (1.04 μm) excitation (Yu et al., 1990) but is of much better quality. Therefore, it is now established that the technique proposed here is practical and more satisfactory.

Figure 3 shows the 840-nm excited Raman spectra in the 290–390-cm^{-1} region of the resting (A), CO-bound half-reduced (B), and CN^--bound resting (C) forms of the enzyme measured with the optical fiber system. The Raman band at 330 cm^{-1} is observable only when the excitation wavelength is located at 800–850 nm and disappears upon reduction of the cytochrome *a*–Cu_A part. Therefore, this band is resonance-enhanced by the 830-nm absorption of the oxidized Cu_A. Figure

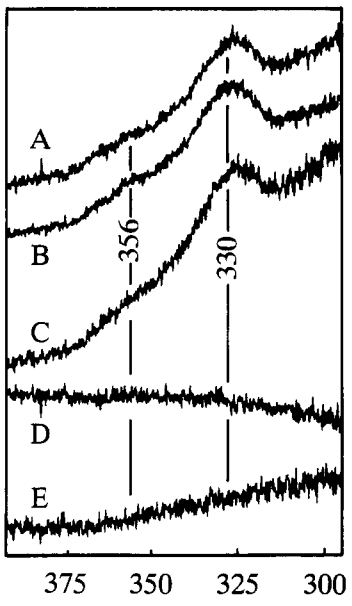

FIGURE 3: Resonance Raman spectra in the 390–290-cm^{-1} region of the resting (A), CO-bound half-reduced (B), and CN^--bound resting forms (C) of cytochrome *c* oxidase excited at 840 nm and their differences: (D) spectrum B – spectrum A; (E) spectrum C – spectrum A. The spectrometer (Ritsu MC-100DG) with the optical fiber device was used.

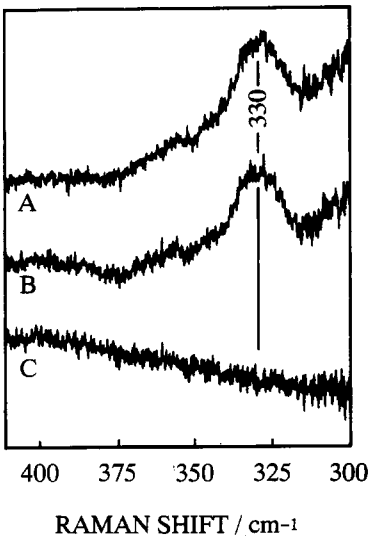

FIGURE 4: Resonance Raman spectra in the 410–300-cm^{-1} region of ferricytochrome *c*-bound cytochrome *c* oxidase (A) and free cytochrome *c* oxidase (B) excited at 840 nm, and their difference, spectrum B – spectrum A. The sample concentration was 65 μM for cytochrome *c* oxidase and 130 μM for cytochrome *c* in 5 mM sodium phosphate buffer.

3 also contains two difference spectra, that is, trace D = spectrum B – spectrum A and trace E = spectrum C – spectrum A. The two difference spectra exhibit no features at 330 cm^{-1}, indicating that the position and shape of this band remain unaltered upon binding of CO and CN^- to cytochrome a_3 as well as upon reduction of the catalytic site.

Figure 4 shows the 830-nm excited Raman spectra of the ferric cytochrome *c*-bound resting (A) and the free resting cytochrome *c* oxidases (B) and their difference (C). In order to make the complex formation of the resting enzyme with cytochrome *c* unquestionable, the buffer concentration was kept at 5 mM and the enzyme concentration was lowered to 65 μM. Complex formation under these conditions was

