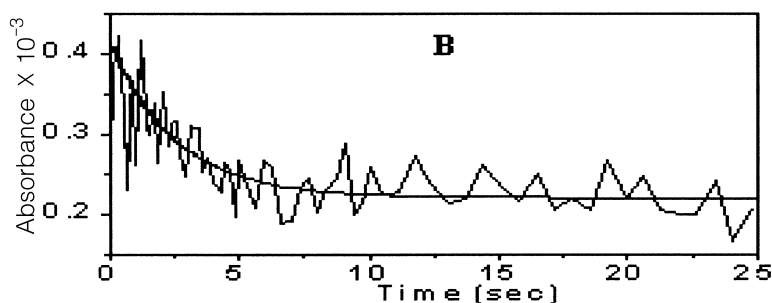Communication

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# Stopped-Flow Fourier Transform Infrared Spectroscopy of Nitromethane Oxidation by the Diiron(IV) Intermediate of Methane Monooxygenase

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Stopped-flow Fourier transform infrared (SF-FTIR) spectroscopy has been used to monitor directly the kinetics of hydroxylation of a substrate by the key di($\mu$-oxo)diiron(IV) enzyme intermediate in the catalytic cycle of the hydroxylase (MMOH) enzyme component of soluble methane monooxygenase (sMMO) isolated from *Methylococcus capsulatus* (Bath).[1,2] The experiments were performed under single-turnover conditions using the alternative substrate deutero-nitromethane ($CD_3NO_2$).[3] This substrate has increased solubility, more intense IR bands, and a relatively slow rate of hydroxylation as compared to the natural substrate methane.

sMMO comprises three proteins. MMOH is a $\alpha_2\beta_2\gamma_2$ heterodimer (251 kDa) containing two functionally independent dinuclear, carboxylate-bridged iron sites at which dioxygen is activated and substrates are hydroxylated.[3–8] A coupling protein (MMOB) regulates activity, and a reductase (MMOR) transfers reducing equivalents from NADH to the hydroxylase using an FAD cofactor and a 2Fe–2S cluster.[1,2] The intermediate form of the hydroxylase enzyme that is thought to insert an oxygen into a C–H bond of substrates is designated Q, which various spectroscopic measurements have assigned as having a di($\mu$-oxo)diiron(IV) core.[9–11] The relatively slow rate of the reaction, observed previously for $CD_3NO_2$ using stopped-flow spectrophotometry to monitor Q decay,[3] brings the kinetics of this single-turnover reaction into a range accessible by SF-FTIR, allowing for the first time direct monitoring of the hydroxylation of a methane-derived substrate. This result provides stronger evidence than that currently in the literature, which only allowed the conclusion that Q may interact directly with the substrate, nitrobenzene in that case.[12] In addition, nitromethane has advantages as an analogue of the natural substrate methane over analogues such as nitrobenzene, because the size of the substrate and products can influence the kinetic profile of the reaction.[13] MMO is of considerable environmental importance in terms of bioremediation by microorganisms and of commercial interest in the development of novel catalysts for the petrochemical industry.

A stopped-flow Fourier transform infrared (SF-FTIR) apparatus capable of operating under strictly anaerobic conditions has been developed to elucidate enzyme mechanisms and related chemistry, in particular, the activation of small molecules by metalloenzymes.[14–16] The apparatus can monitor time-dependent ($>25$ ms) changes in the amplitude of IR bands ($>5 \times 10^{-5}$ absorbance units) against a high background (ca. 0.8 absorbance units) because of solvent and protein amide bands in the $1000-3000$ cm$^{-1}$ range. The stopped-flow circuit and CaF$_2$ cell are essentially the same as described previously.[14–16] Multimixing experiments were performed by using a manually operated, two-syringe, premixing device that allowed Q, generated by the reaction of reduced MMOH with

