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A Comparison of the Resonance Raman Properties of the Fast and Slow Forms of Cytochrome Oxidase†

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ABSTRACT: Resonance Raman (RR) spectra of the "rapid" and "slow" forms (Baker et al., 1987) of resting cytochrome oxidase obtained with Soret excitation at 413.1 nm are reported. There are a number of conspicuous differences between the two forms in the high-frequency region of the RR spectrum which involve changes in Raman intensity arising from a blue shift in the Soret maximum of cytochrome a_3 upon conversion to the slow form. In the low-frequency region a peak present at 223 cm^{-1} in the rapid form shifts to 220 cm^{-1} in the slow form; this peak is assigned as the cytochrome a_3 Fe(III)–N(His-Im) stretch. The slow form of the enzyme possesses greater intensity in RR peaks near 1620 cm^{-1} which have been previously attributed by others to partial photoreduction of the enzyme. We have quantitated the amount of laser-induced photoreduction in these RR spectra by comparison with the spectra of mixed-valence derivatives of the enzyme and find that these 1620-cm^{-1} features are unreliable indicators of photoreduction. The spectra of the fast- and slow-reacting species in H_2O and D_2O have been compared. The fast-reacting form exhibits a 4-cm^{-1} shift, from 223 to 219 cm^{-1}, upon transferring to D_2O in a peak which we assign as the cytochrome a_3 Fe(III)–N(His-Im) stretch. There is a parallel shift in the feature at 1651 cm^{-1} due to the C=O stretch of the formyl group of cytochrome *a*. These deuterium shifts are not observed in the slow form. We suggest that the nature of the difference between the fast and slow forms of the enzyme is a global conformational change involving inter alia the axial histidine ligand of cytochrome a_3 and the cytochrome *a* environment. This conformational change is also responsible for the enhanced Raman intensity observed at 1620 cm^{-1} in the slow form. Some mechanisms whereby such a conformational change might affect the cytochrome a_3 Soret energy are discussed.

Cytochrome oxidase catalyzes the four-electron reduction of dioxygen to water and accepts the required electrons from the cellular respiratory chain via cytochrome *c*. The enzyme also conserves the energy of the cytochrome *c*–O_2 redox couple (~500 mV) by consuming four protons in the mitochondrial matrix space and by active pumping of additional protons to the cytosolic space. This creates a transmembrane ion gradient which is used to drive the synthesis of adenosine 5′-triphosphate (ATP) (Malmström, 1979; Wikström et al., 1981).

Electron paramagnetic resonance (EPR), magnetic circular dichroism (MCD), and magnetic susceptibility measurements have provided information on the electronic structure of the

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paramagnetic ferric, ferrous, and cupric species in the enzyme (Wikström et al., 1981); however, these techniques are relatively insensitive to the detailed stereochemistry and bonding of the hemes and their substituents. On the other hand, resonance Raman (RR) spectroscopy is sensitive to the structure of the prosthetic groups of heme proteins. Cytochrome oxidase has been extensively studied by RR spectroscopy, and peaks which are unique to the individual hemes and indicative of the oxidation state, spin state, and coordination characteristics of the two cytochrome centers have been identified [reviewed in Babcock (1988)]. The separation of the cytochrome a and a_3 RR spectra is possible because of relative enhancement of one cytochrome over the other due to differences in the wavelength of the Soret maxima in different derivatives. Hence, the RR phenomenon is a powerful probe for identifying stereochemical and electronic properties of one cytochrome or the other.

Even so, because of the extreme chemical and spectroscopic complexity of this enzyme, there have been significant problems in methodology and interpretation associated with these RR studies. These problems have sometimes led to apparently conflicting results but more often to conflicting interpretations, from different laboratories. A case in point is the substantial disagreement which exists concerning the extent of, and the appropriate diagnostics for, laser-induced photoreduction in RR studies of oxidized derivatives of this enzyme (Babcock et al., 1981; Ogura et al., 1985; Woodruff et al., 1981, 1982b).

The lability of resting cytochrome oxidase toward photoreduction upon irradiation with Soret-wavelength laser light was first noted by Adar and Yonetani (1978) as an increase in the intensity of the Raman line at 1359 cm^{-1}, the oxidation-state frequency typical of ferrous heme a. This observation has been confirmed by other workers in the field (Adar & Erecinska, 1979; Bocian et al., 1981; Babcock et al., 1981; Woodruff et al., 1981; Ogura et al., 1985). Photoreduction has also been correlated with the appearance of features near 1620 cm^{-1}, which can be observed in the absence of significant changes in intensity in the 1359-cm^{-1} region (Babcock et al., 1981). The correlation of increases of intensity in the 1620-cm^{-1} region with photoreduction has been championed by Kitagawa (Ogura et al., 1985). This correlation is not universally accepted, however, and we have attributed the appearance of these features to the effects of detergents or other agents producing a change in protein conformation (Woodruff et al., 1981, 1982b).