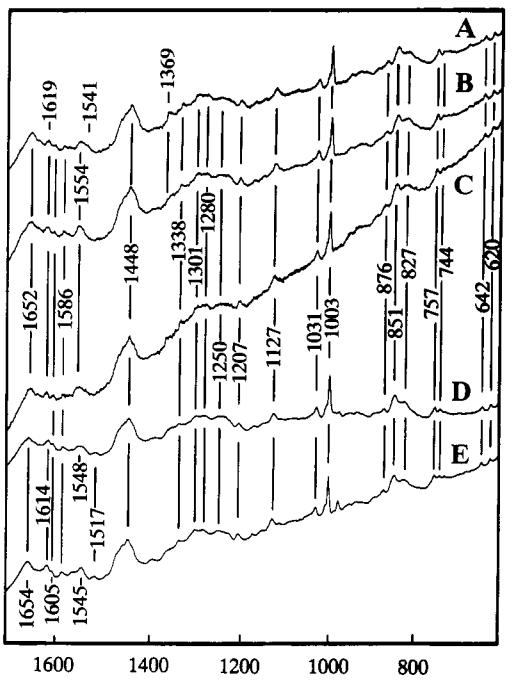

FIGURE 5: Raman spectra in the 1700–600-cm^{-1} region of cytochrome *c* oxidase derivatives excited at 828 nm: (A) resting form (a^{III}, Cu_A^{II}, a_3^{III}, Cu_B^{II}); (B) CN^--bound resting form (a^{III}, Cu_A^{II}, $a_3^{III}CN$, Cu_B^{II}); (C) CO-bound half-reduced form (a^{III}, Cu_A^{II}, $a_3^{II}CO$, Cu_B^{I}), (D) CN^--bound half-reduced form (a^{II}, Cu_A^{I}, $a_3^{III}CN$, Cu_B^{II}); and (E) CO-bound fully reduced form (a^{II}, Cu_A^{I}, $a_3^{II}CO$, Cu_B^{I}).

confirmed by Shinzawa-Itoh and Yoshikawa (unpublished results). Nonetheless, the difference spectrum exhibited no differential feature around 330 cm^{-1} as shown by trace C in Figure 4. This result indicates that the structure of Cu_A site is quite independent from binding of cytochrome *c* to cytochrome *c* oxidase.

Figure 5 shows the 828-nm excited Raman spectra in the 600–1700-cm^{-1} region of the resting (A), CN^--bound resting (B), CO-bound half-reduced (C), CN^--bound half-reduced (D), and CO-bound fully reduced (E) forms of cytochrome *c* oxidase. Most of Raman bands in these spectra are nonresonant protein modes including amide I (1652–1655 cm^{-1}), amide III (1250–1300 cm^{-1}), CH_2 scissoring (1448 cm^{-1}), skeletal C–N stretching (1127 cm^{-1}), and side chain vibrations of aromatic amino acid residues such as Phe (620, 1003, and 1031 cm^{-1}), Trp (757, 876, 1207, and 1338 cm^{-1}), and Tyr (642, 827, 851, and 1207 cm^{-1}). These bands appear to be irrelevant to the redox state of the metal centers. The high intensity of the CH_2 band is partly due to the presence of about 50 molecules of BL-8SY [CH_3–$(CH_2)_{11}$–O–$(CH_2$–CH_2–O)$_8$H] per monomer of the enzyme. There are also a few redox state dependent bands, which include the bands at 1619, 1554, and 1369 cm^{-1} for the resting form and those at 1614, 1545, and 1517 cm^{-1} for the CO-bound fully reduced forms. The intensities of the redox state dependent bands were relatively enhanced upon excitation at 735 nm (not shown), indicating that these bands are not in resonance with the 830-nm band and accordingly are assigned to heme modes. In fact, the observed frequencies for the CO-bound fully reduced form coincide with those obtained by the α-band excited resonance Raman spectra (Bocian et al., 1979; Callahan & Babcock, 1983; Centeno & Babcock, 1991).

Figure 6 shows the 830-nm excited RR spectra of the resting enzyme in H_2O (A) and D_2O (B). The Raman band at 1653 cm^{-1} exhibits no intensity reduction in D_2O and therefore

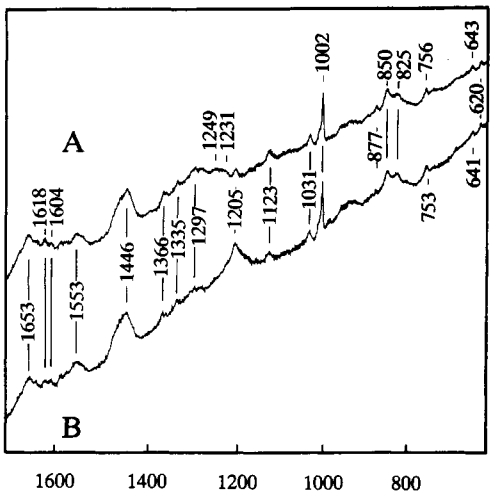

FIGURE 6: Raman spectra in the 1700–600-cm^{-1} region of the resting cytochrome *c* oxidase in H_2O (A) and D_2O (B) excited at 830 nm.

