A Transient Intermediate of the Methane Monooxygenase Catalytic Cycle Containing an $Fe^{IV}Fe^{IV}$ Cluster

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The soluble form of methane monooxygenase (MMO) purified from *Methylosinus trichosporium* OB3b is a three protein component enzyme that catalyzes the reaction[1,2]

$$CH_4 + NADH + H^+ + O_2 \rightarrow CH_3OH + NAD^+ + H_2O$$

Past studies have shown that the oxygen activation and insertion chemistry of MMO occurs on the hydroxylase component,[2-5] which contains two (identical) spin-coupled, oxygen-bridged dinuclear iron clusters.[6-10] Many hydrocarbons are adventitiously oxidized by MMO.[11-15] Our studies of the mechanism of MMO have shown that the cluster is activated for reaction with O_2 during the natural catalytic cycle by reduction to the $Fe^{II}Fe^{II}$ state.[2] Recently, we studied the transient reaction kinetics of the $Fe^{II}Fe^{II}$ hydroxylase with O_2 in the presence of the component B of the MMO system.[16] Among the intermediates detected was a yellow species (λ_{max} = 330 and 430 nm) termed compound Q. Substrates were found to have little effect on the rate of compound Q formation, but they greatly accelerated its decay. The decay rate depended upon both the concentration and the type of substrate present and also matched the formation rate of product bound in the active site. These observations suggest that compound Q may be either the activated form of the enzyme that leads directly to substrate hydroxylation or an immediate precursor. For the present study, we have trapped compound Q by rapid freeze quench techniques for characterization by Mössbauer spectroscopy.

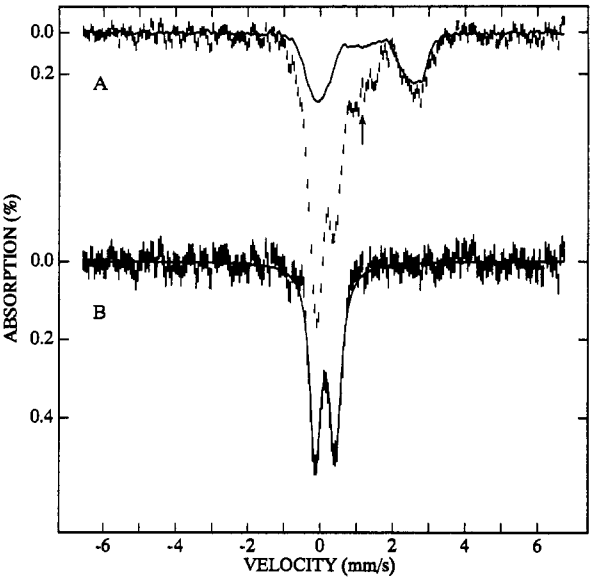

Figure 1. (A) Zero-field Mössbauer spectrum of ^{57}Fe-enriched hydroxylase prepared as described in the text. The solid line is a spectrum of $Fe^{II}Fe^{II}$ hydroxylase freeze-quenched in the absence of O_2. (B) Spectrum, representing compound Q, prepared by subtracting the $Fe^{II}Fe^{II}$ (30%) and $Fe^{III}Fe^{III}$ (25%) species from the experimental data. The solid line is a least-squares fit for ΔE_Q = 0.53 mm/s and δ_{Fe} = 0.17 mm/s.

The Mössbauer sample of compound Q was prepared by rapid mixing of 240 nmol of substrate-free $Fe^{II}Fe^{II}$ hydroxylase[2,8] (sp act. 1200 nmol min^{-1} mg^{-1}) and 480 nmol of component B (sp act. 10 100 nmol min^{-1} mg^{-1}) with a saturated solution of O_2 in 100 mM MOPS buffer, pH 7.7 at 4 °C, using an Update Instruments stopped freeze apparatus. The mixture was allowed to age for 4 s to maximize compound Q formation and was then frozen by being sprayed into isopentane at −140 °C.

