Time-Resolved Resonance Raman Spectroscopy of Transient Species Formed during the Oxidation of Cytochrome Oxidase by Dioxygen

Gerald T. Babcock,*‡ John M. Jean,† Leah N. Johnston,† Graham Palmer,*§ and William H. Woodruff*†

Department of Chemistry
University of Texas at Austin, Austin, Texas 78712
Department of Chemistry, Michigan State University
E. Lansing, Michigan 48824-1322
Department of Biochemistry, Rice University
Houston, Texas 77251
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Cytochrome oxidase catalyzes the rapid and efficient reduction of dioxygen to water. The sequence of events that occurs in this mechanistically complex, four electron/four proton reaction involves oxygen binding at the cytochrome a_3 Fe^{2+}–Cu_B^{1+} site followed by electron transfer from these metals and from the more remote cytochrome a Fe^{2+} and Cu_A^{1+} centers.[1] More detailed information on this process, particularly on the structures of the initial oxygen adduct and of the partially reduced intermediates, has been difficult to obtain owing to the rapid rates of O_2 binding ($\sim 10^8$ M^{-1} s^{-1}) and electron transfer ($\sim 10^5$ to 10^3 s^{-1}) reactions. To overcome this kinetic obstacle, we have combined rapid mixing and flash photolysis methods[2] with time-resolved resonance Raman techniques so that we can use this structure-specific vibrational spectroscopy to study the cytochrome oxidase/oxygen reaction at room temperature. We demonstrate here the feasibility of this approach and provide information on early events in the reduction of O_2.

Cytochrome oxidase was prepared from beef heart[3a] and, following anaerobic reduction with 4 mM sodium ascorbate and 1 μM cytochrome c,[2e] combined with carbon monoxide. The solution was loaded into one syringe and oxygenated buffer (50 mM HEPES, 0.5% Lauryl maltoside, pH 7.4) into a second syringe. These were mounted on a syringe pump and connected to a Gibson-type, four-jet tangential mixer.[4] The solution (oxidase concentration after mixing, 25–40 μM) flowed out of the mixer into the Raman cuvette, usually 1 mm inside diameter quartz capillary, upon which the Q-switched pulses (10-ns duration) of two Quanta Ray Nd:YAG lasers were line focused. The pulse from the first laser was the 532-nm second harmonic of the Nd:YAG fundamental. Its energy was typically 2.5 mJ, sufficient to photolyze the CO cytochrome oxidase complex and initiate the O_2 reduction reaction. Light scattered from the second, time-delayed 416-nm pulse, obtained from the first Stokes stimulated Raman shift by H_2 gas of the 355-nm Nd:YAG third harmonic, was dispersed by a spectrograph and detected by an EG&G/PARC 1420 image-intensified multichannel photodiode array.[5]

Figure 1 shows Raman spectra of cytochrome oxidase at various times after initiation of the oxidation reaction. The 10-ns spectrum