should be assigned to amide I but not to the bending vibration of solvent water, although the bending mode of D_2O gives a strong band at 1205 cm^{-1}. It is noted that the bands at 1653 and 1553 cm^{-1} show no frequency shifts in D_2O. The broad feature around 1300 cm^{-1} in spectrum A is weakened in spectrum B. The 877-cm^{-1} band of Trp residues in spectrum A is weak but seems to be much weaker in spectrum B, and the 756-cm^{-1} band of Trp is shifted to a lower frequency in D_2O, while the 850/825 cm^{-1} doublet of Tyr remained unchanged in D_2O.

DISCUSSION

Assignments of the 330-cm^{-1} Band. Previously, we assigned the broad and intense band at 330 cm^{-1} to a resonance-enhanced Raman band associated with the Cu_A center, since the appearance of this band was exactly parallel with the presence of the absorption band at 830 nm. This assignment is supported by recent observation of the corresponding RR band for a bacterial cytochrome oxidase, cytochrome aco_3 (*Bacillus* YN-2000), having no cytochrome a_3 (Yumoto et al., unpublished results). The 330-cm^{-1} Raman band of cytochrome c oxidase was composed of three components, each of which shows a frequency shift, an intensity change or no change in D_2O. We tentatively assigned the deuteration-insensitive strong band at 330 cm^{-1} to Cu–Cys stretching and the deuteration-sensitive weak band at 356 cm^{-1} (shoulder) to Cu–N(His) stretching vibrations (Takahashi et al., 1991).

The broad feature and relatively low frequency of these Cu–ligand stretching RR bands of cytochrome *c* oxidase are distinct from those of blue copper proteins, which give several sharp peaks between 350 and 450 cm^{-1}. Among RR spectroscopically characterized copper proteins, two are inferred to serve as a possible model of Cu_A. One is for a case of a mononuclear center, that is, a Cu-substituted liver alcohol dehydrogenase under the presence of excess imidazole, which has a copper ion coordinated by two Cys and one His residues and one external imidazole (Maret et al., 1986), and the other is for a binuclear copper, that is, center A of nitrous oxide reductase with a mixed valence binuclear copper center (Kroneck et al., 1988, 1990; Dooley et al., 1987). Unfortunately, both give similar RR spectra, and they are close to that of cytochrome *c* oxidase regarding the absolute frequency and bandwidth. Therefore, we cannot diagnose from the present RR spectra whether the Cu_A center is a mononuclear or binuclear center. Isotope substitution of amino acid residues and Cu_A are very desirable.

Interactions between Cu_A Center and Other Metal Centers. The difference spectra shown in Figures 3 and 4 exhibited no differential pattern. For a Lorentzian band with the intensity of I_0 and the half-height full-width of Γ, the valley to peak height (ΔI) in the difference spectrum expected for a frequency shift of β is $\Delta I = (3\sqrt{3}/3)I_0\beta/\Gamma$ (Kamogawa & Kitagawa, 1989). The smallest frequency shift detectable in these difference calculations would be in the case that ΔI is equal to a noise level, and it is estimated to be 1.7 cm^{-1} for spectra shown in Figures 3 and 4. Therefore, the frequency shifts of the Cu_A–ligand bands upon binding of the ligand to the oxygen reduction site or binding of cytochrome *c* to the enzyme should be less than 1.7 cm^{-1}.

Woodruff et al. (1988) deduced that the Cu–S stretching band of blue copper proteins exhibited a frequency shift by 10 cm^{-1} for the change of its bond length by 0.02 Å. Sanders-Loehr and co-workers (Han et al., 1991) compared the RR spectral patterns of 11 mononuclear copper proteins and pointed out the sensitivity of the Cu–S stretching Raman intensity to the microenvironments around the Cu–S bond. Considering these features, we are tempted to conclude that the Cu_A center of cytochrome *c* oxidase is placed in circumstances quite independent from other parts of the enzyme.

