Spectroscopic Detection of Intermediates in the Reaction of Dioxygen with the Reduced Methane Monooxygenase Hydroxylase from *Methylococcus capsulatus* (Bath)

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Soluble methane monooxygenase (MMO), an enzyme system isolated from methanotrophic bacteria, which catalyzes the conversion of methane to methanol,[2] has three components,[3,4] a hydroxylase (H), a coupling protein (B), and a reductase (R). Methane is oxidized at two dinuclear non-heme iron centers in H[4] following transfer of electrons from NADH via R.[5] B affects the rate, yield, and regioselectivity of substrate hydroxylation by H as well as its redox potentials.[6-9] Previously we reported on spectroscopic[10,11] and X-ray crystallographic[12] studies of MMOH from *Methylococcus capsulatus* (Bath), which revealed the active site structure of the resting state enzyme. In this communication we present the results of stopped-flow and freeze–quench kinetic investigations of the reaction of dioxygen with the reduced hydroxylase, H_{red}, which elaborate upon and extend the findings of parallel work on MMOH from *Methylosinus trichosporium* OB3b.[13,14]

Native MMOH[15] (H_{ox}, $Fe^{III}Fe^{III}$) was reduced to the diiron(II) form (H_{red}) in the presence of B.[18] Concentrations ranged from 30 to 70 μM in H and 60 to 140 μM in B for stopped-flow experiments, while samples 700 μM in H and 1.4 mM in B were used for rapid freeze–quench studies. Solutions of H_{red} and B in one syringe were mixed rapidly with a dioxygen-saturated (~1 mM) 25 mM MOPS, pH 7.0 buffer solution at 4 °C. In the freeze–quench work, ^{57}Fe-enriched protein was allowed to react with dioxygen for fixed time periods (0.025–60 s) before being sprayed into isopentane at −140°C.

As will be described in detail elsewhere,[19] stopped-flow spectrophotometry revealed transient absorptions with λ_{max} values of 350, 420, and 520 nm. When the reaction was monitored at 420 nm, a transient formed and decayed with rate constants of 0.31 ± 0.02 and 0.065 ± 0.017 s^{-1}, respectively, under pseudo-first-order conditions in dioxygen. These kinetic constants are similar to those reported for an intermediate designated compound Q observed in the analogous reaction of $MMOH_{red}$ from *M. trichosporium* OB3b.[14] In Figure 1A,B we present the 4.2 K Mössbauer spectra of rapid freeze–quench samples of H_{red} that had reacted with O_2, the limiting reagent under these conditions, for 155 ms and 3 s, respectively, before freezing. Four spectral components in the form of quadrupole doublets were detected, corresponding to four different states of the dinuclear iron center: H_{ox}, H_{red}, and two intermediates designated L and Q. Components H_{ox} and H_{red}, delineated by brackets in Figure 1, have Mössbauer signals indicative of the oxidized, $Fe^{III}Fe^{III}$, and fully reduced, $Fe^{II}Fe^{II}$, states of the diiron center.[10] For the 155-ms and 3-s samples, respectively, H_{red} accounts for ~60 and 55% of the total iron absorption and H_{ox}, ~10 and 12%. Measurements performed on a control sample revealed the presence of a small amount (~8%) of H_{ox} in the starting solution prior to its reaction with dioxygen. The Mössbauer spectrum corresponding to a newly detected intermediate L (Figure 1C) is most apparent in the 155-ms sample, where it accounts for ~28% of total iron. Also present in both freeze–quench samples is Q, which develops following the decay of L. Approximately 23% of iron absorption in the 3-s sample can be attributed to Q. Preliminary analysis of a time course study[19] reveals that L forms with a first-order rate constant of ~25 s^{-1}, a value in good agreement with that (22 s^{-1}) reported for the disappearance of the $g = 16$ EPR signal of H_{red} from *M. trichosporium* OB3b by rapid freeze–quench EPR spectroscopy.[14] Our analysis also yields rate constants for the decay of L and formation of Q (both ~0.4 s^{-1}), suggesting that L is a kinetically competent precursor of Q.[20] We therefore conclude that H_{red} reacts with dioxygen first to form L, which then decays to Q.

The spectra of compounds L (Figure 1C) and Q (Figure 1D) can be obtained by subtracting the appropriate contributions of the other components from the raw data. Intermediate Q exhibits a spectrum with a slightly broadened high-energy line and can be fitted with two unresolved equal-intensity quadrupole doublets, indicating the presence of two inequivalent iron sites. The least-squares-fit parameters obtained for Q are $\delta = 0.21 \pm 0.02$ mm/s and $\Delta E_Q = 0.68 \pm 0.03$ mm/s for doublet 1, and $\delta = 0.14 \pm 0.02$ mm/s and $\Delta E_Q = 0.55 \pm 0.03$ mm/s for doublet 2. The average isomer shift of 0.18 mm/s and quadrupole splitting of 0.62 mm/s compare well with those ($\delta = 0.17$ mm/s and $\Delta E_Q = 0.53$ mm/s) obtained for the corresponding spectrum of Q from the *M. trichosporium* OB3b hydroxylase, for which the two iron atoms were assumed to be equivalent.[13] The observed isomer shift values are substantially smaller than those expected for a carboxylate-bridged dinuclear high-spin octahedral ferric cluster[21,22] and have been used to assign an Fe(IV) oxidation state, specifically a

