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TIME-RESOLVED RESONANCE RAMAN STUDY OF THE OXIDATION OF CYTOCHROME OXIDASE BY DIOXYGEN

Cytochrome oxidase catalyzes the rapid and efficient reduction of dioxygen to water. The sequence of events which occurs in this mechanistically complex reaction has been difficult to elucidate owing to the rapid rates of O_2 binding ($\sim 10^8 M^{-1} s^{-1}$) and electron transfer ($\sim 10^5$ - $10^3 s^{-1}$) [1]. To overcome this kinetic obstacle, flash photolysis of the carbon monoxide complex of the reduced protein has been used to initiate the oxygen reduction reaction. This «flow-flash» approach was pioneered by GIBSON and GREENWOOD for room temperature spectrophotometry [2], and adapted by CHANCE and coworkers to low temperature optical, EPR and EXAFS spectroscopies [3]. Although these approaches have clarified the kinetics of the reaction [4], basic structural questions concerning the reaction sequence remain unresolved. Owing to this situation and to uncertainties in extrapolating the low temperature results to room temperature [5], we have combined flow-flash and time-resolved resonance Raman techni-

ques to study the cytochrome oxidase/oxygen reaction at room temperature.

Cytochrome oxidase was prepared from beef heart [6a] and, following anaerobic reduction, combined with carbon monoxide. The solution was loaded into one syringe and oxygenated buffer into a second syringe. These were mounted on a syringe pump and connected to a Gibson-type, four-jet tangential mixer [7]. The mixed solution flowed into the Raman cuvette upon which the Q-switched pulses (10 ns duration) of two Quanta Ray Nd:YAG lasers were focussed. The pulse from the first laser was the 532 nm Nd:YAG second harmonic. Its energy was sufficient to photolyze the CO cytochrome oxidase complex and initiate the O_2 reduction reaction. Raman scattered light from the second, time delayed pulse at 416 nm was dispersed and detected by an EG&G/PARC 1420 diode array detector [8]. The delay times between the green photodissociation and violet probe pulses were variable from 10 ns to approximately 1 ms.

Fig. 1 shows Raman spectra of cytochrome oxidase at various times after initiation of the reoxi-

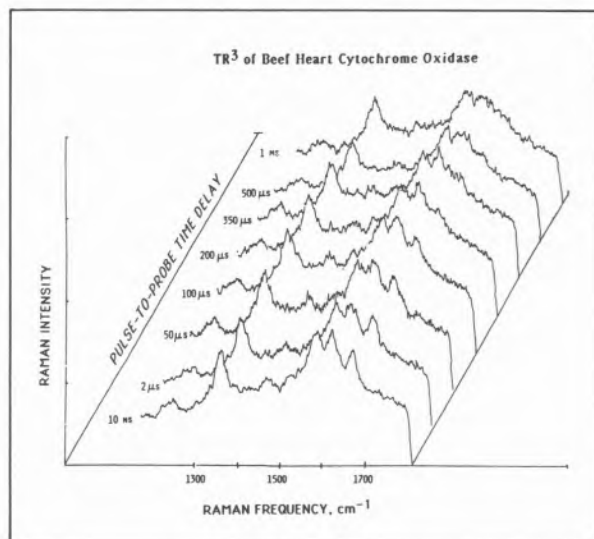


Fig. 1

Raman spectra of cytochrome oxidase at the indicated times following initiation of the oxygen reduction reaction at room temperature

dation reaction. The 10 ns spectrum is that of the photodissociation product of the reduced carbon monoxy enzyme. The oxidation state marker (ν_4 , $1355 cm^{-1}$) and the a_3^{2+} formyl stretching frequency ($\nu_{C=O}$, $1666 cm^{-1}$) [6] are well-resolved

and agree with those recently obtained in an anaerobic study of this species [9]. As the reaction proceeds, the 1355 cm^{-1} mode shifts to higher frequency [10] and the 1666 cm^{-1} mode decreases in intensity, indicating that oxidation of the protein is occurring.

