Photoreductive Titration of the Resonance Raman Spectra of Cytochrome Oxidase in Whole Mitochondria†

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Abstract: A photoreductive titration of the resonance Raman (RR) spectra of cytochrome c oxidase in whole mitochondria was recorded by exploiting the preferential enhancement of the Raman signals of reduced cytochrome oxidase excited at 441.6 nm. When the sample was cooled to about -10 °C, it was possible to slow down the photoreductive effect of the laser and to record RR spectra at various states of reduction. Compared to the earliest recorded scan (most oxidized), the dithionite-reduced sample shows the appearance of new bands at 216, 363, 560, and 1665 cm⁻¹. At intermediate stages of photoreduction, the 216- and 560-cm⁻¹ bands appear before the 363- and 1665-cm⁻¹ bands; photoreduction induces full intensity in the former bands, whereas the latter bands are photoreduced to 50% of the dithionite-reduced intensity. The relative intensities of a doublet at 1609–1623 cm⁻¹ are affected by reduction: the band at 1609 cm⁻¹ is weaker in the earlier scans; in later scans, this band has grown to equal intensity with the 1623-cm⁻¹ band. We conclude that this reductive titration of the RR spectrum of cytochrome c oxidase reflects three states in its reduction. The behavior of the doublet at 1609–1623 cm⁻¹ suggests that the two hemes are nonequivalent but interacting. The band at 216 cm⁻¹ may be indicative of an iron–copper interaction that is affected by the presence of external ligands.

Cytochrome c oxidase is the terminal component in the electron transport chain whose function is to transfer four electrons to oxygen, thereby reducing it to water, and to convert the energy released to chemical energy by phosphorylating ADP to ATP. The enzyme contains four prosthetic groups that are active in its function: two a-type hemes and two copper ions. In spite of much activity in the investigation of this system, a detailed picture of its molecular organization and mechanism of activity has yet to emerge.

Resonance Raman (RR) spectra of many heme proteins [see Warshel (1977), for example] have been examined with the aim of relating the spectroscopic behavior of the hemes to their biological function. In the case of cytochrome c oxidase, it was recognized early that the polarization behavior of the RR spectra was different than that of other hemes and reflected differences in resonance enhancement by excitation in the Soret region rather than the visible absorption bands (Salmeen et al., 1973; Nafie et al., 1973; Friedman & Hochstrasser, 1973).

Salmeen et al. (1973) reported that when exciting cytochrome c oxidase at 441.6 nm, the strongest band appeared as a doublet at 1372 and 1358 cm⁻¹ and suggested that this reflected contributions from the inequivalent hemes a and a₂. Subsequently, Adar & Yonetani (1977), using the 413.1-nm excitation, showed that the laser beam exciting the spectra induced photochemical events which produced reducing equivalents and caused reduction of the enzyme. Thus, if the oxidase preparation used by Salmeen et al. (1973) was not fully oxidized, the strongest band in their spectrum would appear as a doublet; in fact, the markers for oxidized and reduced hemes at 1372 and 1358 cm⁻¹ did appear simultaneously. This is consistent with the data of heme a derivatives of Kitagawa et al. (1977) in which the RR bands of ferric and ferrous hemes fall in the expected regions.

In this work, we took advantage of the capability of the laser beam to produce reducing equivalents and carried out the photoreductive titrations of cytochrome c oxidase in whole mitochondria induced by the 441.6-nm HeCd laser line. Cytochrome oxidase accepts four reducing equivalents which reside on the two a-type hemes and two copper ions.
Near-equilibrium redox titrations indicated that these components in the absence of external ligands are reduced in the order cytochrome a₃, "invisible copper", visible copper, and cytochrome a (Dutton & Wilson, 1974). The reduction of cytochrome a₃ and the invisible copper occurs almost simultaneously because their $E_{m/2}$ values are very close to one another [cytochrome a₃, 0.375 V (Wilson & Dutton, 1970); invisible Cu, 0.34 V (Lindsay et al., 1975)]. The same is true for cytochrome a and the visible copper [cytochrome a, 0.21 V (Wilson & Dutton, 1970); visible Cu, 0.24 V (Erecińska et al., 1971)].

