smaller than the 2.2–2.9 G ^{15}N couplings observed in $^2A_{2u}$ systems such as ZnTPP$^+$.[2] The nitrogen hyperfine coupling in a $^2A_{1u}$ radical has never been measured directly; however, an estimate of 1 G for a ^{15}N coupling would seem reasonable for a purely $^2A_{1u}$ ground state.[2,15,16] Thus the couplings we observe suggest that some mixing of the $^2A_{2u}$ state into the $^2A_{1u}$ ground state may occur. This type of mixing has earlier been proposed by O'Malley and Babcock to account for ^1H couplings observed in the ENDOR spectrum of the oxidized reaction center chlorophyll in the Photosystem I of plant photosynthetic membranes.[17] Recently, Czernuszewicz et al. have addressed this question for MgOEP$^+$ and have concluded from Raman spectra that the A_{2g} vibrational modes mix the $^2A_{2u}$ state into a predominately $^2A_{1u}$ ground state.[7c] While the possibility of orbital mixing and the mechanism by which it may occur remain to be settled, the ENDOR data we have presented here indicate that both the dihalide and diperchlorate cations are predominately $^2A_{1u}$ in character, regardless of their visible absorption spectra.

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(16) Vincow, G. In *Radical Ions*; Kaiser, E. T., Kevan, L., Eds.; Interscience: New York, 1968; pp 151–209.
(17) O'Malley, P. J.; Babcock, G. T. *Proc. Natl. Acad. Sci. U.S.A.* **1984**, 1098–1101.

Time-Resolved Raman Detection of ν(Fe–O) in an Early Intermediate in the Reduction of O_2 by Cytochrome Oxidase

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Cytochrome oxidase contains four redox-active centers per functional unit: cytochromes *a* and a_3 and the copper atoms, Cu_A and Cu_B. Cytochrome *c*, the physiological substrate of cytochrome oxidase, transfers electrons to the cyt *a* and Cu_A sites. These reducing equivalents are transferred to the binuclear cyt a_3···Cu_B center, which binds O_2 and reduces it to H_2O. Although the reaction between O_2 and cytochrome oxidase occurs too quickly to be studied by conventional stopped-flow techniques, Gibson and Greenwood[1] showed that photolysis of the cytochrome a_3^{2+}–CO complex of the enzyme in the presence of O_2 could be used to circumvent this limitation. Babcock et al.[2] adopted this approach and used time-resolved resonance Raman spectroscopy to study the reaction of fully and partially reduced cytochrome oxidase with O_2. Although instrumental limitations restricted data acquisition to the high-frequency (>1000 cm^{-1}) range, they concluded that the reoxidation of cytochrome oxidase proceeds via a cytochrome a_3^{2+}–O_2 complex that resembles oxymyoglobin and oxyhemoglobin. Direct detection of iron/bound oxygen ligand vibrations is necessary to test these conclusions as well as to provide detailed information on subsequent intermediates in the dioxygen reduction reaction. To this end we have developed techniques that allow us to carry out low-frequency Raman detection under flow/flash conditions; here we report ν(Fe^{2+}–O) for an early intermediate in the cytochrome oxidase/dioxygen reaction. Consideration of data on dioxygen adducts of other heme proteins and model hemes indicates that this early intermediate is most likely the cytochrome a_3^{2+}–O_2 complex. Its ν(Fe^{2+}–O_2) frequency is elevated relative to that observed for the other systems, however, from which we conclude that already at 10 μs in the oxidase reaction the O=O bond is weakened, presumably in preparation for its rupture in subsequent steps in the reaction.

The experimental techniques used for the measurements of time-resolved Raman spectra have already been reported[2b] with the exceptions that the scattering volume was a free jet in air and that 427-nm excitation was used as the probe wavelength.[3] The

