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Generation of a Mixed-Valent Fe(III)Fe(IV) Form of Intermediate Q in the Reaction Cycle of Soluble Methane Monooxygenase, an Analog of Intermediate X in Ribonucleotide Reductase R2 Assembly

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The selective conversion of methane to methanol under ambient conditions at the carboxylate-bridged diiron center in the hydroxylase component (MMOH) of soluble methane monooxygenase (sMMO) is a remarkable reaction. Several intermediates in the reaction cycle have been identified kinetically and spectroscopically, including diiron(III) peroxo (H_{peroxo}) and a formally dioxodiiron(IV) (Q) species.[1−3] These intermediates build up and decay following reaction of the fully reduced, diiron(II) form of MMOH with O_2. Similar chemistry occurs during the assembly of the structurally analogous dimetallic center in the R2 subunit of *Escherichia coli* ribonucleotide reductase (RNR), where an intermediate, mixed-valent state Fe(III)Fe(IV) species has been well characterized.[4−6] The paramagnetic properties of intermediate X in R2 have been a valuable asset in its characterization, facilitating study by electron nuclear double resonance (ENDOR) spectroscopy.[4,6] In the present paper, we present the results of rapid freeze quench (RFQ)/radiolytic reduction/Mössbauer spectroscopy which demonstrate that intermediate Q in the MMOH reaction cycle can be cryoscopically reduced to the paramagnetic Fe(III)Fe(IV) state. This species, which we designate Q_X, is closely related to intermediate X in the R2 assembly reaction. Its identification strengthens the spectroscopic link between the O_2 reaction chemistry of MMOH and R2 and paves the way for future ENDOR studies using isotopically labeled dioxygen for the structural characterization of Q.

Low-temperature radiolytic reduction has been applied to the study of carboxylate-bridged diiron proteins[7,8] and models.[9] In a solid matrix, a diamagnetic sample can be reduced by one electron to a paramagnetic species while maintaining a geometry close to that of the diamagnetic form. Annealing the sample to temperatures above 200 K allows the sample to relax to an equilibrium conformation, with concomitant conversion of the electron paramagnetic resonance (EPR) signal to that observed following chemical reduction in solution at 25 °C. This technique has thus far been applied only to the diferric form of non-heme iron proteins and model compounds, generating the mixed-valent Fe(II)Fe(III) form. Intermediate Q, which is diamagnetic at 4 K, has now been made paramagnetic in this manner.

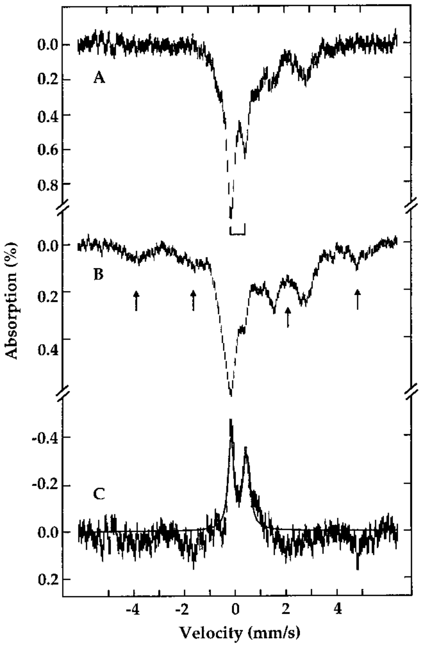

Figure 1. Mössbauer spectra of a rapid freeze quench sample from the reaction of H_{red}/2B with dioxygen at 4 °C. The reaction was quenched at 6 s. Spectra were recorded at 4.2 K with a 50 mT magnetic field applied parallel to the Mössbauer low-energy γ rays. (A) The spectrum recorded before the sample was subjected to high-energy γ irradiation. The position of the doublet attributed to intermediate Q is indicated by a bracket. (B) The spectrum of the sample after high-energy γ irradiation. The signals due to the new paramagnetic species Q_X are designated by arrows. (C) A difference spectrum (B minus A) showing the conversion of a portion of the diamagnetic Q (upward pointing quadrupole doublet) into the paramagnetic Q_X (downward pointing absorption signals). The solid line is a theoretical simulation of Q using the previously reported parameters.[11]