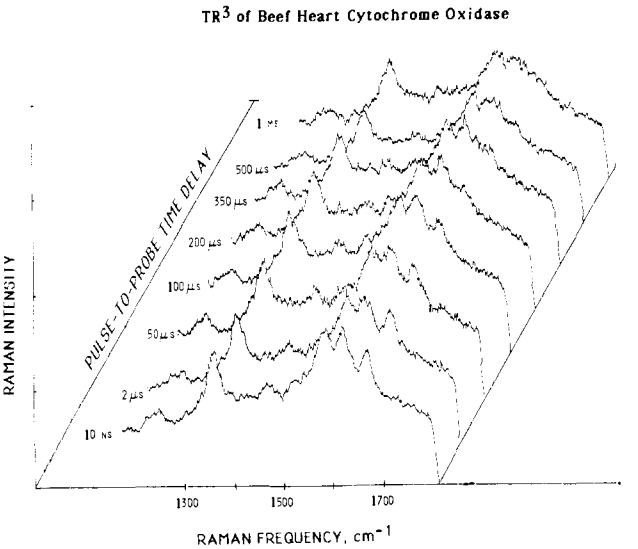

Figure 1. Raman spectra of cytochrome oxidase at the indicated times following initiation of the oxygen reduction reaction at room temperature. Each spectrum was obtained as the average of 115 pump–delay–probe experiments. The energy of the 532-nm photolysis pump pulse was 2.5 mJ, that of the 416 Raman probe pulse was 2.0 mJ; a fairly tight line focus on the sample was used for both beams. The spectrometer entrance slit was 300 μm, which provides a spectral resolution of 9 cm^{-1}. The enzyme concentration after mixing was 35 μM in oxidase; the oxygen concentration was not measured but is estimated at 600 μM after mixing as O_2-saturated buffer was used in the second syringe (above 250 μM O_2 the rate of the reoxidation reaction becomes markedly less dependent on O_2 concentration[2e]).

is that of the photodissociation product of the reduced carbon monoxy enzyme. The oxidation-state marker ($\tilde{\nu}_4$ 1355 cm^{-1}) and the a_3^{2+} formyl stretching frequency ($\tilde{\nu}_{C=O}$ 1666 cm^{-1})[3] are well resolved and, in agreement with a recent anaerobic study of this species,[6] indicate that the reduced deoxy enzyme has been generated. As the reaction proceeds, the 1355-cm^{-1} mode shifts to higher frequency[7] and the 1666-cm^{-1} mode decreases in intensity, indicating that oxidation of the protein is occurring. Control experiments show that the time course of these changes is oxygen concentration dependent.

The data in Figure 1 and the difference spectrum in Figure 2a indicate that little change in the observed vibrational frequencies occurs during the first 50 μs of reaction. This is surprising in view of time-resolved optical data[2] which have clearly established that oxygen addition and, possibly, partial oxidation of the metal centers should have occurred in this time range at the oxygen concentration we have used. The initial oxygen adduct, however, has been suggested to be oxycytochrome a_3,[8,9] i.e., an oxygenated heme a species similar in electronic properties to oxyhemoglobin and oxymyoglobin. If this were the case, then photodissociation of this initial oxygen adduct may occur as photodissociation of oxy-Hb and oxy-Mb is well-known.[10] Evidence that the early

† University of Texas at Austin.
‡ Michigan State University.
§ Rice University.

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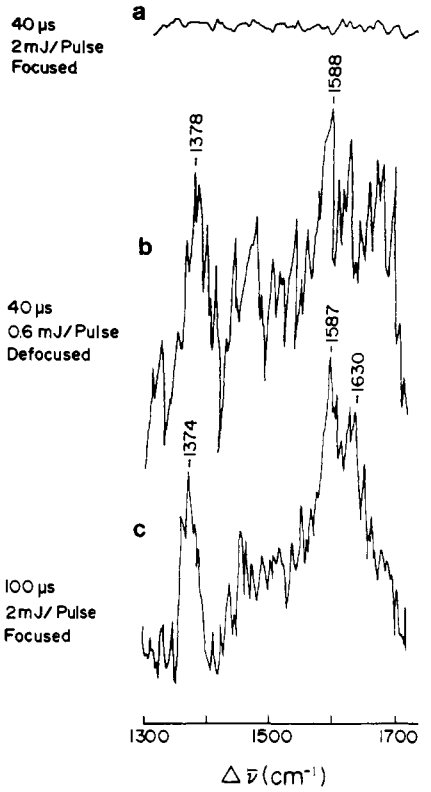

Figure 2. Estimates of the Raman properties of intermediate species formed during the reoxidation of cytochrome oxidase by O_2 at 40 (a,b) and 100 μs (c) after initiation. For spectra (a) and (c) data taken under the high power density conditions of Figure 1 were used. The scaled spectrum of the reduced photodissociated enzyme (the 10-ns spectrum in Figure 1) was subtracted from the experimental spectra so that cancellation of the reduced oxidation-state marker (1355 cm^{-1}) occurred. In the 40-μs 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. A total of 1320 experiments were averaged in (b); the other experimental conditions were the same as those in Figure 1.