The RR spectra of whole mitochondria excited at 441.6 nm can be assigned to cytochrome oxidase by comparison with spectra of the purified enzyme as Salmeen et al. (1973) did with electron transport particles. In the course of the photoreduction, three oxidation states different from the dithionite reduced state can be identified. By association of the changes in RR spectrum during photoreduction, it is hoped that it will be possible to derive information of the redox and geometrical relationships between the components of cytochrome oxidase.

Materials and Methods

Mitochondria were isolated from pigeon breast muscle as described by Erecińska et al. (1973) and stored frozen at -40 °C. Aliquots were thawed, diluted approximately 10-fold in 10 mM phosphate buffer, pH 7.4, and centrifuged for 10 min at 10000 rpm in the Sorvall centrifuge. The pellet was washed twice in 100 mM phosphate buffer, pH 7.4, and finally suspended in the same buffer. Samples were oxygenated with oxygen gas before transferring to capillaries and cooled with nitrogen gas to about -10 °C. It was found that under these conditions it was possible to slow the photoreductive effect of the laser in order to record RR spectra at various states of reduction.

The Raman system consists of a double monochromator designed with concave holographic gratings from Instruments SA and is as described previously (Adar & Erecińska, 1978). The spectra generated here were excited by the 441.6-nm output of the HeCd laser. The laser power at the sample was about 30 mW. Slits were set at 600 μm, corresponding to a band pass of about 8 cm⁻¹. RR bands frequencies were accurate to ±1 cm⁻¹. Because of the intrinsically poor optical quality of mitochondria, the capillaries were illuminated by focusing the light slightly off axis on the surface of the capillary facing the collection lens. The signals were strong enough to scan 100 cm⁻¹/min, more rapidly than usual, in order to maximize the number of observable states.

Results

A photoreductive titration is shown in Figure 1. The earliest scan appears at the bottom of the figure. With the exception of the earliest two scans, the period between starting times was about 17 or 18 min. The first two scans covered only the low-frequency region since we wished to observe early changes in the 216-cm⁻¹ band which appears only in the RR
spectra of cytochrome oxidase and not in heme $a$ (Salmeen et al., 1978). However, the 216-cm$^{-1}$ band did not begin to appear until the fourth scan, and no other significant differences can be identified between scans 1 and 3. Another band at 560 cm$^{-1}$ appears simultaneously with the 216-cm$^{-1}$ band in scan 4 but achieves its full intensity as soon as it appears. What is highly significant is that the 1665-cm$^{-1}$ band, which Salmeen et al. (1978) have associated with the carbonyl group of cytochrome $a_3$, does not appear with the initial scans (1–4) where the intensities of the 216- and 1357-cm$^{-1}$ bands already attained 40% of their dithionite-reduced values but begins to rise in scan 5. A band at 363 cm$^{-1}$ appears with the 1665-cm$^{-1}$ band, both of which are photoreduced to only about 50% of their total dithionite intensity. Dithionite reduction appears to reduce the intensity of the band at 340 cm$^{-1}$, relative to other bands, suggesting a shift of intensity from it to the 363-cm$^{-1}$ band located close-by.

The doublet at 1609–1623 cm$^{-1}$ appears consistently as a doublet in all scans although the relative intensities of the two components shift during reduction; scans 3 and 4 show the 1623-cm$^{-1}$ band stronger, whereas all scans after 6 as well as the dithionite-reduced scan show the two components with more comparable intensities. It should be noted, however, that this behavior was variable from preparation to preparation.

Figure 2 shows the RR spectra of the reduced cyanide and nitrous oxide complexes together with the reduced enzyme without exogenous ligands. Both samples with added ligands have low intensity at 216 and 560 cm$^{-1}$. The bands at 362 and 1665 cm$^{-1}$, which do not show their full dithionite intensity during photoreduction, exhibit very low intensities in the spectra of the liganded forms.

Discussion

Comparison of our RR spectra of reduced whole mitochondria excited at 441.6 nm with those obtained on isolated oxidase with the same excitation (Salmeen et al., 1973, 1978) indicates that these spectra can be assigned to the oxidase; the $b$- and $c$-type ferrous cytochrome bands are not intense because the excitation wavelength does not match their Soret transitions as well. Enhancement of all ferric components will be weaker than that of the ferrous ones also because of poorer overlap between the excitation wavelength and the optical transitions and because of shorter lived intermediate states of the ferric vs. ferrous cytochromes (Adar et al., 1976).