Figure 1A depicts the Mössbauer spectrum of a RFQ sample containing intermediate Q (35% of total iron) as well as other protein species.[10,11] The Mössbauer spectrum of the same sample after irradiation is shown in Figure 1B.[12] A difference spectrum (Figure 1C) illustrates that irradiation converts a portion of Q into a new paramagnetic species, designated Q_X. The amount of Q lost appears as a negative quadrupole doublet (pointing upward) and corresponds to approximately 22% of the total iron in the sample, while the new species accounting for the same portion

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(10) The sample was prepared with proteins from *Methylococcus capsulatus* (Bath) by rapid freeze quench (RFQ) techniques as reported previously.[11] An anaerobic solution of 0.7 mM $MMOH_{red}$ and 1.4 mM MMOB in 25 mM MOPS (pH 7) was mixed rapidly at 4 °C with a dioxygen-saturated solution of 25 mM MOPS (pH 7) with 40% glycerol. The reaction mixture was allowed to age for 6 s before quenching into isopentane at −150 °C. The sample was packed into either a Mössbauer sample cell or a quartz Q band ENDOR tube for spectroscopic measurements. Analysis of the Mössbauer data indicates that the sample contained 32% H_{red}, 10% H_{peroxo}, 35% Q, 14% H_{ox}, and 9% V. Spectral parameters for these species have been reported previously[11] except for V, which is a new diamagnetic diiron(III) component having $\delta = 0.51$ mm/s and $\Delta E_Q = 2.34$ mm/s, parameters similar to those of the (μ-oxo)-diiron(III) form of R2.[1]

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(12) Samples were irradiated by using γ rays from a ^{60}Co source at a dose rate of 0.45 Mrad/h to a total dose of 6 Mrad.

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Table 1. Mössbauer Parameters for Q_X and Related Species[a]

	Q_X		R2-X[b]		$[Fe_2(6\text{-Me-TPA})_2(\mu\text{-}O)_2]^{3+}$[c]	
	Fe_A	Fe_B	Fe_A	Fe_B	Fe_A	Fe_B
δ[d]	0.48(6)	0.14(6)	0.56(3)	0.26(4)	0.48(3)	0.08(3)
ΔE_Q[d]	−0.9(1)	−0.6(1)	−0.9(1)	−0.6(1)	1.6(2)	0.5(1)
η	0.2(5)	0.9(5)	0.5(2)	2.7(3)	1.0(3)	1.0(3)
A_{xx}[e]	−70(4)	26(4)	−74.2(2)	27.5(2)	−64.5(20)	20(3)
A_{yy}[e]	−75(4)	30(4)	−72.2(2)	36.8(2)	−64.5(20)	36.5(15)
A_{zz}[e]	−75(4)	32(4)	−73.2(2)	36.8(2)	−64.5(20)	36.5(15)

[a] Numbers in parentheses are estimated standard deviations in the last significant digits. [b] Data from ref 4. [c] Data from ref 14. 6-Me-TPA = *N*-(6-methyl-2-pyridylmethyl)-*N*,*N*-bis(2-pyridylmethyl)amine. [d] Units mm/s. [e] Units MHz.