The present RR study of cytochrome oxidase was motivated by the observation that, depending upon the preparative protocol, resting cytochrome oxidase has variable spectral and kinetic properties. For example, enzyme prepared according to Volpe-Caughey (1974) [as modified by Yoshikawa et al. (1977)], Yonetani (1961), and Yu et al. (1974) results in preparations possessing a large $g' = 12$ EPR signal. In the original Hartzell–Beinert (HB) preparations (1974) this signal is variable, while in enzyme prepared with a recent modification of the HB procedure it is absent (Baker et al., 1987). Furthermore, the cyanide-binding kinetics of resting cytochrome oxidase are preparation-dependent (Baker et al., 1987). The modified HB enzyme always shows a single, fast exponential ($k \sim 0.06\ s^{-1}$)[1] reaction with cyanide, while most other preparations are dominated by a much slower ($k \sim 0.0002\ s^{-1}$) phase in the kinetics. For this reason we make the operational distinction between "fast" and "slow" enzyme.

The position of the net observed Soret maximum (i.e., $a + a_3$) also varies in different preparations. The conversion of fast enzyme to the slow form leads to a blue shift in the Soret absorbance, with slow enzyme typically demonstrating an absorption maximum at 417 nm compared to 424–425 nm for the fast enzyme. However, the MCD spectra in this region are essentially unchanged, indicating that the absorbance changes are not due to cytochrome a. There are small changes in the visible MCD, including a slight shift in the feature at about 650 nm (which is associated with high-spin cytochrome a_3) with no reduction in intensity.

The present study seeks to establish which RR features may be correlated with the position of the optical Soret peak and the MCD and EPR observations and, thereby, to infer any structural rationale for the spectroscopic and kinetic effects. The spectra of fast and slow enzyme are compared to each other and to the spectra of their deuteriated derivatives.

In the course of these studies, it was observed that the RR spectrum of slow, but not fast, enzyme exhibited the controversial features near 1620 cm^{-1} noted above. This observation offered the opportunity to determine which components of the RR spectra are due to photoreduction and which are not. Comparison of the RR spectra of fast and slow cytochrome oxidase with those of the two mixed-valence species ($a^{2+}a_3^{3+}$·CN and $a^{3+}a_3^{2+}$·CO) allowed us to establish valid RR criteria for, and to quantitate the extent of, photoreduction.

Materials and Methods

Enzyme Preparations. Solubilized beef heart cytochrome oxidase was prepared by a modified procedure of Hartzell and Beinert (Baker et al., 1987). The final precipitate was dissolved in 0.1 M NaH_2PO_4·NaOH containing 0.1% dodecyl maltoside or in 50 mM HEPES·KOH [HEPES = 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid] containing 0.1% dodecyl maltoside. The enzyme, herein referred to as fast enzyme, was maintained at pH 8.0 and had a λ_{max} = 424–425 nm, a single rapid reaction with cyanide ($k \sim 0.06\ s^{-1}$),[1] and no $g' = 12$ EPR signal. Slow enzyme was prepared by incubation of fast enzyme at pH 6.7 overnight on ice. This form of resting oxidase had a λ_{max} = 417 nm, a single slow reaction phase with cyanide ($k \sim 0.0002\ s^{-1}$), and a $g' = 12$ EPR signal whose amplitude was proportional to the amount of slow phase. The total heme a concentration for all samples was ~100 μM. The mixed-valence derivatives were prepared from the fast oxidase preparation; the cyanide derivative was prepared by the addition of a neutralized NaCN solution followed by a few crystals of dithionite. The final species was $a^{2+}a_3^{3+}$·CN, as verified by the absorption spectra and by RR criteria. The formate derivative was prepared by addition of 0.1 M formate to fast enzyme; it was subsquently converted to the mixed-valence derivative by addition of *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine (TMPD) and sodium ascorbate to final concentrations of 0.5 and 3 mM, respectively. The mixed-valence carbon monoxide derivative was prepared by anaerobic incubation with CO in the dark. Laser illumination under RR spectral acquisition conditions induced approximately 100% photolysis of the bound CO. The electron remained on the a_3 heme to give reduced cytochrome a_3 and oxidized cytochrome a. This was confirmed by noting the Raman frequency of the cytochrome a_3 formyl stretch which is located at 1676 cm^{-1} in the oxidized enzyme and at 1666 cm^{-1} when a_3 is reduced. Deuteriated samples were prepared at the desired pD with deuteriated buffer by the exchange method described elsewhere (Baker et al., 1987); approxi-

[1] The values for these rate constants differ from those published by others. We perform cyanide kinetics using high, saturating, levels of cyanide, and the reported rate is the limiting, first-order rate constant. Others [e.g., Naqui et al. (1984)] measure the kinetics at low cyanide concentrations and express the kinetics as a second-order rate.

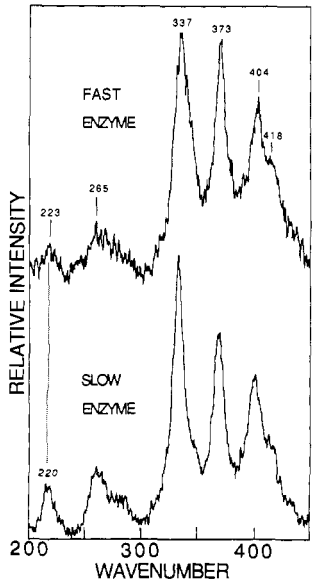

FIGURE 1: RR spectra of fast (top) and slow (bottom) resting cytochrome oxidase between 200 and 450 cm^{-1}. The 3-cm^{-1} shift of the 223-cm^{-1} peak is labeled. Laser wavelength for all reported RR spectra was 413.1 nm with between 35 and 45 mW of laser power incident on the sample.

