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Supplementary Material Available: A listing of synthetic, cleavage, and ligation methods as well as the sequences flanking the ligation sites in pAMP6-9 (3 pages). Ordering information is given on any current masthead page.

Observation of the Cu_A -Ligand Stretching Resonance Raman Band for Cytochrome *c* Oxidase[†]

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Cytochrome *c* oxidase, the terminal enzyme of the respiratory chain of aerobic organisms, contains two heme A groups and two copper atoms as redox-active metal centers.¹ While one copper atom (Cu_B) in the resting enzyme is EPR silent as a result of antiferromagnetic coupling with one of the heme groups ($\text{Cyt } a_3$), the other copper atom (Cu_A) displays an atypical EPR signal in the sense that no hyperfine splittings are clearly resolved and the *g* values are quite low.² Although the reduced hyperfine coupling is reminiscent of type I copper (blue copper) proteins, the systematic investigation of EPR spectra of Cu-containing proteins³ displayed an appreciable difference between Cu_A and blue copper centers. This difference was also manifested by X-ray absorption data,^{4,5a,6a} which indicated that upon reduction of the protein the Cu absorption edge changed from the cupric to cuprous state for blue copper centers but not for Cu_A . The EPR and ENDOR studies on isotope-substituted yeast cytochrome *c* oxidase⁷ established coordination of one cysteine (Cys) and one histidine (His), at least, to Cu_A while EXAFS studies^{5b,6} suggested coordination of two N (or O) and two S (or Cl) atoms to Cu_A .

Resonance Raman (RR) spectroscopy has been used extensively to characterize the blue copper proteins.⁸⁻¹¹ However, excitation

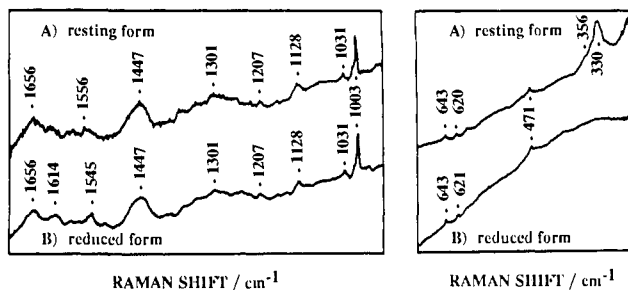


Figure 1. Resonance Raman spectra of the resting form (A) and the fully reduced form (B) of bovine cytochrome *c* oxidase excited at 840 nm. The enzyme concentration was 500 μM (in terms of Cu_A) in 10 mM sodium phosphate buffer, pH 7.4. The sample was contained in a spinning cell (1800 rpm) at about 5 °C. The reduced form was prepared by adding a small amount of dithionite anaerobically to the resting enzyme.

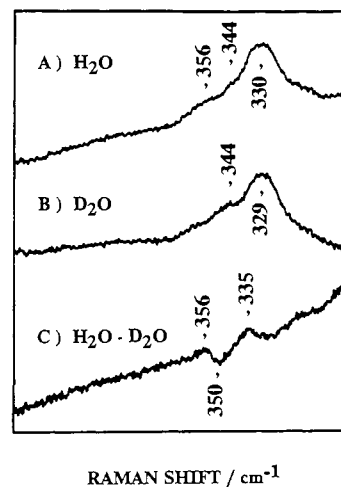


Figure 2. Resonance Raman spectra of the resting form of bovine cytochrome *c* oxidase in H_2O (A) and D_2O (B) excited at 845 nm and their difference spectrum ($C = A - B$). Experimental conditions are the same as those in Figure 1.

of Raman scattering from cytochrome *c* oxidase around 590–610 nm has brought about only heme modes¹² but no Cu_A -associated bands. Although Cu_A of the resting enzyme gives a weak and broad absorption around 830 nm,¹³ observation of Raman spectra in resonance with this absorption has not been successful on account of the low sensitivity of a photomultiplier in a far-red region. Therefore, we constructed a new Raman system using a CCD (charge-coupled device) detector and applied it successfully to observe the Cu_A -ligand RR bands of cytochrome *c* oxidase.

The excitation light was obtained from a Ti-sapphire laser (SP, 3900) pumped by an Ar^+ ion laser (SP, 2045). A CCD (Astromed CCD3200) detector was attached to a single monochromator (JASCO CT-50) which employed a 750-nm blazed grating with 1200 grooves/mm. In order to circumvent the charge-trap problem,¹⁴ the short axis of the CCD tip was turned to the direction

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of light dispersion and software that allows scanning of the spectrometer was prepared. Details of this Raman system will be reported elsewhere.¹⁵ Purification procedures of beef heart cytochrome *c* oxidase has been described previously,^{16a} but the final step of purification adopted was crystallization,^{16b} which removed the fluorescence background in Raman scattering. A deuterated sample was prepared by repeated (three times) filtration through a membrane filter (Amicon YM-5) and dilution by 10-fold (v/v) D₂O buffer.

