Redox Properties of the Hydroxylase Component of Methane Monooxygenase from Methylococcus capsulatus (Bath)

EFFECTS OF PROTEIN B, REDUCTASE, AND SUBSTRATE*

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The reduction potentials of the hydroxylase component of the soluble methane monooxygenase from Methylococcus capsulatus (Bath) have been investigated through potentiometric titrations. The potentials were determined by EPR spectroscopic quantitation of the mixed valent hydroxylase as a function of added sodium dithionite in the presence of appropriate mediators. The reduction of the oxidized Fe(III)-Fe(III) form to the mixed valent Fe(II)-Fe(III) form occurs at 48 mV versus NHE while the potential for the formation of the fully reduced Fe(II)-Fe(II) species from the mixed valent form was determined to be -135 mV. Addition of the substrate propylene to the hydroxylase did not have a major effect on the reduction potentials. Introduction of the protein B and the reductase components, however, completely inhibited reduction of the hydroxylase at potentials as far negative as -200 mV. Addition of propylene to all three methane monooxygenase components greatly facilitated hydroxylase reduction. Under these conditions, the fully reduced form of the protein was obtained at potentials of >150 mV. This high redox potential indicates that the oxidized form of the protein is highly reactive, as required for methane oxidation. The present results reveal aspects of how both protein B and a substrate can regulate electron transfer into and out of the hydroxylase component of methane monooxygenase.

Methane monooxygenase from the methanotrophic bacterium Methylococcus capsulatus (Bath) catalyzes the synthesis of methanol from methane and oxygen, which is the first step in the metabolic pathway of the organism (1). Methane monooxygenase exhibits an extremely broad substrate specificity, reportedly inserting oxygen into a wide variety of alkanes, alkenes, ethers, and alicyclic, aromatic, and heterocyclic compounds (2). In addition, methane monooxygenase from Methylosinus trichosporum OB3b has been shown recently to oxidize haloalkenes, including trichloroethylene (3). This reactivity parallels that of the well studied heme analogue, cytochrome P-450 (4, 5), although the mechanism of oxygen activation may be more complex (6).

Soluble methane monooxygenase from both organisms consists of three components, all of which are necessary for activity (7, 8). The hydroxylase component has a molecular mass of 250 kDa and is comprised of three subunits in an α3β2γ2 configuration (7). The hydroxylase is the site of substrate binding and contains up to two non-heme dinuclear iron cores, the non-heme bridged dinuclear iron core (8). The fully reduced form of the hydroxylase is thought to be responsible for dioxygen activation (9). The reductase, molecular mass of 40 kDa, contains one FAD and one [2Fe2S] cluster with reduction potentials of -260 and -220 mV versus NHE, respectively (10). The oxidized form of the antiferromagnetically coupled [2Fe2S] cluster has no EPR signal, while the reduced form has a resonance at gUM = 1.96 (11). The reductase accepts electrons from NADH and subsequently transfers them to the hydroxylase (12, 13). The third component, protein B, has a molecular mass of 17 kDa, contains no prosthetic groups, and has been proposed to regulate electron transfer between the reductase and hydroxylase (14).

The hydroxylase has been previously characterized, in part by our laboratory, with the aid of optical, Mössbauer, EXAFS, and EPR spectroscopy (8, 9, 15–19). The native oxidized Fe(III)-Fe(III) form is EPR-silent due to antiferromagnetic coupling in the bridged diiron core, while the mixed valent Fe(II)-Fe(III) species exhibits an EPR signal with gUM = 1.83 indicative of a non-heme, bridged diiron center. The fully reduced Fe(II)-Fe(II) form gives rise to a signal at g = 1.5 characteristic of its integer spin system. The EPR spectra of the protein in all three oxidation states usually contain a minor signal at g = 4.3 due to rhombic Fe(III) and a free radical signal at g = 2.01. Each of these signals generally accounts for less than 5% of total iron present in the sample and is due to trace levels of mononuclear iron or protein-associated radical, respectively. EXAFS and Mössbauer data are also consistent with the presence of a bridged dinuclear iron center in the hydroxylase, although the exact nature of the bridge(s) is still unknown. The optical spectrum is essentially featureless above 300 nm in any oxidation state.

