Radiolytic Reduction of Methane Monoxygenase Dinuclear Iron Cluster at 77 K

EPR EVIDENCE FOR CONFORMATIONAL CHANGE UPON REDUCTION OR BINDING OF COMPONENT B TO THE DIFERRIC STATE

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The soluble form of methane monoxygenase (MMO) consists of three components: reductase, hydroxylase (MMOH), and “B” (MMOB). Resting MMOH contains a diferric bis-µ-hydroxodinuclear iron “diamond core” cluster which is the site of oxygen activation chemistry after reduction. Here it is shown that γ-irradiation of MMOH at 77 K results in reduction of the diiron cluster to an EPR active Fe(II)-Fe(III) mixed valence state. At this temperature, the conformation of the enzyme remains essentially unchanged during reduction, so the EPR-spectrum becomes a probe of the conformation of the diferric state. The γ-irradiated MMOH exhibits EPR spectra that differ in lineshape and saturation properties from those of the mixed valence MMOH generated by chemical reduction in solution; annealing the γ-irradiated sample at 230 K yields the spectra of the chemically reduced sample. This demonstrates that the conformation of diferric MMOH in the vicinity of the diiron cluster changes during reduction to the mixed valence state. The analogous experiment for the MMOB-MMOH complex gives a new mixed valence species following γ-irradiation that differs from all previously reported mixed valence species. Thus, binding of MMOB also causes a change in the conformation of diferric MMOH.

It is hypothesized that the structural changes observed for the first time here may involve conversion of the dihydroxo-bridged diamond core structure to one with more readily dissociable bridging oxygen ligands to facilitate reaction with O2 following cluster reduction.

Methane monoxygenase (MMO) catalyzes the chemically difficult monooxygenation of methane to form methanol (1–6).

\[ \text{CH}_4 + \text{O}_2 + \text{NAD(P)}^+ + \text{H}^+ \rightarrow \text{CH}_3\text{OH} + \text{H}_2\text{O} + \text{NAD(P)}^+ \]  

(Eq. 1)

This is the first step in the oxidation of methane by methanotrophic bacteria, a conversion of fundamental importance to the global balance of this potent greenhouse gas. The soluble form of MMO is composed of three different protein components, each of which is required for efficient catalysis in vivo: a monomeric 38-kDa reductase (MMOR) with iron-sulfur (Fe₃S₄) and FAD cofactors, a 248-kDa hydroxylase (MMOH) containing bis-µ-hydroxo-bridged “diamond core” dinuclear iron clusters in an (αβγδ)₄ quaternary structure (7–10), and a monomeric 15-kDa “B” component (MMOB) without cofactors or metals. MMOH is the only component for which the crystal structure is known (7, 9, 10).

MMOH can catalyze the monooxygenation of methane and a broad range of other hydrocarbons without the presence of the other two components if the diiron cluster is nonbiologically reduced or H₂O₂ is supplied as a source of reduced oxygen (11–13). Nevertheless, the kinetics and other properties of the catalysis are dramatically affected by the other two components. MMOR efficiently couples the reaction to NADH oxidation (4). MMOB increases the rate of O₂ reaction with the diferrous cluster by 1000-fold at 4 °C, thereby converting this step from rate-limiting to nonrate-limiting in the catalytic cycle (14) and increasing the steady state initial velocity by up to 150-fold (13, 15). These and other effects of MMOR and MMOB result from formation of a specific complexes with MMOH. There is direct evidence from CD/magnetic CD (16) and EPR (13, 15, 17–19) spectroscopic studies of the diferred and/or mixed valence forms of the cluster that the formation of the MMOB complex causes conformational changes that affect the diiron cluster. For example, the antiferromagnetic coupling is decreased 6-fold in the mixed valence state, causing a dramatic change in the lineshape and the microwave saturation properties of the EPR spectrum when the complex is formed (15, 17).