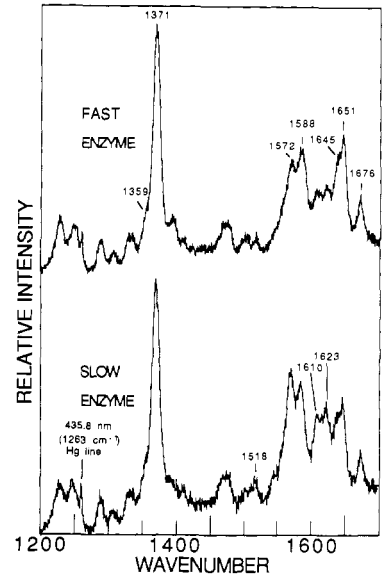

FIGURE 2: RR spectra of the high-frequency region (1200–1700 cm^{-1}) of fast (top) and slow (bottom) resting cytochrome oxidase with the major intensity changes noted.

mately 6-h incubation in D_2O was sufficient for fast enzyme whereas 24 h was utilized for the slow enzyme. All samples were frozen in liquid nitrogen and thawed just prior to the RR experiments.

Instrumentation. RR spectra were obtained on a SPEX 1403 spectrometer equipped with a cooled RCA 31034A photomultiplier tube, an ORTEC 9300 series photon counter, and a Nicolet 1180E Raman data system. Laser excitation was provided by the 413.1-nm line of a Spectra-Physics Kr^+ laser. Spectral scan rates were 1 cm^{-1} s^{-1}, and the spectral slit width was 4 cm^{-1} with laser power between 35 and 45 mW incident on the sample. Typical signal averaging utilized 9 scans in the high-frequency region and as many as 25 scans in the low-frequency region. Samples for the RR experiments were contained in a sealed 1-cm path-length spectrophotometer cuvette thermostated between 8 and 10 °C. Raman scattering was observed in a 135° backscattering geometry in a plane perpendicular to the polarization of the laser beam.

To minimize photoreduction, our sampling technique consisted of approximately 1.0 mL of oxidase in the sealed 1-cm cuvette, in contact on all sides with a cell holder which was cooled to 8 °C. A small opening in the cell holder allowed for the incoming laser light and observation of the scattered radiation. Convection, due to local heating from the laser, sufficiently mixed the sample and prevented significant photoreduction. Spectra were also obtained from stirred samples with identical results. The low levels of laser power noted above were used for all experiments. UV–visible absorption, EPR, and MCD were run before and after the RR experiments to check sample integrity.

Optical spectra were obtained on an IBM 9430 UV–visible spectrophotometer. EPR spectra were recorded at 12K on a Varian E6 spectrometer; the instrumental conditions were as follows: modulation amplitude, 20 G; microwave power, 3 mW; microwave frequency, 9.24 GHz. MCD spectra were obtained with a Jasco 500C spectrometer using a 1.3-T electromagnet.

Results and Discussion

A Comparison of the RR Spectra of Fast and Slow Resting Cytochrome Oxidase. Figures 1 and 2 compare the RR spectra of fast and slow resting cytochrome oxidase in the low- (200–450 cm^{-1}) and high-frequency (1200–1700 cm^{-1}) regions, respectively. Identical spectra have been obtained with three separate preparations of both types of enzyme. The spectrum of slow enzyme shows significant differences when compared to that of fast enzyme, including a large increase in intensity in the doublet peak at 1610 and 1623 cm^{-1} ("1620-cm^{-1}" peak) and intensity enhancement of high-frequency features at 1518 and 1572 cm^{-1} (Figure 2). In the low-frequency region there are also significant differences in intensity between the RR spectra of fast and slow enzyme, with a clear improvement in the signal-to-noise ratio of all the Raman peaks below 500 cm^{-1} due to greater RR intensity in the slow enzyme spectra (Figure 1). The remaining peaks between 500 and 1000 cm^{-1} show few significant differences between fast and slow preparations (data not shown). The RR intensity differences between the fast and slow enzyme spectra can largely be attributed to increased resonance enhancement of cytochrome a_3 modes in slow enzyme relative to fast enzyme. This is due to the blue shift (i.e., toward the laser wavelength) of the position of the Soret absorption band of cytochrome a_3 in slow enzyme relative to fast enzyme (vide infra).

Only one RR mode is found to change frequency upon conversion from fast to slow enzyme. This is the peak found at 223 cm^{-1} in fast enzyme which moves to 220 cm^{-1} in slow enzyme. This peak is most likely to be the iron–histidine [Fe(III)–N(His-Im)] stretch of cytochrome a_3. The corresponding stretch at 210 cm^{-1} has been identified in the reduced enzyme from *Thermus thermophilus* on the basis of iron isotope studies (Ogura et al., 1983, 1984). The corresponding mode in the reduced mammalian enzyme is found at 214 cm^{-1}. The major difficulty with our assignment is that attempts to observe the iron–histidine stretch in high-spin Fe(III) heme model compounds have failed even when chelated heme was studied (Mitchell et al., 1985). However, in this latter study only 5-coordinate derivates were examined; cytochrome a_3 is believed to be 6-coordinate. Furthermore, a similar peak at 216 cm^{-1} has been observed in myoglobin fluoride (Choi & Spiro, 1983). Supporting our assignment is the shift in the 223-cm^{-1} peak that we observe in D_2O (vide infra), there being few if any molecular motions in this frequency range other than the Fe–N(His) stretch which might be expected to change frequency in D_2O.