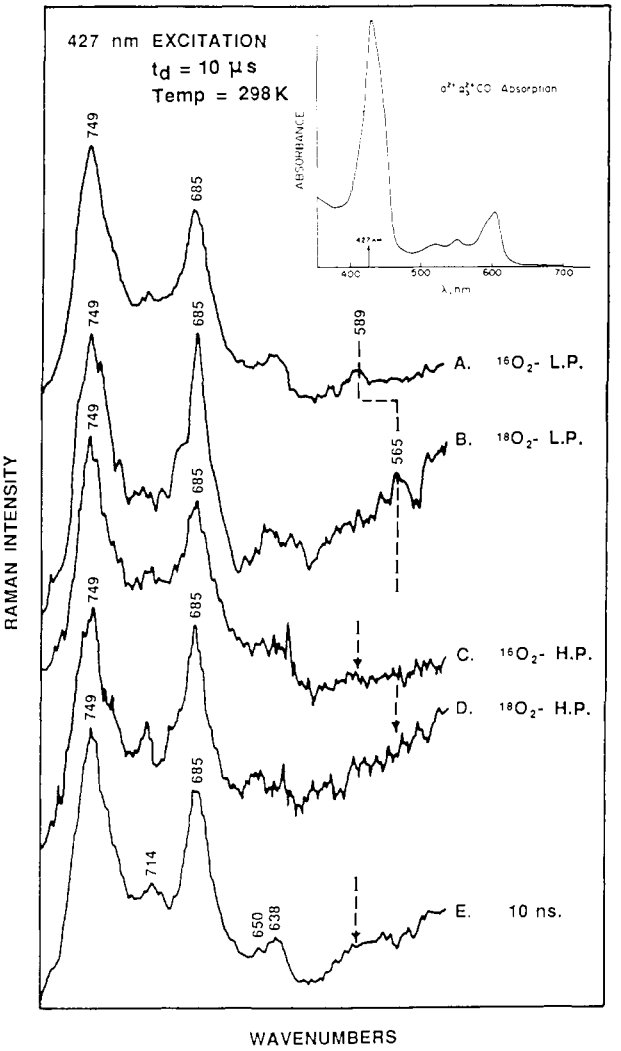

Figure 1. Time-resolved resonance Raman spectra of cytochrome oxidase following initiation of the reaction with oxygen at room temperature. The energy of the 532-nm photolysis pump pulse was 1.3 mJ, sufficient to photolyze the enzyme–CO complex and initiate the O_2 reduction reaction. The energy of the probe beam was 0.3 mJ for spectra A and B[14] and 1.0 mJ for spectra C–E. The repetition rate for both the pump and probe pulses (10-ns duration) was 10 Hz. The pump–probe delay was 10 μs for spectra A–D and 10 ns for transient spectrum E. The accumulation time was 110 min for spectrum A, 70 min for spectrum B, 5 min for spectra C and D, and 15 min for spectrum E.

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(1) (a) Gibson, Q.; Greenwood, C. *Biochem. J.* **1963**, *86*, 541. (b) Greenwood, C.; Gibson, Q. *J. Biol. Chem.* **1967**, *242*, 1782. (c) Greenwood, C.; Hill, B. *Biochem. J.* **1984**, *218*, 913. (d) Hill, B.; Greenwood, C.; Nichols, P. *Biochim. Biophys. Acta* **1986**, *853*, 91. (e) This technique has been modified for low-temperature application by Chance and co-workers and has been used to study the oxidase/O_2 reaction in frozen samples by several groups. See: Chance, B.; Saronio, C.; Leigh, J. S. *J. Biol. Chem.* **1975**, *250*, 9226. Clore, G. M.; Andreasson, L. E.; Karlsson, B.; Aasa, R.; Malmstrom, B. G. *Biochem. J.* **1980**, *185*, 139. Chan, S. I.; Witt, S. N.; Blair, D. F. *Chem. Scr.* **1988**, *28A*, 51. For a review, see: Naqui, A.; Chance, B. *Annu. Rev. Biochem.* **1986**, *55*, 137.

(2) (a) Babcock, G. T.; Jean, J. M.; Johnston, L. N.; Palmer, G.; Woodruff, W. H. *J. Am. Chem. Soc.* **1984**, *106*, 8305. (b) Babcock, G. T.; Jean, J. M.; Johnston, L. N.; Woodruff, W. H.; Palmer, G. *J. Inorg. Biochem.* **1985**, *23*, 243. (c) Babcock, G. T. *Biological Applications of Raman Scattering*; Spiro, T. G., Ed.; Academic Press: New York, 1988; Vol. 3, p 295.