intermediate(s) are, in fact, photolabile is supplied by the 40-μs difference spectrum (Figure 2b) obtained by using a low-intensity, defocused probe pulse.[11] Under these conditions, the principal intermediate at 40 μs is shown to have an oxidation-state marker ($\bar{\nu}_4$) at 1378 cm^{-1} and spin-state marker ($\bar{\nu}_2$)[3] at 1588 cm^{-1}. These frequencies are similar to those reported for oxymyoglobin and oxyhemoglobin. This observation, together with the photolability of the 40-μs transient, provides the best support to date for the suggestion that the reoxidation of cytochrome oxidase involves an oxycytochrome a_3 species at early times in the reaction.

The difference spectrum in Figure 2c (100-μs spectrum minus 10-ns photolysis product spectrum) was obtained under high light intensity conditions and 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. Thus nonphotolabile intermediates are formed within 100 μs of initiation of reoxidation. Whether these species are actually ferric heme *a* intermediates or low-spin ferrous heme *a* complexes with strong π-acid ligand remains to be determined.

The results above establish a number of points regarding the oxidation of cytochrome oxidase by O_2. First, time-resolved resonance Raman, which offers greater molecular insight than optical spectroscopy and does not require paramagnetism in order to visualize intermediates, can be profitably applied to the study of the reaction. Second, the initial intermediate in the reaction mechanism is photolabile and indicates the formation of oxycytochrome a_3 as the precursor of dioxygen reduction. This conclusion may seem to be at odds with low-temperature work reported by Chance et al.[8] who described their compound A as "non photolabile", but their observation most likely results from the fact that oxyheme species have much lower photolysis quantum yields than carbon monoxy heme species. Whether a peroxy-type intermediate[8,12] will be photolabile remains to be established. Third, at reasonably early times in the reaction (~100 μs) heme iron oxidation apparently occurs and the photolysis quantum yield decreases significantly or goes to zero. Fourth, we note the apparent disappearance at longer times (>200 μs) of the 1666/1676-cm^{-1} cytochrome a_3 formyl C=O stretch. This mode is always observed in stable forms of the enzyme,[3] and its absence may suggest unusual reactivity of the formyl group in the turnover dynamics of the enzyme. The structures of these species, as well as those that occur in the mixed valence oxidase/O_2 reaction,[13] are under investigation.

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Registry No. O_2, 7782-44-7; cytochrome oxidase, 9001-16-5.

(11) Control experiments in which the same illumination geometry as that used for Figure 2b was used to study oxymyoglobin showed that the oxy species, rather than the photodissociation product, predominated in the spectrum. The poor signal-to-noise ratio in Figure 2b results from the suboptimal illumination conditions necessary to record the spectrum.

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Copper(II), a Chemical Janus: Two Different (Oxalato)(bipyridyl)copper(II) Complexes in One Single Crystal. Structure and Magnetic Properties

Miguel Julve* and Juan Faus

Departamento de Chimica Inorgânica
Facultad de Ciencias Químicas
Burjasot, Valencia, España

Michel Verdaguer*†

Laboratoire de Spectrochimie des Elément
de Transition, ERA 672
Université de Paris Sud, 91405 Orsay, France

Alain Gleizes

Laboratoire de Chimie de Coordination, C.N.R.S.
31400 Toulouse, France
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In our attempts to tune the exchange interaction between two copper(II) ions through multiatomic bridges[1-4] we obtained single crystals of $[Cu_2(bpy)_2(H_2O)_2(C_2O_4)][(Cu(bpy)(C_2O_4)](NO_3)_2$ (**1**) (bpy = 2,2'-bipyridyl) where both mononuclear and binuclear copper(II) complexes are present in the same cell. This is a rare case where two molecular complexes of copper(II) bound to the same ligands coexist in the same compound with different stoi-

† Permanent address: E.N.S., 92211 Saint Cloud, France.

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