We have studied samples containing compound Q in zero field and in applied magnetic fields of 1.0, 3.0, 6.0, and 8.0 T. The 4.2 K Mössbauer spectrum of Figure 1A contains three species. Approximately 30% of the iron belongs to a broad doublet ($\Delta E_Q \approx$ 2.4–3.1 mm/s and $\delta \approx$ 1.3 mm/s). This doublet belongs to clusters in the Fe^{II}/Fe^{II} state since the same features are observed for anaerobically freeze-quenched $Fe^{II}Fe^{II}$ hydroxylase (solid line in Figure 1A). Assignment of this doublet to the $Fe^{II}Fe^{II}$ hydroxylase is further supported by the high-field spectra (Figure 2), which, for the $Fe^{II}Fe^{II}$ component, exhibit paramagnetic hyperfine features similar to those reported for the (isopentane-free) $Fe^{II}Fe^{II}$ hydroxylase cluster.[6]

A second species exhibits doublets with features previously observed for $Fe^{III}Fe^{III}$ hydroxylase samples; $\Delta E_Q \approx$ 1.05 mm/s and $\delta \approx$ 0.50 mm/s.[6,9] The high-energy lines of this species are indicated by the arrows in Figures 1A and 2. Spectral subtractions suggest that it accounts for roughly 25% of the iron. The 6.0-T spectrum of Figure 2 shows that this species is diamagnetic, in accord with assignment to the native $Fe^{III}Fe^{III}$ state attained after decay of compound Q.

The most abundant species (compound Q) in the spectrum of Figure 1A is revealed after subtraction of the $Fe^{II}Fe^{II}$ (30%) and $Fe^{III}Fe^{III}$ species (25%) from the experimental data,[17] as shown

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(17) The solubility of O_2 limits the concentration of enzyme that can be used in these experiments. Since O_2 is consumed by any residual concentration of the reagents used to reduce the hydroxylase, a slight excess of $Fe^{II}Fe^{II}$ hydroxylase over O_2 probably occurred, leaving some clusters in the $Fe^{II}Fe^{II}$ state after mixing. Kinetic experiments monitored by EPR and optical methods were conducted using lower hydroxylase concentrations, allowing O_2 to be supplied in large excess.[16] Under these conditions, none of the g = 16 signal of the $Fe^{II}Fe^{II}$ hydroxylase was observed at 4 s. Moreover, no other signals that could be associated with compound Q were observed in accord with the diamagnetic nature of this species established by Mössbauer spectroscopy. For the dilute samples, approximately 60% of the diiron clusters were estimated to be in the compound Q state 4 s after mixing by kinetic and product yield analysis as discussed in ref 16.

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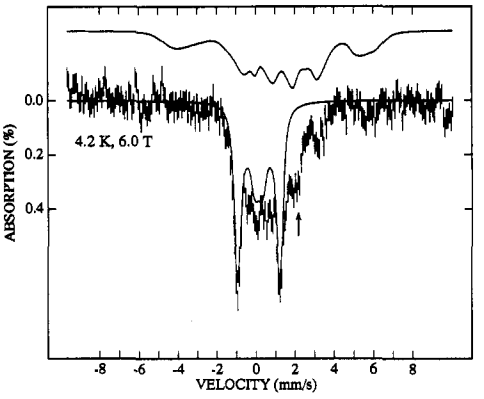

Figure 2. Mössbauer spectrum (4.2 K) of the sample of Figure 1A recorded in a 6.0-T parallel field. The solid line drawn above the data is a spectrum of the $Fe^{II}Fe^{II}$ hydroxylase simulated as described in ref 6. The solid line drawn through the data is a spectrum calculated for compound Q with the assumption that the cluster is diamagnetic ($S = 0$). The excellent match between the calculated and observed splittings shows that the ground state of the cluster is indeed diamagnetic.