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dioxygen in the presence of MMOB, to be injected into a loop (100 $\mu$L volume) that had been incorporated into the main stopped-flow circuit. Subsequent mixing of $CD_3NO_2$ with Q was achieved within ca. 3 s of Q formation. The drive system with a manual premixer and thermostated IR cell with integral mixer were entirely located in an anaerobic and dry glovebox operated under N$_2$ with $<2$ ppm O$_2$ (Belle Technology, Portesham, Dorset, U.K.). The IR cell path length was calibrated to be 33 $\mu$m by fringe pattern analysis. FTIR peak intensity as a function of time was determined by fitting an averaged spectrum similar to that in Figure 1A to a sum of Voigt function line shapes. The peak widths and positions were then constrained, and, for each spectrum of the time course, peak intensities and a baseline function were allowed to float. This approach significantly reduced the "noise" in the time course and eliminated any time-dependent baseline instabilities or contributions from other time-dependent changes. It also ensured that only changes in the specific band of interest were determined.[14,15]

The hydroxylase component, MMOH, was isolated from *Methylococcus capsulatus* (Bath) as previously described.[17,18] Recombinant component B was expressed in *E. coli* and purified using published procedures.[18,19] Both components were exchanged into D$_2$O ($>90\%$) by three sequential concentration and dilution cycles (total time ca. 1 h, at 5 °C) using a Centricon YM-10 centrifugal filter device (Millipore, Bedford, MA). Reduced MMOH was prepared as follows: A solution (1 mL) containing a 1:2 molar ratio of MMOH (110 $\mu$M) and component B (220 $\mu$M) with sodium dithionite (450 $\mu$M) as reducing agent and methyl viologen (450 $\mu$M) as electron-transfer mediator was prepared under anaerobic conditions and then dialyzed in a glovebox overnight against D$_2$O/ MOPS buffer (200 mL, 25 mM, pD 7.0). This solution was premixed with dioxygen saturated D$_2$O/MOPS buffer to generate Q (55 $\mu$M), which was mixed within 3 s with $CD_3NO_2$ (100 mM in 25 mM MOPS/D$_2$O buffer, pD 7.0, 25 °C). Final mixed concentrations in the SF-FTIR cell were [Q] = 27.5 $\mu$M (55 $\mu$M active sites) and [$CD_3NO_2$] = 50 mM. Typically, a spectral resolution of 4 cm$^{-1}$ and a time resolution of 25 ms were used for the measurements. UV–visible stopped-flow kinetic experiments were performed by using a Hi-Tech SF-61 DX-2 double-mixing stopped-flow spectrometer (Hi-Tech Scientific, Salisbury, U.K.) interfaced with a CU-61 control unit installed in an anaerobic glovebox as previously described.[16] Sample preparation and reaction conditions were identical to those described above for SF-FTIR, except that the final mixed concentration of Q was 3.75 $\mu$M with 7.5 $\mu$M of component B in the cell (10 mm path length). Both the SF-FTIR and the SF-UV–visible data were analyzed with the KineticAsyst 3.0 software package (Hi-tech Scientific, Salisbury, U.K.). The data were well fit to a single-exponential function.

The asymmetric NO$_2$ bending vibration at 1557 cm$^{-1}$ of CH$_3$NO$_2$ shifts to 1548 cm$^{-1}$ in $CD_3NO_2$ with an experimentally
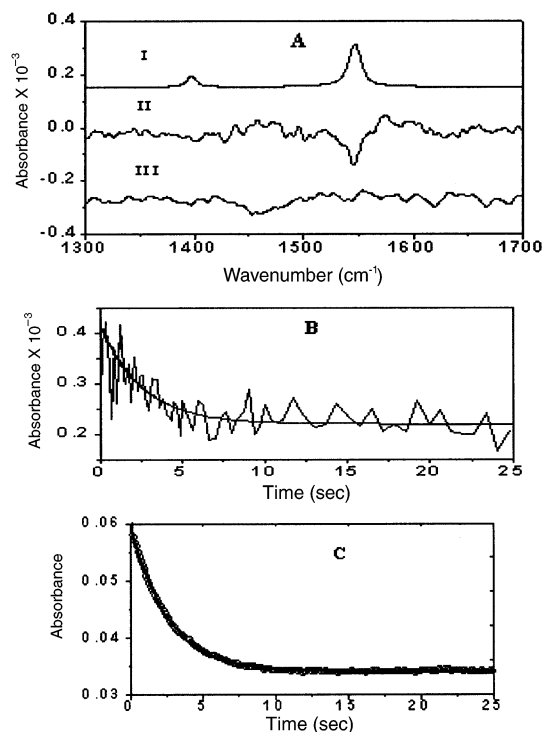
**Figure 1.** (A) Spectra illustrating the consumption of $CD_3NO_2$ by Q. (I) 50 $\mu$M $CD_3NO_2$ in $D_2O$. (II) Time-difference spectra from the reaction. The spectrum measured between 0.3 and 0.7 s has been subtracted from that at 15−20 s. A time-dependent infrared spectrum associated with the decay of Q has been subtracted from the data for clarity. (III) A control time-difference spectrum identical to (II) except that $O_2$ was omitted from the reaction mixture. (B) Time course of $CD_3NO_2$ consumption monitored at 1548 $cm^{-1}$ by SF-FTIR for Q (27.5 $\mu$M, 55 $\mu$M active sites) that was allowed to react with $CD_3NO_2$ (50 mM). Data were fit to a single-exponential function ($k_{obs} = 0.45 \pm 0.07$ $s^{-1}$). The amplitude ($1.7 \times 10^{-4}$ absorbance units) corresponds to the loss of 55 $\mu$M $CD_3NO_2$ equivalent to the active site Q concentration and represents 0.1% of the total $CD_3NO_2$ signal. (C) Decay of Q monitored by SF-UV−visible spectrophotometry at 420 nm when 3.75 $\mu$M (7.5 $\mu$M active sites) Q reacted with $CD_3NO_2$ (50 mM). The data were fit to a single-exponential function ($k_{obs} = 0.39 \pm 0.01$ $s^{-1}$). The amplitude (0.026 absorbance units) corresponds to the reduction of 7.2 $\mu$M Q-active sites. Both experiments were performed at 25 °C, pD 7.0 as described in the text.