The following discussion is based on the assumption that the spectra obtained during photoreductive titrations of the mitochondria represent a series of near-equilibrium redox states of the oxidase in which electrons reduce the highest potential component first. In order to assure that the material in the capillary at the initiation of data collection was oxidized, it was necessary to pass oxygen through a sample before drawing into the capillary. The near-equilibrium description of the system is based on the fact that, even after oxygenation of the sample, the relative concentration of oxygen to protein ($\sim$30 mg of protein/mL) was low. When we cooled the sample with cold
nitrogen gas, the rate of oxidation of cytochrome oxidase by the remaining oxygen was significantly slower than the rate of photoreduction induced by the laser. It should also be pointed out that although it has been shown that intramolecular electron transfer can be very slow in isolated cytochrome oxidase in the absence of cytochrome $c$ (i.e., in the so-called "resting" oxidase; see, e.g., Gibson et al. (1965) and Antonini et al. (1977)) in the intact mitochondria, even containing only trace amounts of cytochrome $c$, there is rapid electron transfer between low and high potential hemes (Wohlrab, 1970). That is, the oxidase state characterized as cytochrome $d^+_a\alpha_3^{3-}$ cannot be formed under these conditions.

As in all experiments involving cytochrome oxidase, the interpretation must be based on some model. In order to avoid ambiguity arising from the assignment of the heme components ($a$ and $a_3$) to sites for ligand binding, our discussion of the photoreduction will be described in terms of the high- and low-potential heme components. In many cases the high-potential component can unambiguously be assigned to cytochrome $a_3$ which by definition is the component that binds CO (Wever et al., 1977; Wilson & Miyata, 1977) and reacts with molecular oxygen (Greenwood et al., 1974; Chance et al., 1973). Although the anaerobic titrations of isolated cytochrome $c$ oxidase carried out by Wilson and co-workers (Wilson & Leigh, 1972; Leigh et al., 1974; Wilson et al., 1976) demonstrated the presence of two distinguishable hemes (i.e., high- and low-potential hemes), those of Babcock et al. (1978) showed a mixture of equal amounts of the two hemes. The results reported here on cytochrome oxidase in situ are in agreement with the former authors. Therefore, the equivalent heme model was used for the interpretation of these RR data which were obtained on whole mitochondria.

The conclusions that can be made at the present time are enumerated below.

(1) The fully oxidized protein, which can be monitored by the presence of a band at $1372\text{ cm}^{-1}$ (Adar & Yonetani, 1978), was never observed in our preparation; apparently photoreduction in whole mitochondria induced by 441.6-nm laser excitation starts so rapidly that we cannot identify any indicators of the fully oxidized form. This is in contrast with the earlier report of the RR spectrum of cytochrome oxidase in its purified form (Salmeen et al., 1973) also excited at this wavelength in which the indicator for oxidized heme was clearly observed. This would imply that either the amounts of reducing equivalent are lower in the isolated preparation or the kinetics of photoreduction are more efficient in whole mitochondria.

(2) Because the excitation wavelength coincides with the Soret maximum of reduced cytochrome oxidase, we have assumed that most of the RR intensity arises from the reduced form, which is consistent with the absence of the $1372\text{ cm}^{-1}$ band.

The appearance of the doublet at $1609-1623\text{ cm}^{-1}$ in even the earliest spectrum is then puzzling if one assumes that only one heme is reduced at this stage. Normally, only one band appears in the RR spectra of heme proteins between 1600 and 1650 cm$^{-1}$. The band is known to shift with oxidation state ($1622$ vs. $1637\text{ cm}^{-1}$ in ferrous vs. ferrie cytochrome $c$) as well as with spin state ($1607$ vs. $1624\text{ cm}^{-1}$ in deoxy- vs. oxy-hemoglobin) (Spiro & Strekas, 1974). If only the high-potential heme is reduced in scan 3, it can be characterized as a high-spin ferrous heme (Babcock et al., 1976; Falk et al., 1977), then the $1609-\text{cm}^{-1}$ band would be correlated with it. The $1623-\text{cm}^{-1}$ band could be assigned to a low-spin ferrous heme. However, the photoreductive behavior would indicate that the other oxidase heme is not reduced at this stage (i.e., the intensity of the $1357\text{ cm}^{-1}$ band in the earliest scans is considerably less than half that of the dithionite scan). Because the $1623-\text{cm}^{-1}$ band appears in such early scans, it can be argued that the hemes are close enough so that electron density is shared between them or that excitonic-like coupling between the similar groups produces scattering into levels that reflect mixed vibrations of the two.