Granting the validity of this assignment, the change in frequency from 223 to 220 cm^{-1} upon conversion from fast to slow enzyme represents a shift in the Fe(III)–N(His-Im) stretching frequency between the two forms of the enzyme. This in turn suggests that the Fe–N bond strength is modulated by conformational or electrostate changes in the vicinity of cytochrome a_3. For example, the lowering in frequency is consistent with decreased hydrogen bonding by the N(3)-H of the histidine residue (Stein et al., 1980; Hori & Kitagawa, 1980; Teraoka et al., 1983; Debois & Lutz, 1981; Kincaid et al., 1979).

The differences in the high-frequency region are illustrated by the spectra shown in Figure 2. The peaks at 1225, 1645, and 1651 cm^{-1} lose *relative* intensity in the slow oxidase spectra. The 1651-cm^{-1} peak has been attributed to the formyl stretch of cytochrome *a* (Babcock & Callahan, 1983a,b); because of the intensity changes noted above, which are expected of cytochrome *a* peaks in slow enzyme due to the blue shift of the cytochrome a_3 Soret (i.e., cytochrome a_3 peaks become relatively more intense), all three of these peaks may now be assigned to cytochrome *a* modes. On the other hand, the peaks at 1518, 1572, 1610, and 1623 cm^{-1} show significant *increases* in relative intensity in spectra of slow enzyme due to the blue shift of the cytochrome a_3 Soret toward the 413.1-nm laser line and may thereby be ascribed to cytochrome a_3. The 1572-cm^{-1} vibration has previously been assigned as the high-spin core-size indicator of cytochrome a_3 (Babcock et al., 1981; Woodruff et al., 1981).

The changes in relative intensity noted above are due to changes in the wavelength maximum of the Soret absorbance which occur during the fast to slow transition, there being a 6–7-nm blue shift in the position of the Soret maximum of cytochromes $a + a_3$ (Baker et al., 1987).

A number of possible reasons for this shift can be excluded by the presently available spectroscopic data. First we note that the Soret MCD is unchanged upon conversion to the slow form (Baker et al., 1987). Since this MCD is due to cytochrome *a*, it is quite clear that the optical properties of this center are unaffected and that essentially all of the spectral changes in the absorption spectrum can be attributed to cytochrome a_3. We next note that neither heme center is reduced as judged both by the location of the Raman oxidation state marker (this work) and the absence of any intensity at 450 nm in the MCD spectrum (Baker et al., 1987). Finally, we find that the Raman core-size marker for cytochrome a_3 is unchanged as is the near-infrared MCD (Baker et al., 1987); thus, cytochrome a_3 is still high spin and 6-coordinate. Consequently, the observed shift in optical absorption of cytochrome a_3 cannot be attributed to changes in redox state, spin state, or coordination number of this cytochrome.

Several possibilities remain. A body of evidence developed here (vide infra) and elsewhere (Baker et al., 1987) suggests the transition from fast to slow enzyme involves a significant protein conformational change involving both hemes. A conformational change might affect heme π–π^* energies either by imposing constraints upon the heme structure itself or by changing factors extrinsic to the heme.

Many of the spectroscopic peculiarities of heme *a*, when compared to hemes *b* and *c*, can be understood on the basis of raising of the *x*, *y* degeneracy of the heme *a* π system. This raising of degeneracy results from the π interaction of the heme molecular orbitals with the vinyl and formyl substituents on pyrroles II and IV of the porphyrin macrocycle (Woodruff et al., 1982). This interaction is modulated by the orientation of these substituents with respect to the heme plane, being maximum when the molecular system is coplanar. Reducing this interaction by removing either or both substituents from coplanarity will increase the symmetry of the heme and result in a change in the Soret energy. However since these transition energies are determined by two-electron terms (Weiss et al., 1966), it is not a simple matter to predict the direction of the change. Thus control of the coplanarity of these substituents by the protein structure is one mechanism whereby protein conformation might control the Soret wavelength. We note, however, that the position of the formyl mode ascribed to cytochrome a_3 remains at 1676 cm^{-1} during the fast to slow transition; if the formyl had become uncoupled from the porphyrin, its frequency should have reverted to that found with isolated formyl groups at about 1690 cm^{-1} (Zwarich et al., 1971). Consequently, if this mechanism is at play, it would seem that the orientation of the vinyl substituent is being affected.

Extrinsic factors which might be sensitive to conformation include axial ligation, polarizability of the heme environment, or charge distribution in the vicinity of the heme. As the observed changes in the Soret maximum can be reversed under conditions in which the enzyme still retains poor reactivity with cyanide (Baker et al., 1987), it seems unlikely that there has been a change in heme ligation. Similarly, a change in polarizability sufficiently large to produce the observed 7-nm shift would seem to require an extreme change in conformation; in fact, the shift is slightly larger than the shifts observed upon complete transfer of heme model compounds from a purely nonpolar medium to a purely aqueous medium (Bomberg & Kassner, 1982). However, the appearance of a negative charge (or, equivalently, disappearance of a positive charge) at the heme periphery might well be sufficient to destabilize the optical excited state by ca. 0.5 kcal, thus producing the observed change. Such a change might be produced by movement of a propionate side chain or of a charged amino acid side chain or, less likely, modification of an amino acid (e.g., hydrolysis of asparagine to aspartate). It is relevant that addition of formate to fast enzyme leads to an immediate shift in the Soret maximum to 417 nm with no change in any other measured property (Palmer et al., 1988). With catalase it is believed that formate does not bind to the heme but to a site on the protein (Hershberg & Chance, 1975).