While the effects of MMOB on the mixed valence and diferrous states of MMOH have been extensively studied, very little information has been obtained concerning the structural consequences of MMOB binding to resting diferrec MMOH. Studies using Mössbauer (17) and EXAFS (8, 20) spectroscopies have failed to detect any change in the diiron cluster that could account for the dramatic effects of MMOB on catalysis. Another approach to this problem is to make use of the structurally sensitive EPR spectrum of the mixed valence state of the enzyme to probe the resting diferrec state through application of radiolytic one-electron reduction at 77 K. Many enzymes and proteins have been shown to harbor oxygen-bridged dinuclear
Low Temperature EPR spectra of mixed valence form of MMOH in buffer/glycerol (1:1). Samples were reduced chemically in solution (a) or radiolytically at 77 K (b, c); d, difference spectrum obtained by subtraction of spectrum b from spectrum c as described in the text. The spectrum shown in a is decreased somewhat in intensity due to partial saturation by microwave power. It is presented in this way to facilitate comparison with the samples produced by X-irradiation (b-d). Instrument settings: modulation frequency, 100 kHz; modulation amplitude, 10 G; gain, $5 \times 10^3$; microwave power, 10 mW; temperature, 6.4 K (a), 11.9 K (b), and 3.8 K (c).
Radiolytic Reduction of MMOH at 77 K

**TABLE I**

| Species          | $g_1$ | $g_2$ | $g_3$ | $P_{1/2}$ | Ratio |
|------------------|-------|-------|-------|-----------|-------|
| MMOH alone       | 1.94  | 1.86  | 1.79  | 20 mW (7 K) | 65    |
| II               | 1.85  | 1.75  | 1.7   | >100 mW (4 K) | 35    |
| MMOH$^{\text{mv}}$ | 1.95  | 1.86  | 1.76  | 0.03 mW (7 K) |       |
| I, II$^b$        | 1.94  | 1.86, 1.75 |      | 15 mW$^d$ (3.8 K) | 25    |
| III              | 1.90  | 1.79  | 1.59  | 100 mW (3.8 K) | 75    |
| MMOH-MMOB        | 1.86  | 1.77  | 1.60  | 15 mW (3.8 K) |       |

$^a$ Temperature is shown in parentheses.

$^b$ Samples prepared by chemical reduction in solution. MMOH$^{\text{mv}}$ is mixed valent MMOH.

$^c$ Species with similar spectra to Species I and II from MMOH alone.

$^d$ Measured at $g = 1.86$.

is observed for both ribonucleotide reductase R2 protein from *E. coli* and hemerythrin at similar irradiation doses (21, 22). The intensity of Species II increases with applied microwave power of up to 100 mW at 4 K (Fig. 1c). In contrast, at 4 K Species I becomes saturated at a microwave power $P = 2$ mW. In Fig. 1c recorded at 3.8 K and 10 mW, Species II is therefore enhanced. The approximate lineshape of Species II ($g_1 = 1.85$, $g_2 = 1.75$, and $g_3 = 1.70$) was obtained by subtraction of the spectrum of Fig. 1b from that of Fig. 1c to yield a null base line at $g = 1.94$ as shown in Fig. 1d. Approximate quantitation in the temperature range 3.6–12 K shows that the species giving rise to Species I represents 65 ± 20% and Species II represents 35 ± 15% of the mixed valence species. The characteristics of the observed species are summarized in Table I.