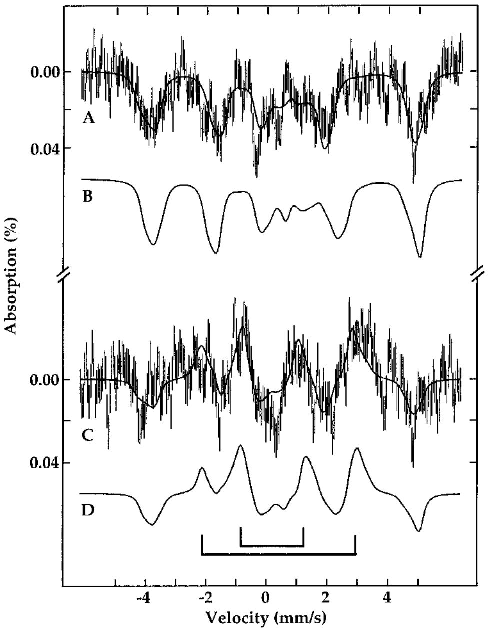

Figure 2. Mössbauer spectral comparison of Q_X and R2-X. (A) 4.2 K spectrum of Q_X prepared from the spectrum in Figure 1B by removing the contributions of H_{red} (40% of total iron absorption), H_{peroxo} (9.5%), Q (13.5%), V (8.5%),[10] and H_{ox} (6.5%). The solid line plotted through the experimental data is a theoretical simulation of Q_X obtained by using the parameters listed in Table 1. The corresponding theoretical spectrum of R2-X[4] is shown in B. (C) A difference spectrum (parallel minus perpendicular) showing the field direction dependence of Q_X. The solid line plotted through the experimental data is a theoretical simulation of this dependence obtained by using the parameters listed in Table 1. The corresponding difference spectrum for the R2-X intermediate[4] is shown in D. The positions for the two sets of $\Delta m = 0$ lines corresponding to the two Fe sites are indicated by brackets at the bottom of the figure.

appears as a magnetic spectrum in the positive direction (pointing downward). Detailed analysis of the spectra indicates that high-valent Q was reduced preferentially over other species during irradiation, except for a small portion (7.5% of the total iron) of H_{ox} which was apparently fully reduced to the diferrous form.

Because the Mössbauer parameters of the other components are known,[11,13] their contributions can be removed from the spectrum of the irradiated sample to reveal the features of Q_X (Figure 2A). Its properties are very similar to those of R2-X (Figure 2B), formally an Fe(III)Fe(IV) species.[4] The close resemblance of the electronic structures of Q_X and R2-X is further revealed by the field direction dependence of their Mössbauer spectra. For an isotropic $S = {}^1/_2$ system, such as the exchange-coupled Fe(III)Fe(IV) state of R2-X, the weak-field spectrum depends strongly on the direction of the applied field in relation to the direction of the Mössbauer γ rays. The $\Delta m = 0$ transition lines acquire their minimum and maximum intensities, respectively, when the field is oriented in a parallel and perpendicular direction. In the irradiated sample, all species except for Q_X are either diamagnetic or have an integer spin, and their spectra do not change with the direction of a weak applied field. Consequently, a difference spectrum (Figure 2C) recorded with a 50 mT field applied parallel and perpendicular to the γ rays will cancel the contributions from all other species, revealing the field direction dependence of Q_X. Two sets of $\Delta m = 0$ lines (marked by brackets) corresponding to two distinct Fe sites are clearly visible. This difference spectrum is very similar to the analogous one of R2-X (Figure 2D).[4] Preliminary analysis of these Q_X spectra yields Mössbauer parameters comparable to those reported for R2-X and a related model compound (Table 1).[4,14]

The structure of intermediate Q has been inferred from a variety of physical and theoretical studies,[11,13,15−18] but owing in part to its diamagnetism is less well defined than that of intermediate X of RNR-R2. The rhombic nature of the EPR spectrum of R2-X at 35 GHz (Q band) has been exploited by ENDOR spectroscopy to afford a very detailed picture of the structure, as well as the fate of oxygen atoms incorporated during its formation from reduced R2 and O_2.[4,6] Such studies on Q would prove invaluable in its characterization. The 35 GHz EPR spectrum of a RFQ sample of Q quenched into a sample tube and then irradiated exhibits an intense signal at $g = 2$ due to free radicals generated by the high energy γ rays (data not shown). This signal completely obscures the Q_X EPR features, as would be expected if its dispersion from $g = 2$ were as small as that of R2-X.[19] Efforts are underway to reduce the intensity of the free radical signal so that the EPR spectrum of Q_X may be revealed.

In conclusion, this work, together with recent experiments on wild-type and mutant forms of R2,[20] demonstrates the first spectroscopic link between sMMO intermediate Q and RNR R2 intermediate X. As such it provides strong support for the supposition[1,21,22] that the reaction chemistry of the diiron centers in the two proteins is quite analogous. The present application of the radiolytic reduction technique extends its utility to high-valent iron species and facilitates detailed ENDOR studies of intermediate Q. Knowledge of the structure of Q is crucial for understanding the sMMO reaction cycle.

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