Effect of Deuteriation on the RR Spectra of Fast and Slow Enzyme. The RR spectral differences between fast and slow enzyme were further investigated by observing the isotopic shifts accompanying deuteriation of all labile and solvent-accessible protons of the protein. The spectra of fast cytochrome oxidase in H_2O and D_2O between 200 and 450 cm^{-1} are shown in Figure 3. A distinct peak appears at 418 cm^{-1} in the D_2O spectrum compared to a weak shoulder near the same frequency (on the high-frequency side of the 404-cm^{-1} peak) in the H_2O spectrum. In the D_2O spectrum, the 404- and 418-cm^{-1} intensities are approximately equal. This same effect is observed in the low-frequency region of slow enzyme (Figure 4). The 418-cm^{-1} peak in the D_2O spectra is probably the same vibration, enhanced in intensity, as the shoulder at ca. 418 cm^{-1} in the H_2O spectra. We make this assertion because there is no evidence of a peak shifted by the H/D mass effect; that is, there is no peak at or below ($\sqrt{2} \times 418$) cm^{-1} in the H_2O spectra which disappears upon deuteriation, indicating that it might be isotope shifted to 418 cm^{-1}. Thus, in this instance, deuterium substitution affects RR intensities rather than frequencies. The reasons for this are obscure. It may be that the stronger hydrogen bonding upon deuteriation of the protein causes minor conformational changes which

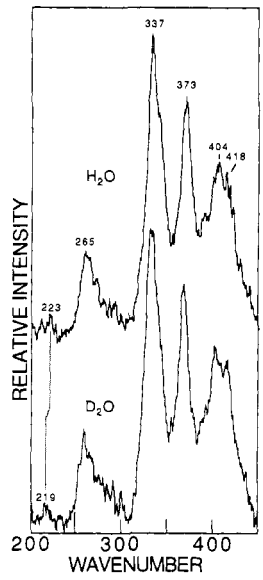

FIGURE 3: Comparison of the RR spectra of fast cytochrome oxidase in H_2O (top) and D_2O (bottom). The deuterium effect on the 223-cm^{-1} peak is labeled.

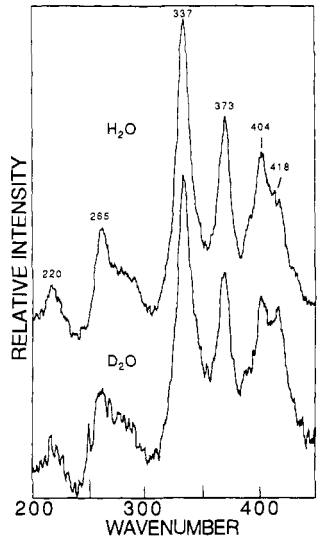

FIGURE 4: Slow enzyme in H_2O (top) and D_2O (bottom) between 200 and 450 cm^{-1}.

affect the vibronic activity of this mode specifically. Some intensity changes upon deuteriation are also noted in the high-frequency region (vide infra). In any case, we associate all of these low-frequency features with cytochrome a_3 because of their increased RR intensity in slow enzyme with the blue-shifted cytochrome a_3 Soret band.

Another spectral change upon deuteriation is an apparent downshift of 4 cm^{-1} between the H_2O and D_2O spectra of the peak present at 223 cm^{-1} in the fast enzyme. Because of the weakness of this feature, the precise magnitude of the deuterium shift must be considered uncertain, but a nonzero (negative) deuterium shift is indicated. An Fe(III)–N(His-Im) stretch should shift in the observed direction if the labile protons [C(2) and N] on the histidine imidazole ring are deuteriated. It should be noted that the 214-cm^{-1} Fe(II)–N-(His-Im) stretch in spectra of *reduced* cytochrome a_3 (Ogura et al., 1983) does not shift in frequency upon incubation of the protein in D_2O (Argade et al., 1986). However, infrared studies on deuteriated CO derivatives of reduced cytochrome oxidase (Einarsdôttir, 1986) reveal that the cytochrome a_3 environment in fully reduced enzyme is remarkably isolated

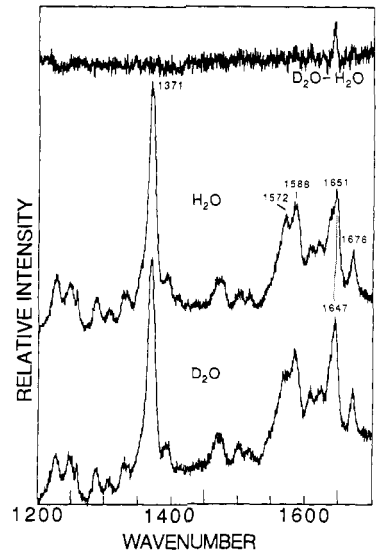

FIGURE 5: Spectra of fast enzyme in H_2O (middle) and D_2O (bottom) between 1200 and 1700 cm^{-1} along with the spectral subtraction (D_2O – H_2O, top). The downshift of the formyl stretch of cytochrome *a* is labeled.

from deuterium effects. Accordingly, imidazole deuteriation may only occur in oxidized forms of the enzyme.