Although Species I seems similar to the chemically reduced mixed valence MMOH (Fig. 1a) significant differences were observed in their relaxation properties at 7 K. At that temperature, the half-microwave saturation power ($P_{1/2}$) for Species I and the EPR spectrum of chemically reduced mixed valence MMOH (both measured at $g = 1.86$) were found to be ~20 and 0.03 mW, respectively. These results suggest that mixed valence species of MMOH produced by γ-irradiation at 77 K and by chemical means at room temperature have distinct diiron cluster structures. Under the assumption that mixed valence species formed at 77 K retain ligation geometry very close to that in the original difer- ric center, we conclude that the resting diferric enzyme has at least two structures, each of which is different from the structure of the mixed valence diiron cluster produced by chemical reduction. It is possible that the diferrous sites in the oxidized and postequilibrium mixed valence states have different ligation geometries or even ligand structures. However, it must be noted that EPR spectra from diiron clusters are very sensitive to small changes in the zero field splitting parameters ($D$) for each iron, the exchange coupling parameter ($J$), and the symmetry of the coordination environments of each iron, so even subtle changes in structure might cause the observed differences (21, 26–29).

The constrained Species I and II are expected to relax to an equilibrium state when the sample is warmed. Accordingly, upon annealing the sample of mixed valence MMOH produced at 77 K for 3 min at 230 K, both EPR Species I and II disappeared and an EPR spectrum identical to that obtained for the chemically reduced mixed valence MMOH shown in Fig. 1a appeared (data not shown).³

³ Previously, EPR signals with the characteristics of the mixed valence state produced by chemical reduction have been noted following x-ray absorption studies of initially difereric MMOH (8, 30). These samples probably underwent reduction at low temperature as reported here, but they represent the relaxed states because they were transferred to EPR tubes at or above the annealing temperature.

Effect of MMOB on the Mixed Valence EPR Signals of the Iron Cluster of MMOH—The presence of MMOB affects the EPR properties of the mixed valence state of MMOH produced either by standard chemical reduction or by γ-irradiation at 77 K. As previously observed, the chemically reduced complex of mixed valence MMOH and MMOB (1:4) gives rise to a broad EPR signal from a single species with $g$ values at 1.86, 1.77, and 1.60 shown in Fig. 2a, at 3.8 K as well as at 12 K.

Fig. 2b shows the 7 K EPR spectrum (only observed below 20 K) of the MMOH-MMOB complex (1:4) in buffer, 50% glycerol after γ-irradiation at 77 K. In this spectrum, resonance features centered at $g = 1.94, 1.86$, and 1.75 are clearly observed, which are similar to those observed for Species I and II shown in Fig. 1b, albeit at significantly decreased intensity. In addition, there are some new features that are readily observed at a higher magnetic field. To separate out these new features, the Fig. 1b spectrum was subtracted from the spectrum shown in Fig. 2b to give a null base line at $g = 1.86$; this is the spectrum shown in Fig. 2c ($g_1 = 1.90, g_2 = 1.79, g_3 = 1.59$, Species III). Quantitation under nonsaturating conditions of the mixed valent species in Fig. 2b indicates that they represent 15–20% of the clusters present. Species III has a different lineshape than the chemically reduced mixed valence MMOH-MMOB complex shown in Fig. 2a. Moreover, Species III is not saturated by microwave powers as high as 100 mW, even at 3.8 K (data not shown), whereas the spectrum shown in Fig. 2a has a $P_{1/2}$ of approximately 15 mW at this temperature (15). Approximate quantitation in the temperature range 3.6–12 K of the species shown in Fig. 2b suggests that Species III represents 75 ± 15% of the mixed valent iron clusters present (see Table I).

Under our experimental conditions, all of the MMOH should be complexed with MMOB (15, 18). Indeed, saturation of the MMOH-MMOB complex is evident from the single species observed in Fig. 2a (15). Therefore, it is likely that the species similar to Species I and II, which is present in Fig. 2b, also represents a MMOB complexed form of MMOH. This suggests that the diiron cluster in about 25% of diferric MMOH is not altered significantly by MMOB.

As noted above for the 77 K γ-irradiated MMOH, annealing the sample at 270 K for 2 min causes the mixed valence signals of Fig. 2b and c to disappear and a spectrum identical to that of the chemically reduced mixed valence spectrum of Fig. 2a to appear (data not shown).
The chemically reduced mixed valence state does not appear to be composed of significantly different species. Thus, it appears that one-electron reduction results in a conformational change that causes the two different subspecies of diferric MMOH to become more similar. However, any change that resulted in similar J and D values for the clusters would result in similar EPR spectra.