in Figure 1B. At 4.2 K, compound Q contributes one well-defined doublet with $\Delta E_Q = 0.53$ mm/s and $\delta = 0.17 \pm 0.02$ mm/s; the same value for ΔE_Q was observed at 90 K. The isomeric shift of compound Q is significantly smaller than that of the $Fe^{III}Fe^{III}$ state of the hydroxylase and other iron–oxo proteins ($\delta_{Fe^{III}} = 0.45$–0.55 mm/s),[18,19] suggesting that the iron of compound Q is at the Fe^{IV} oxidation level. The observed isomeric shift is close to those reported for both the Fe^{IV}=O hemes (0.03–0.10 mm/s)[20] and the ferryl intermediate derived from a model complex for iron–oxo proteins (0.11 mm/s at 4.2 K)[21] described by Que and co-workers.[22] A control sample, quenched 0.3 s after mixing with O_2, contained ca. 70% of the $Fe^{II}Fe^{II}$ species, ca. 20% of compound Q, and ca, 10% of the $Fe^{III}Fe^{III}$ state, in reasonable agreement with our transient kinetic studies.[16]

Spectra recorded at 4.2 and 50 K in applied magnetic fields up to 8.0 T show that the Fe^{IV} sites of compound Q are diamagnetic, as demonstrated by the spectral simulation shown in Figure 2. The observed diamagnetism rules out the possibility that the Fe^{IV} sites reside in $Fe^{III}Fe^{IV}$ clusters because this mixed-valence state would be paramagnetic. It follows that the Fe^{IV} spectrum originates from clusters containing two (indistinguishable) Fe^{IV} sites. The ΔE_Q and δ_{Fe} values of compound Q are compatible with either intermediate-spin[22] ($S = 1$) Fe^{IV} or high-spin[23,24] ($S = 2$) Fe^{IV} sites.[25] Since both of these spin states are paramagnetic, the observed diamagnetism implies that two Fe^{IV} sites with the same spin state are antiferromagnetically coupled; analysis of the high-field data suggests a lower limit for the exchange coupling constant of $J > 60$ cm^{-1} ($H = JS_1 \cdot S_2$).

In combination with the transient kinetic analysis reported elsewhere,[16] the spectroscopic studies of compound Q presented here represent the first isolation and characterization of a high-valent iron–oxo species capable of hydrocarbon oxidation in either non-heme or heme protein oxygenase chemistry. Both iron sites of compound Q are clearly at a higher oxidation level than the transient species recently reported for ribonucleotide reductase,[28] the latter consisting of a protein radical that is exchange coupled to an $Fe^{III}Fe^{III}$ cluster. The oxidation level of compound Q is also substantially above that of a μ-1,2 peroxo bridged diiron model complex ($\delta_{Fe^{III}} = 0.52$ mm/s).[29] The oxidation level of compound Q appears to be formally equivalent to that of the proposed activated intermediate of cytochrome P450,[30] in accord with our previous proposal for the involvement of $Fe^{IV}Fe^{IV}$ in the reactive state of the hydroxylase.[1-5] Most interesting is the observation that the Mössbauer spectra of the two iron sites in the $Fe^{IV}Fe^{IV}$ cluster of compound Q are indistinguishable, suggesting that the activated oxygen is bound to the cluster symmetrically. For example, a single oxygen atom might bridge between two cluster irons or two equivalent oxygen atoms may be present in any of several possible configurations of a diferryl unit.

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(25) Although the isomeric shift of compound Q would be compatible with low-spin Fe^{III}, other observations argue against an antiferromagnetically coupled low-spin $Fe^{III}Fe^{III}$ pair. First, low-spin Fe^{III} complexes generally have $\Delta E_Q > 1.5$ mm/s. Second, none of the more than two dozen structurally relevant dinuclear $Fe^{III}Fe^{III}$ complexes reviewed by Kurtz[19] has a low-spin ferric site. Third, EXAFS studies,[10] ENDOR data,[7] and comparison of the hydroxylase amino acid sequence with those of other iron–oxo proteins suggest a 1-N/5-O or 2-N/4-O ligand structure for each site.[26,27] We are unaware of any low-spin ferric complex with N/O ligation that has fewer than three nitrogen ligands. The oxygen-rich environment of the ligand structure also argues against assigning the spectrum of compound Q to two low-spin ($S = 0$) Fe^{IV} sites. To date, no low-spin Fe^{IV} complex has been reported.

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