It has been argued recently that exciton interaction is unlikely. Burke et al. (1978) have collected data on the $\mu$-oxo dimer of iron tetracynoporhphine that bring our earlier assignments (Adar & Srivastava, 1975) into question. However, the RR data of the mitochondrial hemes still can be interpreted in terms of excitonic interactions. In particular, without exciton effects it is difficult to account for the shifts in centers of gravity of bands and for their persistence in samples known not to have enough of the components reduced. This effect was also noted earlier in a study of cytochrome $c$ (Adar & Erečińska, 1977).

It is also worth noting that similar splittings have been observed in crystals of inorganic materials due to the presence of more than one molecular vibrating unit in the crystallographic unit cell [see, for example, Carter (1976) and Ross (1972)]. However, even in these systems, it is difficult to quantitatively account for these effects.

(3) The reduction of the invisible copper can be seen in the appearance of the $216\text{ cm}^{-1}$ band. Since our data on the reduced cyanide and NO samples show vanishingly low intensity at $216\text{ cm}^{-1}$, one can infer that the ligand binds in such a way as to disturb the coupling between the high-potential heme and the invisible copper which is reflected in the band at $216\text{ cm}^{-1}$, in agreement with the suggestion of Salmeen et al. (1978).

(4) The $1665-\text{cm}^{-1}$ band which begins to appear only after the $216-\text{cm}^{-1}$ marker band for copper would then be assigned to the low-potential heme. This is in contradiction to Salmeen et al. (1978) who identified this band with cytochrome $a_3$ (i.e., the high-potential component) because of its disappearance in the partially reduced cyanide and formate complexes in aerobic steady state ($d^+_a\alpha_3^{3-}-L$). It should be pointed out, however, that the redox state of their samples may also have been perturbed by the exciting laser beam. In fact, their RR spectrum of the partially reduced cytochrome oxidase in the presence of cyanide is indistinguishable from our spectrum of reduced oxidase in the presence of cyanide presented in Figure 2. The absence of the $1665-\text{cm}^{-1}$ band in the RR spectra of the reduced NO and HCN complexes would thus suggest that a conformational change in the protein induced by ligand binding to the reduced form of the enzyme affects a rotation of the carbonyl group out of the heme plane of the low-potential cytochrome. This is based on the suggestion of Salmeen et al. (1973) that the carbonyl is conjugated with the porphyrin $\pi$ system when they are coplanar, providing a mechanism for the resonance enhancement of the carbonyl vibration. The assignment is, in fact, supported by RR data of other hemes with nonsaturated side groups which document their effects on the RR vibrations (Adar, 1975).

It can be argued that these data could be interpreted in terms of a model of cytochrome oxidase in which the hemes are reduced simultaneously (Malmsröm, 1974; Nicholls & Chance, 1974). However, the fact that bands begin to appear at different stages of photoreduction cannot be easily explained by the simultaneous appearance of the several components. Thus, the appearance of the $216-\text{cm}^{-1}$ band is complete by scan 8 while the $1665-\text{cm}^{-1}$ band only begins to rise in scan 7.
addition, the relative intensities of the 1609–1623 cm⁻¹ bands change during the titration, which is also inconsistent with simultaneous reduction of the two heme components to equal extents. In fact, the observation of these two bands even in the earliest scans where the cytochrome oxidase is less than half-reduced (based on the intensity of the 1357 cm⁻¹ band) argues in favor of the proposal that the hemes can exhibit spectroscopic interactions because of their orientation on the mitochondrial membrane.

References