The spectra of the H_2O and D_2O samples of fast enzyme in the high-frequency region (1200–1700 cm^{-1}) along with the spectral subtraction (D_2O – H_2O) are shown in Figure 5. One peak in this region exhibits a frequency shift upon deuteriation. This is the peak assigned as the C=O formyl stretch of cytochrome *a*, located at 1651 cm^{-1} in the H_2O spectrum and 1647 cm^{-1} in the D_2O spectrum. This 4-cm^{-1} shift, originally observed by Copeland and Spiro (1986), was reasonably interpreted as resulting from the strengthening of a hydrogen bond involving the formyl group when a deuteron is substituted for a proton. This stronger hydrogen bond in turn slightly weakens the formyl C=O bond, resulting in the observed frequency downshift. Alternatively, the frequency shift could be a simple mass effect including the H-bonded proton.

The RR intensity of the 1651-cm^{-1} (H_2O) peak also increases in intensity relative to the nearby features upon deuteriation, as does the previously mentioned 418-cm^{-1} peak. This may suggest either that deuteriation increases the excited-state displacement of the C=O coordinate or that a D_2O-induced conformational change increases the conjugation of the formyl with the heme. We find no other significant intensity or frequency changes in this region of the spectrum.

In contrast to the behavior of fast enzyme, exchange of protons by deuterons has no significant effect on the *frequencies* of peaks in the low-frequency region of the slow enzyme spectrum (Figure 6). In particular, no deuterium shift is observed for the 220-cm^{-1} peak. It appears that deuterium substitution does not occur on the imidazole ring of the Fe-(III)–N(His-Im) structure of cytochrome a_3 in slow enzyme. However, as in the D_2O spectrum of fast enzyme, there is an increase in the *intensity* of the 418-cm^{-1} peak.

Furthermore, and again in contrast to the result with fast enzyme, no significant deuterium shift is observed in the formyl stretch at 1651 cm^{-1} in the slow enzyme – D_2O spectrum (Figure 6). Thus, neither the cytochrome *a* formyl group nor, as discussed above, the axial histidine ligand of cytochrome a_3 in slow enzyme exchanges with deuterium over a period of 24 h. These sites in slow enzyme must be in environments which are inaccessible to exchange with solvent protons over this time scale. While the slow cyanide-binding rate clearly indicates a restrictive conformation around cytochrome a_3 in

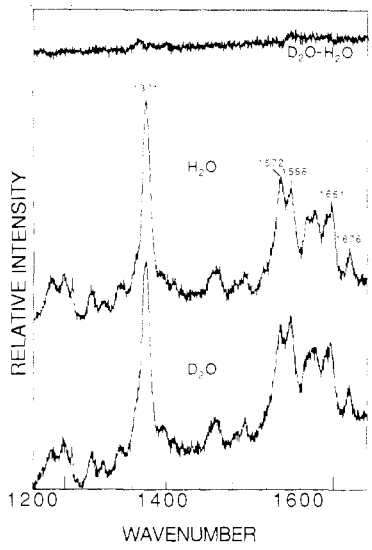

FIGURE 6: High-frequency region of slow enzyme in H_2O (middle) and D_2O (bottom) and the spectral subtraction ($D_2O - H_2O$, top).

slow oxidase, the apparent inaccessibility of both cytochromes a and a_3 to solvent protons is surprising.

These results, coupled with the blue shift of the cytochrome a_3 Soret band noted earlier, provide additional insights into the nature of the differences between fast- and slow-reacting cytochrome oxidase. The facts suggest a protein conformational change which affects both cytochrome a and the cytochrome a_3 reaction site, thereby giving the slow reaction with cyanide and engendering the observed spectroscopic changes. We have suggested that the 223-cm^{-1} peak is the Fe(III)–N-(His-Im) stretch of cytochrome a_3. Accordingly, the frequency change in this mode observed upon conversion of fast to slow enzyme implies that the conformational change involves the axial histidine of cytochrome a_3. The blue shift of the Soret is additional evidence for a conformational change around this cytochrome. There is a clear contrast between the deuteriation behavior of fast and slow enzyme, with only the former showing deuterium shifts in the 223-cm^{-1} cytochrome a_3 peak and in the 1651-cm^{-1} feature assigned to cytochrome a formyl. This demonstrates that the conformational change affects cytochrome a as well as cytochrome a_3. The nature of the conformational change is such as to render both cytochromes inacessible to solvent protons in slow enzyme. The Soret blue shift may suggest that protein either modifies the orientations of the formyl, vinyl, or propionate substituents of cytochrome a_3 or changes the positions of one or more charged amino acids in immediate juxtaposition to the heme.

Evaluation of Photoreduction in Cytochrome Oxidase. The spectra in Figures 1 and 2 were collected under identical conditions including 40–45 mW of laser power incident on the sample. When the laser power is increased to 60–65 mW, spectra of both fast and slow enzyme show noticeable photoreduction with intensity developing in the 1359-cm^{-1} RR peak (indicating reduced cytochrome a or a_3 compared to 1371 cm^{-1} for oxidized heme a). Photoreduction is also apparent in these experiments as a shoulder near 440 nm in electronic absorption spectra and by the appearance of intensity at 450 nm in the MCD spectra of samples examined after the RR experiment. It is important to note that with 60–65 mW the extent of photoreduction observed in slow enzyme was greater than that for the fast enzyme, but the total extent of photoreduction did not exceed 10% in either case (vide infra).