The data in Fig. 1 and the difference spectrum in Fig. 2a indicate that little change in the observed vibrational frequencies occurs during the first $50\text{ }\mu\text{s}$. This observation is surprising in view of time-resolved optical data [2,4] which have clearly established that oxygen addition and, possibly, partial oxidation of the metal centers should have occurred in this time range. The initial oxygen adduct, however, has been suggested to be an oxygenated heme *a* species similar in electronic properties to oxyhemoglobin and oxymyoglobin [3,4,11], which may photodissociate as do the oxygenated O_2 transport proteins [12]. Accordingly, the high light intensity used to record the spectra in Fig. 1 may have photodissociated the oxygen adduct. Evidence that this, in fact, occurs is supplied by the $40\text{ }\mu\text{s}$ difference spectrum (Fig. 2b) obtained by using a low intensity, defocused probe pulse [13]. Under these conditions, the intermediate(s) at $40\text{ }\mu\text{s}$ are shown to have an oxidation state marker ($\bar{\nu}_4$) at 1378 cm^{-1} and spin state marker ($\bar{\nu}_2$) [6] at 1588 cm^{-1} . These frequencies are similar to those reported for oxymyoglobin and oxyhemoglobin. This observation, together with the photolability of the $40\text{ }\mu\text{s}$ transient, provides the best evidence to date that the reoxidation of cytochrome oxidase involves an oxycytochrome a_3 species at early times in the reaction.

The difference spectrum in Fig. 2c ($100\text{ }\mu\text{s}$ spectrum minus 10 ns spectrum) was obtained under high light intensity conditions. As opposed to the $40\text{ }\mu\text{s}$ spectrum obtained under these conditions, frequencies characteristic of oxidized, low- or intermediate-spin heme *a* species ($\bar{\nu}_4$, 1374 cm^{-1} ; $\bar{\nu}_2$, 1587 cm^{-1}) are apparent demonstrating that non-photolabile intermediates are formed at $100\text{ }\mu\text{s}$.

The results establish a number of points [14] regarding the reoxidation of cytochrome oxidase by O_2 . First, time-resolved resonance Raman, which is more general and offers greater structural insight than other spectroscopic probes, can be profitably applied to the study of the reaction. Second, the initial intermediates in the reaction

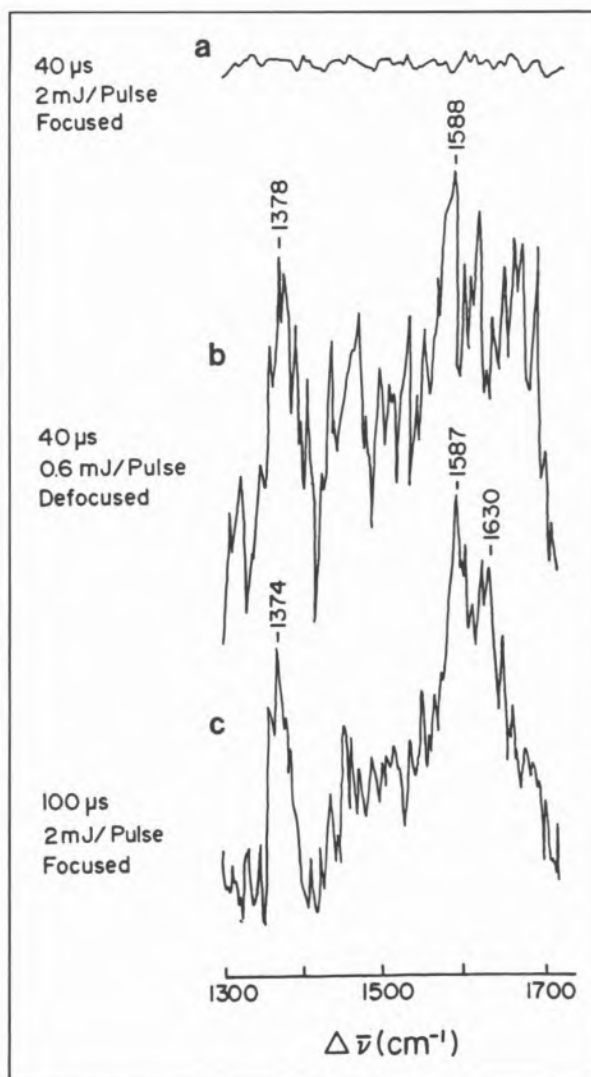


Fig. 2

Estimates of the Raman properties of intermediate species formed during the reoxidation of cytochrome oxidase by O_2 at $40\text{ }\mu\text{s}$ (a,b) and $100\text{ }\mu\text{s}$ (c) after initiation. For spectra (a) and (c) data taken under the high power density conditions of Fig. 1 were used. The scaled spectrum of the reduced photodissociated enzyme (the 10 ns spectrum in Fig. 1) was subtracted from the experimental spectra so that cancellation of the reduced oxidation state marker (1355 cm^{-1}) occurred. In the $40\text{ }\mu\text{s}$ spectrum of (b), the energy of the photolysis pulse was 2.5 mJ , but the probe energy was decreased to 0.6 mJ . A loose line focus at the sample plus a beam mask were used to decrease probe power density further. We estimate that the probe power density used to record spectrum (b) was 30-fold less than in (a), or 1-2 photons per molecule of enzyme in the illuminated volume.