The dinuclear iron core in methane monooxygenase may have features in common with those in other iron oxoproteins such as hemerythrin, ribonucleotide reductase, and purple acid phosphatase. Accurate models for the structural, physical, and chemical properties of the diiron centers in these proteins have been a focus of attention in our laboratory (20, 21). The iron core in each protein functions differently. A compelling question yet to be answered from studies with both the models and the proteins themselves is just how nature has selectively tuned each active site to carry out its particular function. The highly specialized reactivity most likely results from modifications in the coordination environment of the bridged pair of iron atoms.

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2 J. G. DeWitt, J. G. Bentzen, A. C. Rosenberg, B. Hedman, J. Green, S. Pilkington, G. C. Papaethymiou, H. Dalton, K. O. Hodgson, and S. J. Lippard, submitted for publication.
Substrate Tuning of Methane Monoxygenase Redox Potentials

To probe factors that might control the functional properties of the dinuclear iron center in methane monoxygenase from *M. capsulatus* (Bath), we have studied the effect of the reductase and protein B components as well as substrate on the reduction potentials of the hydroxylase by monitoring the EPR spectrum of the mixed valent form at various potentials.

During the course of this work, we found values for the reduction potentials that differ significantly from those previously reported for protein from the same organism (17). Moreover, we discovered that addition of substrate to the complex formed by the hydroxylase, protein B, and the reductase dramatically increased the redox potentials. We describe our findings in this communication.

**Experimental Procedures**

Growth of the *M. capsulatus* (Bath) organism, purification of the three methane monoxygenase components, and assays were carried out as reported previously (9, 22). Specific activity (propylene) for the hydroxylase ranged from 75 to 150 milliunits/mg, but all preparations resulted in nearly identical EPR spectra.

Titrations were performed in a manner similar to that described elsewhere (17). Solutions typically contained about 5 ml of 100 μM hydroxylase in 50 mM MOPS, pH 7.0 (Sigma), and 12 mediators (100 μM each) (17). The resulting solution was repeatedly evacuated and back-filled with oxygen-free argon, then transferred with a gas-tight syringe into an apparatus described elsewhere (23). To perform experiments with substrate present, propylene (Matheson, polymer grade) saturated with buffer was continuously passed through the titration vessel. A redox combination electrode (Pt/Ag/AgCl, Corning) was used to monitor the potential. With constant stirring, small volumes of typically 1 to 3 μl of a 0.1 M sodium dithionite (Aldrich) solution were used to lower the potential. After a 10-min period, determined in a time course study to be sufficient for the system to reach equilibrium, a 300-μl sample was removed with a gas-tight syringe and then injected into an argon-filled, quartz EPR tube. The tube was then immediately frozen in cold isopentane (−50 °C) and then immersed in liquid N2.

For experiments performed with protein B and reductase, a mixture of the two proteins was added in a ratio that maximized hydroxylase activity, each at approximately 200 μM, or twice the concentration of the hydroxylase. To determine the effect of protein B and reductase on the redox properties of the hydroxylase, a mixture of these two proteins was added either before addition of reductant or after the fully reduced form of the hydroxylase was produced.

All EPR spectra were recorded at X-band on a Bruker model ESP 300 spectrometer with an Oxford Instruments EPR 9000 liquid helium cryostat set at 9 K. The g < 2 signal was quantitated under nonsaturating conditions by double integration of the first derivative spectrum for comparison to a frozen solution of copper perchlorate (1 mM CuCl2, 2 mM NaClO4, 0.01 M HCl). Transition probabilities were corrected for g-value anisotropy (24). The percentage of mixed valent species was calculated from the concentration of spin relative to the concentration of iron present in the sample. To compare different titrations the mixed valent signals were normalized to the maximum signal observed for each individual titration.