determined extinction coefficient of 0.97 $mM^{-1}$ $cm^{-1}$. This IR band was used to monitor $CD_3NO_2$ hydroxylation during a single-turnover reaction with Q. The consumption of $CD_3NO_2$ is clearly apparent in the ∼17−0.5 s time-difference spectrum (Figure 1A, trace II). No change in intensity was seen in control experiments where $O_2$ was excluded (Figure 1A, trace III). The time course data (Figure 1B) are fit by a single exponential with $k_{obs} = 0.45 \pm 0.07$ $s^{-1}$ and an amplitude of $1.76 \times 10^{-4}$ absorbance units. This amplitude corresponds to the consumption of 55 $\mu$M $CD_3NO_2$, which is stoichiometric with the concentration of Q active sites. The decay of Q was monitored by SF-UV−visible spectrophotometry at 420 nm (Figure 1C). The single-exponential data returned $k_{obs} = 0.39$ $s^{-1}$ and an amplitude of 0.026 absorbance units which, assuming $\epsilon_{420} = 7200$ $M^{-1}$ $cm^{-1}$, equates to 7.2 $\mu$M active sites in agreement with 7.5 $\mu$M calculated from the initial MMO concentration. Note that $\epsilon_{420}$ is quoted per mole of MMO, which contains 2 active sites. These results are consistent with previous SF-UV−visible results that first demonstrated[3] saturating kinetics for Q-decay with $CD_3$-$NO_2$ as a substrate. The previously determined values of $k_2 = 0.66$ $s^{-1}$ and $K_d = 18 \pm 2$ mM predict a $k_{obs} = 0.48 \pm 0.01$ $s^{-1}$ at the nonsaturating concentration of $CD_3NO_2$ (50 mM) used in the present

experiments. In the present study, however, the solvent was $D_2O$ at 25 °C, whereas previous work was performed in $H_2O$ at 20 °C.[3] We have not been able to detect by SF-FTIR the appearance of the final products of the reaction, formaldehyde and nitrite, which are presumably formed via a $DOCD_2NO_2$ intermediate. Detection is difficult because the nitrite band at 1237 $cm^{-1}$ is weak, broad, and located at the edge of the $D_2O$ absorption window, and the formaldehyde bands are within the protein amide envelope. Hence, we are unable at this point to confirm by SF-FTIR whether these products are produced under single-turnover conditions. However, we have identified formaldehyde and nitrite as products under steady-state assay conditions using established colorimetric methods.[20,21]

In conclusion, we have demonstrated that SF-FTIR is a powerful technique for investigating the MMOH enzyme mechanism, particularly when the substrate cannot be observed by more established techniques, such as stopped-flow spectrophotometry, that operate in the UV−visible region. Here, we have combined both techniques to compare the kinetics of the decay of Q, a di-($\mu$-oxo)diiron(IV) form of the enzyme, with the rate of consumption of the alternative substrate $CD_3NO_2$. The near identity of the observed first-order rate constants for these two processes provides compelling evidence that Q reacts with substrate and that hydroxylation of $CD_3NO_2$ and reduction of Q to the diiron(III) state occur concomitantly.