The third significant finding in this study is that MMOB has an effect on the MMOH diiron site in the oxidized state and that this effect is different than that previously observed for the mixed valence state (15). Again, no other technique has revealed this structural change between the oxidized and mixed valence states. It is in accord with the redox potential measurements (18) that showed that chemical reduction and MMOB binding are thermodynamically coupled, implying that some structural change occurs during reduction to the mixed valence state or when the MMOH-MMob complex forms. The current results suggest that a change in the cluster structure is at least part of the overall changes associated with reduction to the mixed valence state and MMOB binding.

The overall effect of the binding of MMOB to MMOH seen in the γ-irradiated samples is to shift the distribution of non-equilibrium mixed valence species to a form (Species III) more similar to the minority species observed for MMOH alone (Species II). As discussed above, this would be consistent with a shift from a predominant diamond core structure to a mono-bridged oxygen bridged structure, which might be the first step in preparing the cluster to bind O2 (14). However, EXAFS studies of the diferric MMOH and the MMOH-MMob complex show no change in the distribution of diamond core and mono-bridged structures (8). Nevertheless, one electron chemical reduction of the MMOH-MMob complex does cause a 6-fold weaker coupling as expected for a protonated, mono-bridged structure (15), and the crystal structure of the diferrous state shows that one bridging oxygen from the diamond core is lost at some stage of the reduction process (7).

One possibility is that forming the complex of diferri mammalian MMOH with MMOB causes small structural changes that result in weakening, but not loss, of the diamond core structure, such as a second protonation of one of the bridging hydroxides or deprotonation of one of the cluster ligands (Fig. 3). This weakening of the bridging ligands may result in the conversion to a mono-bridged structure when the complex is chemically reduced in solution or when the irradiated MMOH is allowed to anneal. However, prior to reduction or annealing, the Fe-Fe distances would be primarily determined by the number of bridging oxygen ligands, and thus only minor changes in the

**FIG. 3.** Working model of the effect on the diferri cluster of MMOB binding to MMOH. The figure represents the ligation of the iron cluster of MMOH based on the crystal structure (10) and EXAFS data (8) of the *M. trichosporium* MMOH and the data presented in this work, and the dotted lines represent weakened interactions.
EXAFS spectra would be observed. We have shown in previous studies that, of all of the effects of MMOB on MMO catalysis, the acceleration of the reaction of diferrous MMOH with O₂ is the most significant. The basis for this acceleration is unknown. The results presented here show that structural changes in the MMOH diiron cluster occur as a result of formation of the MMOH-MMOB complex before reduction of MMOH to the oxygen-reactive diferrous state, thereby underscoring the potential importance of this complex in initializing catalysis.