**Results**

Three titrations were carried out on the hydroxylase component of methane monoxygenase. Representative EPR spectra are shown in Fig. 1. The mixed valent signal increased in intensity as the potential was lowered, reached a maximum at approximately −50 mV versus NHE, then decreased as potential was lowered. At roughly −100 mV the fully reduced, g = 15, signal was detected and grew in intensity as the potential was further diminished. The reversibility of the system was confirmed by back titration at several potentials. The maximum intensity of the mixed valent signal corresponded to 85% of the total iron present. This yield most likely reflects the maximum attainable because of the inaccuracies introduced by using a copper standard for the integrations.

A plot of the relative intensities of the mixed valent signal versus potential is shown in Fig. 2. The potentials for stepwise reduction of the hydroxylase diiron(III) core, shown in Equation 1, were obtained by fitting the data in this plot of mixed valent intensities versus potential to Equation 2. This expression was derived from the two individual Nernst equations governing the reductions and the mass balance equation.

**Equation 1**

\[ E_{1}^{o} \] Fe(III).Fe(III) → Fe(II).Fe(III) → Fe(II).Fe(II)

**Equation 2**

\[ [Fe(II).Fe(III)] = \frac{100}{\exp(-nF/RT)} \times [E_{1}^{o}) + \exp((nF/RT)(E - E_{1}^{o})/1)] \]

The best fit, where \( n = 1, F \) is Faraday constant, \( R \) is gas constant, and \( T = 298 \) K, yielded potentials of 48 and −135 mV versus NHE for \( E_{1}^{o} \) and \( E_{2}^{o} \), respectively, with estimated errors of ±5 mV. \( E_{1}^{o} \) and \( E_{2}^{o} \) can also be used to compute the relative amounts of all three redox species present at a given potential. Results of such a calculation are also depicted in Fig. 2. The disproportionation constant for the mixed valent species was computed to be 8.04 × 10⁻³.

A plot of the EPR intensity of the mixed valent hydroxylase carried out in the presence of added propylene as a substrate is also shown in Fig. 2. A fit of these data to Equation 2 revealed only a slight diminution of the reduction potentials to values of 30 versus 48 mV and −156 versus −135 mV for \( E_{1}^{o} \) and \( E_{2}^{o} \), respectively. While the potential changes are...
small, they do suggest that substrate binding at the active site perturbs the environment of the iron atoms. Addition of protein B and reductase, however, produced a significant change, illustrated in Fig. 3A. For all potentials investigated down to -200 mV, no mixed valent nor any fully reduced signal was detected, implying that no reduction occurred. When protein B and reductase were added to the fully reduced form of the hydroxylase, the EPR signal of the latter persisted.

A dramatic change resulted when propylene was introduced as substrate to all three methane monooxygenase components. Reduction of the hydroxylase to its fully reduced state was observed upon initial introduction of reductant, even at potentials as high as 150 mV. Representative spectra are displayed in Fig. 3B. The reduction potentials of the hydroxylase under all conditions investigated are summarized in Table I.

**DISCUSSION**

As reported previously (17), the redox potentials for the stepwise reduction of the hydroxylase component of methane monooxygenase can be determined by monitoring the appearance and disappearance of the \( g < 2 \) EPR signal intensity characteristic of the mixed valent form (Equation 1). Our values (\( \mathcal{E}^*_{1} = 48 \text{ mV} \) and \( \mathcal{E}^*_{2} = -135 \text{ mV} \)) for the potentials differ from the earlier ones (350 and 25 mV, respectively (17)).