To establish the relative contributions of oxidized cytochromes a and a_3 to the peak at 1371 cm^{-1} and of reduced a and a_3 to the peak at 1359 cm^{-1}, we have recorded the Raman

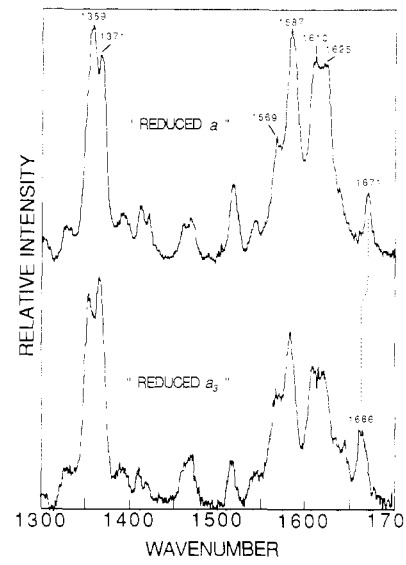

FIGURE 7: Comparison of the RR spectra of the mixed-valence cyanide derivative (top), representing reduced cytochrome a, and the mixed-valence carbon monoxide derivative (bottom), representing reduced cytochrome a_3.

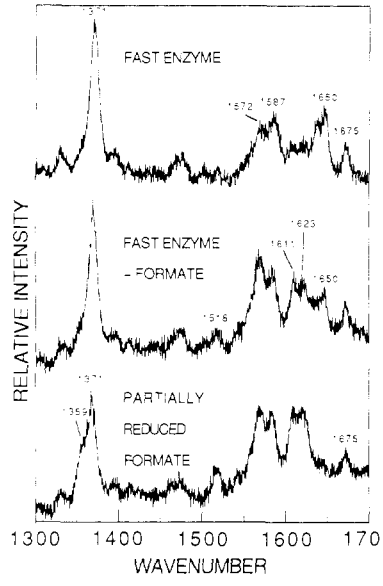

FIGURE 8: Comparison of the RR spectra of fast cytochrome (top), the formate adduct (center), and the mixed-valence formate derivative (bottom).

spectra of three mixed-valence cytochrome oxidase derivatives. Reduction of enzyme pretreated with cyanide leads to a species having the composition $a^{2+}a_3^{3+}$·CN; in this derivative the peak height of the line at 1359 cm^{-1} is a little larger than that at 1372 cm^{-1} (Figure 7, upper trace), although, due to differences in peak width, the integrated intensities of these two features are in the ratio 1.77:1.

In the derivative just described cytochrome a_3 has been converted to the ferric low-spin state, a consequence of coordination by the cyanide which is a strong-field ligand. A second derivative in which a_3 is maintained high spin was obtained with sodium formate.[2] Addition of sodium formate to rapid cytochrome oxidase leads to an immediate blue shift of the Soret maximum to a value, 417 nm, typical of the slow enzyme (Palmer et al., 1988). This enzyme–formate complex already exhibits additional Raman intensity at 1620 cm^{-1} (Figure 8, middle trace). This spectrum is unaffected by

[2] This experiment was suggested by a reviewer.

subsequent addition of the strong oxidant $Os(bpy)_3^{3+}$, while spectra of a separate sample placed in a spinning sample holder and cooled by a stream of cold N_2 gas [cf. Ogura et al. (1985)] was not significantly different. Conversion to the mixed-valence state ($a^{2+}a_3^{3+}$·$HCOO^-$) by addition of TMPD and ascorbate led to the spectrum shown in Figure 8 (bottom) with substantial amounts of heme *a* in both oxidized and reduced states and some increase in intensity around 1620 cm^{-1}; the ratio of intensities of the two oxidation state markers is about 0.67. This ratio differs from that obtained with the cyanide mixed-valence derivative because the Soret maximum of a_3 in the formate derivative (~417 nm) is much closer to the excitation wavelength (413.1 nm) than is the Soret maximum of a_3 in the cyanide derivative (427 nm) and thus undergoes much greater resonance enhancement. The Soret maximum of a^{2+} appears to be the same in the two derivatives. Previous spectra of this derivative obtained with 406.7-nm excitation (Babcock et al., 1981) give little evidence for the 1359-cm^{-1} feature of a^{2+}, suggesting that the resonance enhancement of this species is obscured by the contribution of a_3^{3+}·$HCOO^-$ when 406.7-nm excitation is employed.

A third derivative with the composition $a^{3+}a_3^{2+}$·CO (see Materials and Methods) is photodissociated in the laser beam to yield the species $a^{2+}a_3^{2+}$. Under these conditions, most of the a_3 is present in the ferrous state, as judged by the frequency of the formyl C=O mode at 1666 cm^{-1} (Figure 7, bottom). The presence of a small shoulder shows that a small amount of the a_3^{3+} mode is present at 1676 cm^{-1}; we estimate this is much less than 10%. It follows that more than 90% of cytochrome *a* is still in the ferric state. Consequently, the lines representing the ferric and ferrous hemes are essentially due to cytochromes *a* and a_3, respectively, with the 1359-cm^{-1} line of a_3^{2+} about 10% smaller than the 1371-cm^{-1} line of a^{3+} (Figure 7, lower trace). In this derivative the ratio of the integrated intensities is 0.86:1.0. The absence of a clear feature at 1651 cm^{-1} due to a^{3+} appears to be a characteristic of the mixed-valence species.