Wikstrom (1989) demonstrated that application of a membrane potential to the resting enzyme present in the membrane causes partial reversal of oxygen reduction. This implied the presence of some interaction between the proton pumping site and oxygen reduction site. If the Cu_A site serves as the proton pumping gate as proposed by Chan and Li (1990), the ligand binding or redox change at the oxygen binding site would be accompanied with some conformational changes at the Cu_A site. However, the results shown in Figure 3 suggest independence of the Cu_A conformation from the changes of redox or ligation states of the cytochrome a_3–Cu_B center. Blair et al. (1983) reported that the 830-nm absorption is not affected by the ligand binding and redox changes of the cytochrome a_3–Cu_B center. Furthermore, a bacterial quinol oxidase (cytochrome *o*), which is homologous to mitochondrial cytochrome *c* oxidase, pumps protons but lacks Cu_A (Babcock & Wikstrom, 1992). The present observation as well as the cited facts are incompatible with the Cu_A gate hypothesis.

Cytochrome *c* oxidase and cytochrome *c* are known to form a strong complex under low ionic strength. The complex formation is based on electrostatic interactions between positive charges of basic residues of cytochrome *c* and negative charges of acidic residues of cytochrome *c* oxidase. Chemical cross-linking experiments (Bisson et al., 1978, 1980) demonstrated that a cytochrome *c* binding site existed in subunit II, and accordingly Holm et al. (1987) proposed that cytochrome *c* resides in close proximity to Cu_A in the complex. On the other hand, studies with electron absorption (Michel & Bosshard, 1984) and resonance Raman spectroscopy (Hildebrandt et al., 1990) suggested direct interactions between cytochrome *c* and cytochrome *a*.

The results shown in Figure 4 indicate independence of the 330-cm^{-1} band from the complex formation between ferric cytochrome *c* and the resting enzyme. If the conjecture by Holm et al. (1987) were correct, appreciable changes would be expected to occur in the vibrational spectrum of the Cu_A center on the basis of the sensitivity of the Cu–S stretching RR bands to the protein structures revealed for blue copper proteins (Han et al., 1991). The present observation rather

suggests that the binding site of cytochrome *c* is not close to the Cu_A center. This is compatible with the recent kinetic study (Pan et al., 1991b) which showed that the rate of the complex formation of cytochrome *c* oxidase with cytochrome *c* was not influenced by depletion of Cu_A from the enzyme.

Nonresonant Protein Modes. The far-red excitation of Raman scattering from cytochrome *c* oxidase brought about nonresonant protein vibrations besides preresonant heme modes. The Raman spectra shown in Figures 5 and 6 contain such protein bands, which have not been observed with visible excitation, but the appearance of all the Raman bands of the huge enzyme do not allow a detailed discussion of structural changes of a protein skeleton or of aromatic side chains of the enzyme upon the redox and ligation changes. Nonetheless, the following information can be drawn from the observed spectra.

(1) All the observed protein modes exhibited neither frequency shifts nor intensity changes upon the redox changes of the metal centers. This is in consequence with the results from solid-state NMR studies which detected no difference in protein signals between the resting and fully reduced states (Tuzi et al., 1992). Presumably, the redox-dependent conformational change of the protein is localized, and its range is considerably small compared with a large molecular size of this enzyme.

(2) The 756- and 877-cm^{-1} bands of Trp displayed appreciable changes upon deuteration. The former arises from a breathing-like vibration of the indole ring and gives rise to a small downshift (5 cm^{-1}) in D_2O for an aqueous Trp solution, while the latter involves motion of the N_1H bond of the indole ring and shows a large downshift in D_2O (20 cm^{-1}) for an aqueous Trp solution (Takeuchi & Harada, 1986). The intensity of the 877-cm^{-1} band is known to be sensitive to microenvironments around Trp residues (Kitagawa et al., 1979), and the sensitivity is attributed to hydrogen bonding of N_1H with surrounding residues (Takeuchi & Harada, 1986). It is important to note that the Trp residues responsible for Raman bands are deuterated in D_2O and therefore are in contact with solvent water, while amide I band at 1653 cm^{-1} exhibited no deuteration shift.