**Table 1**

| Iron oxoprotein | Additive(s) | \( \mathcal{E}^*_{1} \) | \( \mathcal{E}^*_{2} \) |
|----------------|-------------|-----------------|-----------------|
| MMO hydroxylase | Propylene | 48 | -135 |
| MMO hydroxylase | Protein B | 30 | -156 |
| MMO hydroxylase | Reductase | s | s |
| MMO hydroxylase | Protein B | >150 | >150 |
| MMO hydroxylase | Reductase Propylene | 110 | 310 |

* No reduction was observed under these conditions (see text).

We have no obvious explanation for the discrepancy, in part because of the lack of experimental detail in Ref. 17. For example, values for activity and iron content of the hydroxylase were not specified nor was it clear that the potentials were measured by an electrode. By using the present potential values for the MMO hydroxylase and \( \mathcal{E}^*_{2} \) of 80 mV for phenazine methosulfate we calculate the maximum attainable yield of the mixed valent species at this potential to be 22%. This number is consistent with the difficulty observed in obtaining yields of the mixed valent hydroxylase from *M. trichosporum* greater than 10–20%, based on total iron, when using phenazine methosulfate as a sole mediator and a stoichiometric amount of dithionite (8). Similarly, our redox potentials could explain why, at 100 mV, the spin concentration of the *M. capsulatus* hydroxylase was only 0.27/protein molecule (17). Finally, the present results are more in accord with an earlier report for *M. capsulatus* that formation of the mixed valent hydroxylase occurs “at redox values around 0 mV” and that the fully reduced form can be obtained “below -100 mV” (25).

Several synthetic model complexes containing diiron o xo and related units are known, but only a few of these display reversible or quasi-reversible redox behavior connecting the Fe(II)-Fe(II), Fe(II)-Fe(III), and Fe(II)-Fe(III) states (26-28). A comparison of the structures and redox behavior of the dinuclear iron centers in the proteins and model compounds leads to some interesting observations. Hemerythrin contains an o xo-bridge in the fully oxidized state, whereas methane monooxygenase does not (19). The diiron center in hemerythrin has reduction potentials of \( \mathcal{E}^*_{1} = 110 \) and \( \mathcal{E}^*_{2} = 310 \text{ mV versus NHE} \) (26). Since \( \mathcal{E}^*_{1} \) is less than \( \mathcal{E}^*_{2} \), the mixed valent form of the protein is unstable to disproportionation to the diiron(II) and diiron(III) species (29). A mixed valent form of the B2 protein of ribonucleotide reductase, which also contains an o xo-bridged dinuclear iron center, has not been observed (26), which result may imply that \( \mathcal{E}^*_{1} \) is less than \( \mathcal{E}^*_{2} \). The mixed valent form of the oxo-bridged model complex \([\text{Fe}_{2}O(O_{2}CCH_{3})_{6}(\text{Me}_{3}TACN)]^{+}\), where MeTACN is the facially capping ligand 1,4,7-trimethyl-1,4,7-triazacyclonane, is also unstable with respect to disproportionation (27). Thus the oxo-bridged models and proteins may share the common feature that \( \mathcal{E}^*_{1} \) is less than \( \mathcal{E}^*_{2} \). These observations indicate that the structure of the core has a direct influence on the redox behavior.