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Table I. Vibrational Frequencies for Dioxygen-Bound Complexes[a]

	ν(Fe–O), cm^{-1}	ref
cytochrome oxidase	589	this work
Hb	567	11
Mb	570	9
HRP III	562	9
Im (heme *a*)Fe^{2+}–O_2	576	7
(TMP)Fe–O–O–Fe(TMP)	574	6
(Pip)(TPP)Fe^{2+}–O_2	575	12
(TPP)Fe^{2+}–O_2	509	12

[a] Abbreviations: Hb, hemoglobin; Mb, myoglobin; HRP, horseradish peroxidase; Im, imidazole; Pip, piperazine; TPP, *meso*-tetraphenylporphyrin.

absorption spectrum of the enzyme–CO complex (inset Figure 1) shows a Soret maximum at 430 nm as expected.[4]

Time-resolved resonance Raman spectra of cytochrome oxidase at 10 μs subsequent to carbon monoxide photolysis in the presence of O_2 are shown in Figure 1A–D. Spectrum E is that of the photodissociation product of the reduced carbonmonoxy enzyme (pump–probe delay = 10 ns). Spectrum A, obtained with a low-energy, defocused beam (0.3 mJ), is similar to the 10-ns spectrum with the exception that a new mode appears at 589 cm^{-1}. Figure 1B shows that the 589-cm^{-1} mode in the $^{16}O_2$ spectrum is downshifted to 565 cm^{-1} when the experiment is repeated with $^{18}O_2$. This allows us to assign it as the iron–oxygen stretching motion in the cytochrome $a_3{}^{2+}$–O_2 complex, as the 24-cm^{-1} shift is in agreement with that expected from the two-body harmonic oscillator approximation for Fe^{2+}–O_2. Spectra C and D were obtained with relatively high energies (1 mJ), and the absence of modes located at 589 cm^{-1} (Figure 1C, $^{16}O_2$) and 565 cm^{-1} (Figure 1D, $^{18}O_2$) indicates photodissociation of the oxy ligand, as was observed in the high-frequency experiments[2,5] and further supports our assignment of the 589-cm^{-1} mode in the cytochrome oxidase/O_2 complex.

The most reasonable assignment of the 589-cm^{-1} mode is that it arises from a cytochrome $a_3{}^{2+}$–O_2 complex. Such an assignment is consistent with the photolability of this species,[2b] but more important, it is in reasonable agreement with $\nu(Fe^{2+}–O_2)$ frequencies observed in other heme Fe^{2+}–O_2 complexes. Table I summarizes several of these frequencies; the 589-cm^{-1} mode for the oxidase intermediate is similar to, but slightly higher than, $\nu(Fe^{2+}–O_2)$ for several dioxygen-bound heme species. Several further points can be made from Table I. First, the $\nu(Fe^{2+}–O_2)$ in the oxidase intermediate is 13 cm^{-1} higher than that of the imidazole–heme *a* Fe^{2+}–O_2 complex, despite the fact that the model compound reproduces the immediate coordination sphere that is expected to occur around the iron in the protein environment. We regard this increase as mechanistically significant, as discussed below. Second, the oxidase species has a frequency that is close to the 574 cm^{-1} observed for the iron–oxygen stretching frequency for the five-coordinate μ-peroxo dimer reported by Nakamoto and co-workers.[6] Despite the similarity in those two frequencies, we nevertheless favor a cyt $a_3{}^{2+}$–O_2 structure for the intermediate we detect. The basis for this lies in our expectation that cytochrome a_3 will retain its proximal histidine ligand during catalysis and that this trans ligand will significantly perturb the iron–oxygen stretching frequency in a peroxy a_3 species relative to the five-coordinate model compound.[7] A similar frequency perturbation in the iron–oxygen stretching frequency as a result of trans-ligand effects is apparent in Table I when one compares the five-coordinate (TPP)Fe^{2+}–O_2 complex (ν(Fe–O) = 509 cm^{-1}) to the six-coordinate (Pip)(TPP)Fe^{2+}–O_2 species (ν(Fe–O) = 575 cm^{-1}) and is also apparent in heme ferryl oxo species.[7]

As noted above, the iron–oxygen stretching frequency in the oxidase intermediate is elevated relative to the other systems for which data exist. In fact, it is the highest $\nu(Fe^{2+}–O_2)$ reported for heme iron–dioxygen complexes. This suggests that a stronger Fe–O bond exists in the oxidase intermediate than is normally encountered. Because an inverse relationship between Fe–O and O=O bond strengths is expected, as we argue elsewhere,[7] a weaker O=O bond is thus likely in the cytochrome a_3–dioxygen adduct. Such an observation is consistent with the dioxygen bond cleaving and reducing functions of the oxidase. It also suggests the subsequent formation of a bona fide peroxy intermediate prior to O=O bond cleavage, as has been long suspected in the oxidase catalytic cycle.[2a,8,13]