Amide I is usually located at 1685, 1674–1665, 1668–1662, and 1655–1645 cm^{-1} for a non-hydrogen-bonded nonregular structure, antiparallel β-sheet, hydrogen-bonded nonregular structure, and α-helix, respectively (Frushour & Koenig, 1975; Harada & Takeuchi, 1986). The observed amide I frequency of cytochrome *c* oxidase (1653 cm^{-1}) is categorized to that of α-helix. Since amide I of α-helix usually undergoes a downshift by 12–15 cm^{-1} upon deuteration (Harada & Takeuchi, 1986), the fact that there is no apparent deuteration shift for amide I of cytochrome *c* oxidase indicates that penetration of D_2O molecules into an inner part of the protein is extremely slow.

(3) Amide III usually appears at 1300–1265, 1253–1243, and 1240–1229 cm^{-1} for α-helix, hydrogen-bonded nonregular structure, and antiparallel β-sheet, respectively (Harada & Takeuchi, 1986). The weak amide III intensity in the frequency region between 1235 and 1240 cm^{-1} and the stronger intensity around 1300 cm^{-1} suggest a small population of the β-sheet and a larger population of α-helix, respectively (Lippert et al., 1976). The large content of α-helix is consistent with the results from circular dichroism (Urry et al., 1967), solid-state NMR (Tuzi et al., 1992), and the primary structure (Frey et al., 1985) as well as the amide I Raman band described above.

Lippert et al. (1976) proposed a method for estimating the secondary structure distribution of proteins from Raman spectra. This method refers to relative intensities of amide III in H_2O and amide I in D_2O, and its application to the present spectra gave the ratio of α-helix:β-sheet:nonregular structure = 0.37:0.20:0.43. Although the α-helix content is in agreement with that of the CD study (Urry et al., 1967), this may be underestimated since it did not undergo a deuteration shift, and the content of nonregular structure may be overestimated. Recent results from solid-state NMR estimate the α-helix content to be as high as 0.6 (Tuzi et al., 1992).

In conclusion, our new system with a CCD detector enabled us to observe the far-red excited Raman spectra of cytochrome *c* oxidase. This yielded the Cu_A-associated resonance Raman bands and some protein vibrations, which cannot be detected in the visible RR spectra of heme proteins. Unexpectedly, the Cu_A center was found to be quite independent from other redox centers and also from binding of cytochrome *c*. Some Trp bands underwent the H/D exchange in D_2O but the skeletal amide groups, which are mainly in the α-helix, did not.

REFERENCES

Babcock, G. T. (1988) in *Biological Applications of Raman Spectroscopy* (Spiro, T. G., Ed.) Vol. 3, pp 293–346, Wiley & Sons, New York.

Babcock, G. T., & Wikstrom, M. (1992) *Nature (London) 356*, 301–309.

Beinert, H., & Palmer, G. (1964) *J. Biol. Chem. 239*, 1221–1227.

Beinert, H., Griffiths, D. E., Wharton, D. C., & Sands, R. H. (1962) *J. Biol. Chem. 237*, 2337–2346.

Beinert, H., Shaw, R. W., Hansen, R. E., & Hartzell, C. R. (1980) *Biochim. Biophys. Acta 591*, 458–470.

Bisson, R., Azzi, A., Gutweniger, H., Colonna, R., Montecucco, C., & Zanotti, A. (1978) *J. Biol. Chem. 253*, 1874–1880.

Bisson, R., Jacobs, B., & Capaldi, R. A. (1980) *Biochemistry 19*, 4173–4178.

Blair, D. F., Martin, C. T., Gelles, J., Wang, H., Brudvig, G. W., Stevens, T. H., & Chan, S. I. (1983) *Chem. Scr. 21*, 43–53.

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

Buse, G., Meinecke, L., & Bruch, B. (1985) *J. Inorg. Biochem. 23*, 149–153.

Callahan, P. M., & Babcock, G. T. (1983) *Biochemistry 22*, 452–461.

Centeno, J. A., & Babcock, G. T. (1991) *J. Raman Spectrosc. 22*, 111–117.