Figure 1 shows the 840-nm-excited RR spectra of the resting enzyme (A) and its fully reduced form (B). They are distinct from the 600-nm-excited RR spectra reported by others¹² and contain protein modes such as amide I (1656 cm⁻¹), CH₂ scissoring (1447 cm⁻¹), and the side-chain modes of Phe residues (1003 cm⁻¹). A heme mode also appears at 1556 cm⁻¹ for the resting form and at 1545 cm⁻¹ for the reduced form. This band became stronger when the excitation wavelength was shifted to 735 nm. Although amide II is expected in this frequency region, it is generally weak in Raman scattering and, moreover, the 1556-cm⁻¹ band does not disappear in D₂O contrary to the property of amide II. Besides these protein and heme modes, a broad and intense band is observed at 330 cm⁻¹ for the resting form. This band disappears in the reduced form while the protein modes show little change upon the redox change. Although a fluorescence band appears in the reduced form in the low-frequency region and the background becomes higher, a weak protein band at 471 cm⁻¹ is identified similarly to the oxidized form. Therefore, if the 330-cm⁻¹ band were present in the reduced form, it could be observed under this background level. The 330-cm⁻¹ band is observable with the mixed-valence enzyme in which cytochrome *a* and Cu_A are oxidized, but not with one with the opposite redox combination. Therefore, it is reasonable to assign the band at 330 cm⁻¹ to the moiety associated with the 830-nm absorption, that is, the Cu_A^{II} center.

Figure 2 compares the RR spectra in the 500–300-cm⁻¹ region of the resting enzyme in H₂O (A) and D₂O (B). Although this was excited at 845 nm to examine another excitation wavelength, a spectrum similar to Figure 1A, obtained with 840-nm excitation, was observed. The difference spectrum (trace C), which shows one derivative pattern around 353 cm⁻¹ and one positive peak at 335 cm⁻¹, indicates the presence of at least three components in the broad band; the 356, 335, and 330-cm⁻¹ bands show a frequency shift, an intensity change, and no change in D₂O, respectively.

The visible absorption of blue copper proteins around 600 nm is considered to arise from a sulfur (Cys) to Cu^{II} charge-transfer transition,¹⁷ and accordingly, RR spectra obtained with 600-nm excitation can be attributed mainly to the Cu^{II}-S stretching mode.^{8b,10,11} The 830-nm absorption of Cu_A is also considered to arise from a similar transition of the Cu_A center,¹³ and hence we tentatively assign the most intense and deuteration insensitive band at 330 cm⁻¹ to a mode mainly associated with a Cu^{II}-S(Cys) stretching vibration of Cu_A and the weak and deuteration sensitive band at 356 cm⁻¹ to the Cu-N(His) stretching vibration. The Cu-S stretching bands of blue copper proteins with a single cysteine ligand exhibit complicated patterns of D₂O shifts,^{8b,11b} which are explained in terms of vibrational mixing of the Cu-S with Cu-N stretching modes. Normal coordinate calculations of the Cu center of blue copper proteins indicate significant mixing of the Cu^{II}-S with the Cu^{II}-N stretching modes.^{8b,18} Therefore, it is highly likely that Cu_A-associated RR bands are mixed modes of the Cu-N and Cu-S stretching vibrations. Recently, coordinations of Cys-196, Cys-200, His-204, and His-161¹⁹ or Met-

207²⁰ of subunit II to Cu_A have been suggested.

The frequency of the Cu-S stretching band of Cu_A is distinctly low compared with the corresponding frequencies of blue copper proteins (350–450 cm⁻¹). This might be explained by the longer Cu-S bond as revealed by EXAFS.⁶ It is observed from the MCD, EXAFS, and sequence analysis²¹ that, among various copper proteins so far characterized, only nitrous oxide reductase has the Cu_A type copper,²¹ and in fact, the present RR spectrum bears close similarity to that of nitrous oxide reductase.²²

The function of Cu_A of cytochrome *c* oxidase is considered to be delivery of electrons, together with Cyt *a*, from cytochrome *c* to a Cyt *a*₃-Cu_B center,^{1,23} and possibly service as a gate of a proton pump.²⁴ However, the mechanism still remains to be experimentally clarified. The present technique combined with the flow apparatus for time-resolved measurements²⁵ is expected to provide a new means for studying the Cu_A site of cytochrome *c* oxidase.

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Photosensitized Decarboxylative Michael Addition through *N*-(Acyloxy)phthalimides via an Electron-Transfer Mechanism

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Although photosensitized electron-transfer reactions have recently attracted considerable attention in organic photochemistry, application of this methodology to organic synthesis has been limited to fewer examples. We have recently reported a method of photodecarboxylation using *N*-(acyloxy)phthalimides which can be readily obtained from carboxylic acids.¹ This method may be applicable to decarboxylative radical type Michael addition. Hitherto, an elegant decarboxylative radical addition to electron-deficient olefins has been reported by Barton and his co-workers.² However, in their method, the 2-pyridylthio group is always introduced to the α -position of the electron-deficient group because of the high affinity of alkyl radicals toward the sulfur atom of the thiocarbonyl group. In order to introduce hydrogen to this position (Michael addition), we have examined several redox systems using *N*-(acyloxy)phthalimides. We now report a new and efficient radical chain method for this conversion using the redox combination of Ru(bpy)₃Cl₂ and 1-benzyl-1,4-dihydro-nicotinamide (BNAH) in aqueous solvents with visible-light excitation.

Irradiation of a THF-water (7:3, 40 mL) solution of Ru(bpy)₃Cl₂ (ca. 10 mg), *N*-(acyloxy)phthalimide (**1a**, 0.58 mmol),

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