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(3) The details of the design and construction of the mixer and jet will be published elsewhere: Varotsis, C.; Babcock, G. T., in preparation. The 427-nm, 10-ns pulses were obtained by pumping stilbene with the third harmonic of a Quanta Ray DCR2A Nd:YAG laser.
(4) Vanneste, W. H. *Biochemistry* **1966**, *5*, 838.
(5) Varotsis, C.; Babcock, G. T., unpublished results.
(6) Paeng, I. R.; Shiwaku, H.; Nakamoto, K. *J. Am. Chem. Soc.* **1988**, *110*, 1995.
(7) Oertling, W. A.; Kean, R. T.; Wever, R.; Babcock, G. T. *J. Am. Chem. Soc.*, submitted.
(8) Blair, D. F.; Witt, S. N.; Chan, S. I. *J. Am. Chem. Soc.* **1985**, *107*, 7389.
(9) Van Wart, H.; Zimmer, J. *J. Biol. Chem.* **1985**, *250*, 8372.
(10) Wikström, M.; Krab, K.; Saraste, M. *Cytochrome Oxidase, A Synthesis*; Academic Press: New York, 1981.
(11) Brunner, H. *Naturwissenschaften* **1974**, *61*, 129.
(12) Nakamoto, K.; Paeng, I. R.; Kuroi, T.; Iosbe, T.; Oshio, H. *J. Mol. Struct.* **1988**, *189*, 293.
(13) Wikström, M. *Proc. Natl. Acad. Sci. U.S.A.* **1981**, *78*, 4051.
(14) A loose point focus at the sample was used to decrease the probe power density. We estimate that the probe power density used to record spectra A and B was 12-fold less than in spectra C–E.

The Geometry of Reactive Intermediates by Nutation NMR Spectroscopy: The *tert*-Butyl Cation

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The use of superacidic liquid or solid matrices at low temperature permits the study of very reactive carbocations for extended periods of time,[1] and variable temperature NMR studies under these conditions have provided a wealth of dynamic and qualitative structural information.[1,2] By contrast, determinations of the geometry (bond lengths and angles) of carbocation salts by diffraction methods are rare. This is not from lack of effort.[3,4d] Reports of the few successful X-ray studies of reactive carbocations provide ample evidence of the difficulty in obtaining suitable single crystals.[4] To our knowledge, X-ray structures of only two carbocations that are not stabilized by heteroatoms or π-system delocalization have been reported, and both of these have numerous

(1) Recent reviews include the following: (a) Olah, G. A.; Surya Prakash, G. K.; Sommer, J. *Superacids*; Wiley: 1985. (b) Vogel, P. *Carbocation Chemistry*; Elsevier: 1985. (c) Ahlberg, P.; Jonsall, G.; Engdahl, C. *Adv. Phys. Org. Chem.* **1983**, *19*, 223.
(2) Saunders, M.; Chandrasekhar, J.; Schleyer, P. v. R. *Rearrangements in Ground and Excited States*; Academic Press: 1980; pp 1–53.
(3) Dinnocenzo, J. P. Ph.D. Thesis, Cornell University, 1985.
(4) (a) Childs, R. F.; Faggiani, R.; Lock, C. J. L.; Mahendran, M. *J. Am. Chem. Soc.* **1986**, *108*, 3613–3617. (b) Montgomery, L. K.; Grendze, M. P.; Huffman, J. C. *J. Am. Chem. Soc.* **1987**, *109*, 4749–4750. (c) Laube, T. *Angew. Chem., Int. Ed. Engl.* **1986**, *25*, 349. (d) Laube, T. *Angew. Chem., Int. Ed. Engl.* **1987**, *26*, 560. (e) For a review of earlier work on more stable ions, see: Sundaralingam, M.; Chwang, A. K. In *Carbonium Ions*; Olah, G. A., Schleyer, P. v. R., Eds.; Wiley: New York, 1976; Vol. 5, Chapter 39.

0002-7863/89/1511-6440$01.50/0