Chan, S. I., & Li, P. M. (1990) *Biochemistry 29*, 1–12.

Deckert, V., & Kiefer, W. (1992) *Appl. Spectrosc. 46*, 322–328.

Dooley, D. M., Moog, R. S., & Zumft, W. G. (1987) *J. Am. Chem. Soc. 109*, 6730–6735.

Fee, J. A., Antholine, W. E., Fan, C., Gurbiel, R. J., Werst, M., Surerus, K., & Hoffman, B. M. (1992) *J. Inorg. Biochem. 47*, 12.

Frey, T. G., Kuhn, L. A., Leigh, J. S., Jr., Costello, M. J., & Chan, S. H. P. (1985) *J. Inorg. Biochem. 23*, 155–162.

Frushour, B. G., & Koenig, J. L. (1975) *Adv. Infrared Raman Spectrosc. 1*, 35–97.

Gelles, J., Blair, D. F., & Chan, S. I. (1986) *Biochim. Biophys. Acta 853*, 205–236.

Griffiths, D. E., & Wharton, D. C. (1961) *J. Biol. Chem. 236*, 1850–1856.

Han, J., Adman, E. T., Beppu, T., Codd, R., Freeman, H. C., Huq, L., Loehr, T. M., & Sanders-Loehr, J. (1991) *Biochemistry 30*, 10904–10913.

Han, S., Ching, Y., & Rousseau, D. L. (1990a) *Proc. Natl. Acad. Sci. U.S.A. 87*, 2491–2495.

Han, S., Ching, Y., & Rousseau, D. L. (1990b) *Nature (London) 348*, 89–90.