It is important to notice that in all of these mixed-valence derivatives there is a marked increase in the Raman intensity in the 1610–1623-cm^{-1} region of the spectrum. It is thus clear that either heme center can contribute to the modes at ca. 1620 cm^{-1} when in the divalent oxidation state and that any assignment of these features uniquely to cytochrome *a* is incorrect.

The availability of the relative RR intensities of each heme center in the two common oxidation states allows the quantitation of the proportion of reduced heme *a* present in a given enzyme sample. By resolution of the Raman spectrum from 1350 to 1380 cm^{-1} into 1359- and 1371-cm^{-1} components, the fraction of reduced heme *a* present can be evaluated. Clearly, if no reduction has occurred, the ratio (1359/1371) of intensities of the two lines is zero; if some intensity is found at 1359 cm^{-1}, comparison of this ratio with that observed with each of the two mixed-valence derivatives described above establishes the extent of reduction in the sample.

Using this approach, we find that spectra obtained using an incident laser power of 65 mW were consistent with the presence of 6–8% reduced heme in fast enzyme and 10% reduced heme in slow enzyme. At lower laser powers, e.g., 40–45 mW, the amount of the 1359-cm^{-1} component was sufficiently small that it could not be reliably quantitated with the curve-resolving capabilities of our data system (Figures 2 and 7).

To evaluate whether the intensity in the doublet located at ca. 1620 cm^{-1} is reliably correlated with the extent of reduction (or photoreduction) of either cytochrome, as has been previously suggested by Babcock (Babcock et al., 1981) and by Kitagawa (Ogura et al., 1985), we have compared the integrated intensities of the 1620-cm^{-1} feature with that present at 1359 cm^{-1}. We emphasize that this analysis is based upon the relative intensities of the 1620- and 1359-cm^{-1} features for the *same* heme in the *same* oxidation state. Therefore, the following arguments are independent of resonance enhancements of these peaks due to the red shift of the Soret band upon reduction.

In the mixed-valence derivatives described earlier, the ratio (1620/1359) is 1.7 when *a* is reduced and a_3 is low spin (the cyanide derivative), 2.3 when *a* is reduced and a_3 is high spin (the formate derivative), and 1.5 when a_3 is reduced (the photodissociated CO derivative). By contrast, when spectra are recorded with an incident laser power of 65 mW (and the small level of photoreduction described above is present), the value for this ratio is 8.6 in oxidized, fast enzyme and 8.3 in slow enzyme. It is quite clear that the intensity of the 1620-cm^{-1} feature is much larger than would be expected from the observed level of reduction. It follows that the observation of Raman intensity in the 1620-cm^{-1} region may result from effects other than reduction (or photoreduction) at one or other of the two heme centers.

In summary, while laser-induced photoreduction can unquestionably be forced to occur in all samples of oxidized cytochrome oxidase, the RR spectra of our oxidized enzyme preparations (fast and slow) exhibit minimal reduction of either the *a* or a_3 cytochrome. Spectra of samples which are unequivocally photoreduced invariably show intensity in the 1359-cm^{-1} oxidation-state marker when 413.1-nm laser excitation is used. This feature is weak or nonexistent in our oxidase spectra. Thus, it seems clear that certain spectral features previously ascribed by others (Babcock et al., 1981; Ogura et al., 1985) to significantly photoreduced preparations are in fact spectra of the species we refer to as the slow resting form of cytochrome oxidase [see, for example, Woodruff et al. (1981, 1982a)].

Conclusions

The availability of two preparations of cytochrome oxidase, each of which behaves homogeneously but quite differently in their reaction with cyanide, has allowed us to use RR spectroscopy to obtain valuable insights into the structural changes which occur during the fast-to-slow transition. We have also been able to provide criteria for defining when exposure to laser light causes photoreduction of cytochrome oxidase. It appears that the manifestations of photoreduction in the RR spectra of this enzyme are widely misunderstood and that many of the claims for photoreduction, in the absence of significant 1359-cm^{-1} intensity, should be reexamined (Babcock et al., 1981; Ogura et al., 1985). Nevertheless, it is clear that bona fide photoreduction can occur under some circumstances, and the criteria we have developed will allow for correct assessment of the extent to which it has occurred.

The changes between fast and slow cytochrome oxidase in reactivity toward ligands, in visible/Soret absorption spectra, in EPR spectra, in MCD spectra, in low-frequency RR peak positions, in the relative intensity of high-frequency peaks, and in the responses of both low- and high-frequency peaks to D_2O make it clear that structural changes occur at both cytochromes *a* and a_3, when this transformation occurs. Accordingly, the protein conformational changes which accompany this transformation must be considered global in character. Some tentative conclusions are drawn as to the structural nature of these changes and their effects upon the redox

centers. Studies of fast and slow cytochrome oxidase are continuing in order to uncover their precise nature. Although it may be that slow enzyme has no physiological significance, the characterization of the structural modifications responsible for the fast-to-slow transition may well reveal valuable information concerning the heme environments and the related chemistry of cytochrome oxidase.

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