REFERENCES
1. Dalton, H. (1980) Adv. Appl. Microbiol. 26, 71–87
2. Lipscomb, J. D. (1994) Annu. Rev. Microbiol. 48, 371–399
3. Liu, K. E., and Lippard, S. J. (1995) Adv. Inorg. Chem. 42, 263–289
4. Wallar, B. J., and Lipscomb, J. D. (1996) Chem. Rev. 96, 2625–2657
5. Andersson, K. K., and Graslund, A. (1995) Adv. Inorg. Chem. 43, 359–408
6. Hansen, R. S., and Hanson, T. E. (1996) Microbiol. Rev. 60, 439–471
7. Rosenweig, A. C., Nordlund, P., Takahara, P. M., Frederick, C. A., and Lippard, S. (1995) Chem. Biol. 2, 409–418
8. Shu, L., Liu, Y., Lipscomb, J. D. and Que, L., Jr. (1996) J. Biol. Inorg. Chem. 1, 297–304
9. Rosenweig, A. C., Frederick, C. A., Lippard, S. J., and Nordlund, P. (1995) Nature 376, 537–543
10. Elango, N., Radhakrishnan, R., Froland, W. A., Wallar, B. J., Earhart, C. A., Lipscomb, J. D., and Oihlendorf, D. H. (1997) Protein Sci. in press
11. Fox, B. G., Froland, W. A., Dege, J. E., and Lipscomb, J. D. (1989) J. Biol. Chem. 264, 10023–10033
12. Andersson, K. K., Froland, W. A., Lee, S.-K., and Lipscomb, J. D. (1991) New J. Chem. 15, 411–415
13. Froland, W. A., Andersson, K. K., Lee, S.-K., Liu, Y., and Lipscomb, J. D. (1992) J. Biol. Chem. 267, 17588–17597
14. Liu, Y., Nesheim, J. C., Lee, S.-K., and Lipscomb, J. D. (1995) J. Biol. Chem. 270, 24462–24465
15. Fox, B. G., Liu, Y., Dege, J. E., and Lipscomb, J. D. (1991) J. Biol. Chem. 266, 540–550
16. Pulver, S., Froland, W. A., Fox, B. G., Lipscomb, J. D., and Solomon, E. I. (1993) J. Am. Chem. Soc. 115, 12409–12422
17. Fox, B. G., Hendrich, M. P., Surerus, K. K., Andersson, K. K., Froland, W. A., Lipscomb, J. D., and Münck, E. (1993) J. Am. Chem. Soc. 115, 3668–3701
18. Paulsen, K. E., Liu, Y., Fox, B. G., Lipscomb, J. D., Münck, E., and Stankovich, M. T. (1994) Biochemistry 33, 713–722
19. Hendrich, M. P., Fox, B. G., Andersson, K. K., Debrunner, P. G., and Lipscomb, J. D. (1992) J. Biol. Chem. 267, 261–269
20. DeWitt, J. G., Rosenzweig, A. C., Salifoglou, A., Hedman, B., Lippard, S. J., and Hodgson, K. O. (1995) Inorg. Chem. 34, 2505–2515
21. Davydov, R., Kuprin, S., Graslund, A., and Ehrenberg, A. (1994) J. Am. Chem. Soc. 116, 11120–11128
22. Davydov, R., Sahlin, M., Kuprin, S., Graslund, A., and Ehrenberg, A. (1996) Biochemistry 35, 5571–5576
23. Ansa, R., and van Rijndwijk, T. (1975) J. Magn. Reson. 19, 308–315
24. Hales, B. J. (1993) Methods Enzymol. 227, 384–395
25. Fox, B. G., Surerus, K. K., Münck, E., and Lipscomb, J. D. (1988) J. Biol. Chem. 263, 10553–10556
26. McCormick, J. M., Reem, R. C., and Solomon, E. I. (1991) J. Am. Chem. Soc. 113, 9066–9079
27. Dominaar, E. L., Dng, X.-Q., Gismelseed, A., Bill, E., Winkler, H., Trautwein, A., Nasri, H., Fischer, J., and Weiss, B. (1992) Inorg. Chem. 31, 1845–1854
28. DeRose, V. J., Liu, K. E., Kurtz, D. M., Jr., Hofman, B. M., and Lippard, S. J. (1993) J. Am. Chem. Soc. 115, 6440–6441
29. Thomann, H., Bernardo, M., McCormick, J. M., Pulver, S., Andersson, K. K., Lipscomb, J. D., and Solomon, E. I. (1993) J. Am. Chem. Soc. 115, 8881–8882
30. DeWitt, J. G., Bentsen, J. G., Rosenzweig, A. C., Hedman, B., Green, J., Filkinson, S., Papasifthismiou, G. C., Dalton, H., Hodgson, K. O., and Lippard, S. J. (1991) J. Am. Chem. Soc. 113, 9219–9235
31. Que, L., Jr., and Dong, Y. (1996) Acc. Chem. Res. 29, 190–196