Harada, I., & Takeuchi, H. (1986) in *Spectroscopy of Biological Molecules* (Hester, R. E., & Clark, R. J. K., Eds.) pp 113–175, Wiley & Sons, London.
Harris, T. D., Schnoes, M. L., & Seibles, L. (1989) *Anal. Chem. 61*, 994–998.
Hildebrandt, P., Heimburg, T., Marsh, D., & Powell, G. L. (1990) *Biochemistry 29*, 1661–1668.
Holm, L., Saraste, M., & Wikstrom, M. (1987) *EMBO J. 6*, 2819–2823.
Hu, V. W., Chan, S. I., & Brown, G. S. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 3821–3825.
Kamogawa, K., & Kitagawa, T. (1989) in *Vibrational Spectra and Structure* (Bist, H. D., Durig, J. R., & Sullivan, J. F., Eds.) Vol. *17B*, pp 447–472, Elsevier, Amsterdam.
Kamogawa, K., & Kitagawa, T. (1990) *J. Phys. Chem. 94*, 3916–3921.
Kitagawa, T., Azuma, T., & Hamaguchi, K. (1979) *Biopolymers 18*, 451–465.
Knoll, P., Singer, R., & Kiefer, W. (1990) *Appl. Spectrosc. 44*, 776–782.
Kroneck, P. M. H., Antholine, W. A., Riester, J., & Zumft, W. G. (1988) *FEBS Lett. 242*, 70–74.
Kroneck, P. M. H., Antholine, W. E., Kastrau, D. H. W., Buse, G., Steffens, G. C. M., & Zumft, W. G. (1990) *FEBS Lett. 268*, 274–276.
Li, P. M., Gelles, J., Chan, S. I., Sullivan, R. J., & Scott, R. A. (1987) *Biochemistry 26*, 2091–2095.
Lippert, J. L., Tyminski, D., & Desmeules, P. J. (1976) *J. Am. Chem. Soc. 98*, 7075–7080.
Maret, W., Shiemke, A. K., Wheeler, W. D., Loehr, T. M., & Loehr, J. S. (1986) *J. Am. Chem. Soc. 108*, 6351–6359.
Martin, C. T., Scholes, C. P., & Chan, S. I. (1988) *J. Biol. Chem. 263*, 8420–8429.
Michel, B., & Bosshard, H. R. (1984) *J. Biol. Chem. 259*, 10085–10091.
Ogura, T., Takahashi, S., Shinzawa-Itoh, K., Yoshikawa, S., & Kitagawa, T. (1990a) *J. Am. Chem. Soc. 112*, 5630–5631.
Ogura, T., Takahashi, S., Shinzawa-Itoh, K., Yoshikawa, S., & Kitagawa, T. (1990b) *J. Biol. Chem. 265*, 14721–14723.
Ogura, T., Takahashi, S., Shinzawa-Itoh, K., Yoshikawa, S., & Kitagawa, T. (1991) *Bull. Chem. Soc. Jpn. 64*, 2901–2907.
Pan, L. P., Li, Z., Larsen, R., & Chan, S. I. (1991a) *J. Biol. Chem. 266*, 1367–1370.
Pan, L. P., Hazzard, J. T., Lin, J., Tollin, G., & Chan, S. I. (1991b) *J. Am. Chem. Soc. 113*, 5908–5910.
Peisach, J., & Blumberg, W. E. (1974) *Arch. Biochem. Biophys. 165*, 691–708.
Pemberton, J. E., & Sobocinski, R. L., (1989) *J. Am. Chem. Soc. 111*, 432–434.
Pemberton, J. E., Sobocinski, R. L., & Sims, G. R. (1990) *Appl. Spectrosc. 44*, 328–330.
Powers, L., Blumberg, W. E., Chance, B., Barlow, C. H., Leigh, J. S., Jr., Smith, J., Yonetani, T., Vik, S., & Peisach, J. (1979) *Biochim. Biophys. Acta 546*, 520–538.
Powers, L., Chance, B., Ching, Y., & Angiolillo, P. (1981) *Biophys. J. 34*, 465–498.
Saraste, M. (1990) *Q. Rev. Biophys. 23*, 331–366.
Scott, R. A., Schwartz, J. R., & Cramer, S. P. (1986) *Biochemistry 25*, 5546–5555.
Stevens, T. H., Martin, C. T., Wang, H., Brudvig, G. W., Scholes, C. P., & Chan, S. I. (1982) *J. Biol. Chem. 257*, 12106–12113.
Takahashi, S., Ogura, T., Shinzawa-Itoh, K., Yoshikawa, S., & Kitagawa, T. (1991) *J. Am. Chem. Soc. 113*, 9400–9401.
Takeuchi, H., & Harada, I. (1986) *Spectrochim. Acta 42A*, 1069–1078.
Tuzi, S., Shinzawa-Itoh, K., Erata, T., Naito, A., Yoshikawa, S., & Saito, H. (1992) *Eur. J. Biochem. 208*, 713–720.
Urry, D. W., Wainio, W. W., & Grebner, D. (1967) *Biochem. Biophys. Res. Commun. 27*, 625–631.
Varotsis, C., & Babcock, G. T. (1990) *Biochemistry 29*, 7357–7362.
Varotsis, C., Woodruff, W. H., & Babcock, G. T. (1989) *J. Am. Chem. Soc. 111*, 6439–6440 [correction (1990) *J. Am. Chem. Soc. 112*, 1297].
Wang, Y., & McCreery, R. L. (1989) *Anal. Chem. 61*, 2647–2651.
Wikstrom, M. (1977) *Nature (London) 266*, 271–273.
Wikstrom, M. (1989) *Nature (London) 338*, 776–778.
Wikstrom, M., Krab, K. K., & Saraste, M. (1981) *Cytochrome Oxidase: A Synthesis*, Academic Press, New York.
Woodruff, W. H., Dyer, R. B., & Schoonover, J. R. (1988) in *Biological Applications of Raman Spectroscopy* (Spiro, T. G., Ed.) Vol. 3, pp 413–438, Wiley & Sons, New York.
Yoshikawa, S., Choc, M. G., O'Toole, M. C., & Caughey, W. S. (1977) *J. Biol. Chem. 252*, 5498–5508.
Yoshikawa, S., Tera, T., Takahashi, Y., Tsukihara, T., & Caughey, W. S. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 1354–1358.
Yu, N. T., Nie, S., & Chang, C. K. (1990) *12th International Conference on Raman Spectroscopy* (Durig et al., Eds.) pp 902–903, Wiley, Chichester.
Yumoto, I., Takahashi, S., Kitagawa, T., Fukumori, Y., & Yamanaka, T. (1993) *J. Biochem. (Tokyo)* (submitted).