Addition of protein B and the reductase to the oxidized hydroxylase component blocks the transfer of electrons to the dinuclear iron core. This result accounts for the previous observations that a mixture of hydroxylase and reductase oxidizes NADH with no substrate turnover but that addition of protein B to this mixture results in NADH consumption only when substrate is oxidized (14). The same report stated that the rate of NADH oxidation without protein B was independent of substrate and occurred at only 40% of the rate in the complete system (hydroxylase, protein B, reductase, and substrate). This finding indicates that reduction of the hydroxylase is somewhat limited even without protein B. Studies of methane monooxygenase from *M. trichosporium* indicate that a large excess (17-fold equivalents) of protein B can inhibit reduction of hydroxylase in the absence of reductase (30). Our observations for the *M. capsulatus* enzyme reveal that electron transfer is completely inhibited with the addition of protein B and reductase at ratios that maximize hydroxylase activity, approximately 2:2:1 molar quantities of reductase:protein B:hydroxylase. These results, together with those from the previous studies (14), indicate that both protein B and the reductase may be necessary to inhibit efficiently reduction of the hydroxylase. This inhibition could...
either be thermodynamic, for example by alteration of the iron coordination environment in some way, or kinetic, through blocking access of reductant. The finding that the addition of protein B and reductase to the fully reduced hydroxylase does not reoxidize the enzyme implies that the inhibition of electron transfer is kinetically controlled. This explanation is attractive in view of evidence that stable complexes form among the three protein components (30). Moreover, when reductase was added to the mixed valent form of the M. trichosporium hydroxylase and component B, the characteristic EPR spectrum of the complex formed between these proteins remained unchanged (30). This result further indicates that the present failure to observe electron transfer from mediators to the hydroxylase in the presence of the reductase and protein B is a kinetic phenomenon. Because we limited our redox titration to potentials greater than \(-210\) mV (Fig. 3), no EPR signal characteristic of the reduced reductase \((g = 1.96)\) was observed.

Perhaps the most interesting discovery of the present investigation is that, upon addition of propylene to the three components of methane monoxygenase, electrons are transferred to the hydroxylase even at the highest potentials monitored, \(>150\) mV. Electron transfer into the dinuclear iron center of the complete system is thus exquisitely regulated, being allowed to occur only when substrate is present. In addition, reduction to the catalytically active Fe(II)-Fe(II) form occurs at a potential at least \(0.25\) V greater than for the hydroxylase alone. These findings are consistent with the steady-state kinetic analysis of the methane monoxygenase complex reported previously, where electron transfer was observed to occur only after substrate is bound (31). Moreover, such an increase in reduction potentials is similar to behavior seen with cytochrome P-450, where small changes in the coordination sphere of the heme iron due to the binding of the substrate can raise the reduction potential from \(-300\) to \(-170\) mV (32).

The radically different behavior of the methane monoxygenase complex in the presence and absence of substrate has several possible implications. As stated earlier, binding of protein B and reductase prevents reduction of the hydroxylase, probably kinetically. Binding of substrate to the hydroxylase not only reverses this effect but also renders the hydroxylase more electron-deficient and hence easier to reduce. This thermodynamic effect would occur only when all three components are present, and the greater driving force could explain the faster rates of NADH oxidation observed when protein B is available. The increased affinity for electrons of the hydroxylase component of the complete methane monoxygenase in the presence of substrate could be due in part to enhanced electrophilicity of the diiron(III) core through proton transfer or hydrogen bond formation involving one or more ligands in the coordination sphere. Moreover, since the hydroxylase-protein B-reductase complex (30) appears to be responsible for the inhibition of electron transfer without substrate, the enhancement with substrate might involve a conformational change at the dinuclear iron center. Experiments to test this hypothesis are currently in progress.

Regulation of electron transfer as just described would be beneficial to the organism in many ways. The obvious advantage is that, when no substrate is present, no NADH consumption takes place, therefore preventing the wasteful consumption of reducing equivalents (14). If reduction were allowed to occur in the absence of substrate, there would be a greater chance that the enzyme might be inactivated through generation of the highly reactive, high energy species required for the oxygenation reaction. From an energetic standpoint, the shift to higher reduction potentials with substrate present is consistent with the core becoming more electron-deficient. This potential shift would increase the reactivity of an activated iron core. Without protein B and reductase, such a species would be lower in energy because of the lowered reduction potentials, which could explain why single turnover experiments with the hydroxylase generally show lower yields (9). This argument implies that the higher energy activated hydroxylase iron core that is generated in the presence of protein B and reductase is required for efficient substrate oxidation. The nature of this chemical activation is under investigation.

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