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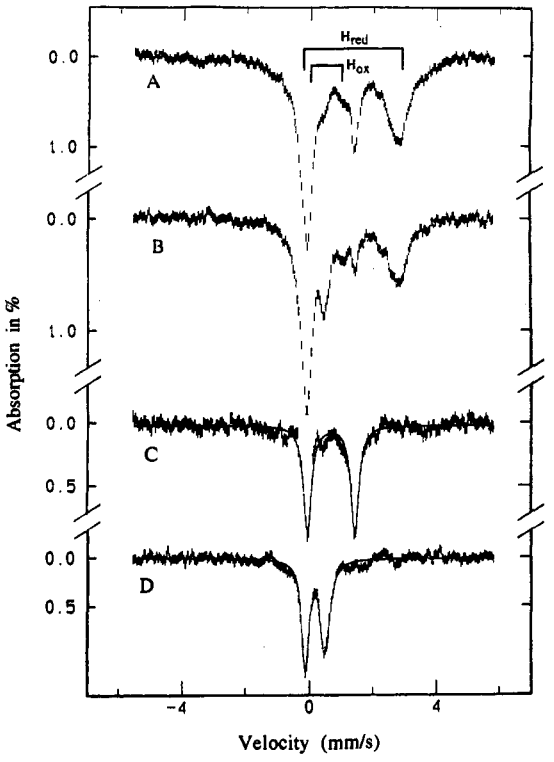

Figure 1. Mössbauer spectra at 4.2 K of rapid freeze–quenched samples from the reaction of ^{57}Fe-enriched reduced MMO hydroxylase with O_2. (A) Spectrum taken 155 ms after mixing. (B) Spectrum taken 3 s after mixing. (C) Spectrum of intermediate L prepared from the spectrum in A by removing 55, 5, and 10% contributions of diferrous, Q, and diferric species, respectively, from total iron absorption. (D) Spectrum of intermediate Q prepared similarly from a spectrum taken 8 s after mixing. In this case, we subtracted 50, 5, and 13% contributions of diferrous, L, and diferric components. Data were recorded in the presence of a 50-mT magnetic field applied parallel to the γ-beam. The solid lines in C and D are least-squares fits to the experimental data.

symmetric ferryl species.[13] It is important to point out, however, that the observed average isomer shift for Q, although small, is greater than those of previously characterized heme and non-heme Fe(IV) complexes.[23-26]

The new intermediate, compound L, exhibits a sharp (line width = 0.30 mm/s), symmetrical quadrupole doublet (Figure 1C), indicating that the two iron atoms are in identical coordination environments. A Mössbauer spectrum of the 155-ms sample recorded in the presence of a 4-T parallel applied field revealed L to be diamagnetic. Consistent with this observation, EPR measurements of corresponding rapid freeze–quench samples displayed no signal associated with L. The weak field Mössbauer spectrum of L shown in Figure 1C is best fitted with parameters of $\delta = 0.66 \pm 0.02$ mm/s and $\Delta E_Q = 1.51 \pm 0.03$ mm/s. These parameters are very unusual and have never been reported for carboxylate–bridged diiron clusters. In particular, the isomer shift is significantly greater than the 0.45–0.55 mm/s range generally observed for carboxylate-bridged diiron(III) clusters[21,22,27] and substantially smaller than the 1.1–1.3 mm/s range found for diiron(II) clusters.[22,28] Based on chemical considerations and on the kinetic data, which indicate L to be the the first intermediate in the reaction of H_{red} with dioxygen, we propose that L is a diiron(III) peroxide.[29] The few known diiron(III) peroxide complexes, however, have $\delta = 0.52$–0.54 mm/s.[31-33] Since δ increases with increasing coordination number and electronic charge,[34] the high δ value of L may indicate six-coordination and considerable peroxide-to-iron charge transfer or, possibly, seven-coordination. Such an assignment would be in accord with the active site composition of H,[12] with the iron atoms in identical $FeNO_5$ or $FeNO_6$ coordination environments, each comprising one histidine, one monodentate glutamate, one bidentate glutamate, a bridging hydroxide, and an η^1,η^1- or η^2,η^2-bridging peroxide ligand.

In conclusion, low-temperature optical spectroscopic and freeze–quench Mössbauer experiments of MMOH from *M. capsulatus* (Bath) reveal the formation of intermediates L and Q in the reaction of H_{red} with dioxygen. Most significant is the new intermediate designated compound L to leave room for additional short-lived species between H_{red} and Q. Compound L forms early in the reaction sequence and appears to be a precursor of Q. We have distinguished two inequivalent iron sites in the Mössbauer spectrum of Q, which suggests that the activated oxygen need not be bound symmetrically to the iron atoms. Finally, we note that the spectral properties of L and Q have not yet been accurately replicated in any known, crystallographically characterized diiron carboxylate complex. The present results should reinforce the incentive to prepare the appropriate model compounds.

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