**Note Added after ASAP Posting.** The version published ASAP 8/23/2003 contained typographical errors in the caption for Figure 1. The final Web version published 8/26/2003 and the print version are correct.

**References**

(1) Merkx, M.; Kopp, D. A.; Sazinsky, M. H.; Blazyk, J. L.; Muller, J.; Lippard, S. J. *Angew. Chem., Int. Ed.* **2001**, *40*, 2782−2807.
(2) Wallar, B. J.; Lipscomb, J. D. *Chem. Rev.* **1996**, *96*, 2625−2657.
(3) Ambundo, E. A.; Friesner, R. A.; Lippard, S. J. *J. Am. Chem. Soc.* **2002**, *124*, 8770−8771.
(4) Colby, J.; Stirling, D. I.; Dalton, H. *Biochem. J.* **1977**, *165*, 395−402.
(5) Dalton, H. *Adv. Appl. Microbiol.* **1980**, *26*, 71−87.
(6) Andersson, K. K.; Froland, W. A.; Lee, S.-K.; Lipscomb, J. D. *New J. Chem.* **1991**, *15*, 411−415.
(7) Fox, B. G.; Borneman, J. G.; Wackett, L. P.; Lipscomb, J. D. *Biochemistry* **1990**, *29*, 6419−6427.
(8) Stahl, S. S.; Francisco, W. A.; Merkx, M.; Klinman, J. P.; Lippard, S. J. *J. Biol. Chem.* **2001**, *276*, 4549−4553.
(9) Lee, S.-K.; Fox, B. G.; Froland, W. A.; Lipscomb, J. D.; Münck, E. *J. Am. Chem. Soc.* **1993**, *115*, 6450−6451.
(10) Liu, K. E.; Valentine, A. M.; Wang, D.; Huynh, B. H.; Edmondson, D. E.; Salifoglou, A.; Lippard, S. J. *J. Am. Chem. Soc.* **1995**, *117*, 10174−10185.
(11) Shu, L.; Nesheim, J. C.; Kauffmann, K.; Münck, E.; Lipscomb, J. D.; Que, L., Jr. *Science* **1997**, *275*, 515−518.
(12) Lee, S.-K.; Nesheim, J. C.; Lipscomb, J. D. *J. Biol. Chem.* **1993**, *268*, 21569−21577.
(13) Brazeau, B. J. Lipscomb, J. D. *Biochemistry* **2003**, *42*, 5618−5631.
(14) Thorneley, R. N. F.; George, S. J. In *Nitrogen Fixation in Bacteria: Cellular and Molecular Biology*; Triplett, E., Ed.; Horizon Scientific Press: Wymondham, U.K., 2000; pp 81−99.
(15) George, S. J.; Ashby, G. A.; Wharton, C. W.; Thorneley, R. N. F. *J. Am. Chem. Soc.* **1997**, *119*, 6450−6451.
(16) George, S. J.; Allen, J. W. A.; Ferguson, S. J.; Thorneley, R. N. F. *J. Biol. Chem.* **2000**, *275*, 33231−33237.
(17) Willems, J.-P.; Valentine, A. M.; Gurbiel, R.; Lippard, S. J.; Hoffman, B. M. *J. Am. Chem. Soc.* **1998**, *120*, 9410−9416.
(18) Gassner, G. T.; Lippard, S. J. *Biochemistry* **1999**, *38*, 12768−12785.
(19) Coufal, D. E.; Blazyk, J. L.; Whittington, D. A.; Wu, W. W.; Rosenzweig, A. C.; Lippard, S. J. *Eur. J. Biochem.* **2000**, *267*, 2174−2185.
(20) Griess, J. P. *Ber. Dtsch. Chem. Ges.* **1879**, *12*, 426−429.
(21) Nash, T. *Biochem. J.* **1953**, *55*, 416−421.

JA036081R