mechanism are photolabile. This conclusion may seem to be at odds with low temperature work reported by CHANCE *et al.* [3] who described their compound A as «non-photolabile», but their ob-

servation most likely results from the fact that oxyheme species have much lower photolysis quantum yields than carbon monoxy heme species. Third, at reasonably early times in the reaction ($\sim 100 \mu\text{s}$) the system evolves to a state in which is photostable. Fourth, we note the apparent disappearance at later times ($> 200 \mu\text{s}$) of the $1666/1676 \text{ cm}^{-1}$ cytochrome a_3 formyl C=O stretch. This mode is always observed in stable forms of the enzyme [6] and its absence may suggest unusual reactivity of the formyl group in the turnover dynamics of the enzyme.

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STEADY-STATE KINETICS OF CYTOCHROME *c* OXIDASE IN PHOSPHOLIPID VESICLES: THE EFFECTS OF pH, IONIC STRENGTH AND D₂O

The steady-state kinetics of cytochrome *c* oxidase (E.C. 1.9.3.1) reconstituted into phospholipid vesicles has been investigated with a spectrophotometric method, using the high activity region of the reaction between reduced cytochrome *c* and the enzyme. k_{cat} and k_{cat}/K_m were evaluated at high ionic strength ($I=0.5$) in the pH range 5.2-8.6, and the effects of varied ionic strength and D₂O were studied in a more limited pH range. Throughout the investigation, the zwitterionic buffers Hepes [4-(2-hydroxyethyl)-1-piperazine-ethane sulfonic acid] and Mes [2-(*N*-morpholino) ethane sulfonic acid] were used at a concentration of 0.05 M. The ionic strength was changed by inclusion of various amounts of K₂SO₄. The orientation was found to be 65% cytochrome *a* on the outside of the vesicle. Consequently, 65% of the total concentration was used when the kinetic parameters were calculated.

Principal results and conclusions are as follows: At high ionic strength ($I=0.5$) k_{cat} increases with decreasing pH from 35 s⁻¹ at pH 8.6 to 1000 s⁻¹ at pH 5.2. This result is qualitatively in accordance with one earlier study, with the enzyme in detergent solution, whereas most other investigators have reported a pH maximum for k_{cat} . The pH dependence of k_{cat} cannot be simulated with less

than three pK_a values, which suggests at least three sites, each increasing the activity on protonation. Our estimated pK_a values (7.8, 6.8 and 4.5) are within experimental uncertainty the same as those found in detergent solution.

Variation of the ionic strength from $I=0.05$ to $I=0.5$ has little effect on k_{cat} but k_{cat}/K_m , and thus K_m , is a function of ionic strength. The velocity at a given cytochrome *c* concentration generally decreases with increasing ionic strength. At low pH (6.2) the effect seems to be more complicated as our results show a maximum of k_{cat}/K_m around $I=0.25$. k_{cat}/K_m was essentially constant at high ionic strength ($I=0.5$) in the pH range 5.2-8.6, *i.e.* K_m also increases with decreasing pH. This suggests that the pH effect is mainly on the rates of catalytic steps, rather than on the combination between enzyme and substrate. It is not obvious which particular steps are affected.

The effects of D₂O were studied in the pD range 5.8-7.2. Taking into account both the difference between pH and pD and the D₂O-induced shift in pK_a ($pK_a^D = pK_a^H + 0.54$) we found that D₂O decreases k_{cat} by a factor of about 2. k_{cat}/K_m is, on the other hand, approximately the same in H₂O and D₂O. From the D₂O-data we conclude that an intramolecular proton transfer step is at least partially rate limiting. This contradicts the common assumption that the product dissociation step of the reaction is rate limiting.

Based on our results and a minimal mechanism involving 13 consecutive steps in the catalytic cycle of cytochrome oxidase, we argue that an intramolecular step which involves both electron and proton transfer is rate limiting. The step is suggested to be important in the function of cytochrome oxidase as a proton pump.