Oxygen Kinetic Isotope Effects in Soluble Methane Monooxygenase*

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Soluble methane monooxygenase (sMMO) contains a nonheme, carboxylate-bridged diiron site that activates dioxygen in the catalytic oxidation of hydrocarbon substrates. Oxygen kinetic isotope effects (KIEs) have been determined under steady-state conditions for the sMMO-catalyzed oxidation of CH3CN, a liquid substrate analog. Kinetic studies of the steady-state sMMO reaction revealed a competition between fully coupled oxygenase activity, which produced glycolonitrile (HOCH2CN) and uncoupled oxidase activity that led to water formation. The oxygen KIE was measured independently for both the oxygenase and oxidase reactions, and values of 1.0152 ± 0.0007 and 1.0167 ± 0.0010 were obtained, respectively. The isotope effects and separate dioxygen binding studies do not support irreversible formation of an enzyme-dioxygen Michaelis complex. Additional mechanistic implications are discussed in the context of previous data obtained from single turnover and steady-state kinetic studies.

The soluble methane monooxygenase system (sMMO)† found in certain methanotrophic bacteria catalyzes the oxidation of methane to methanol according to Reaction 1 (1, 2).

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

Reaction 1

This reaction is the first essential step of a metabolic pathway that allows these bacteria to use methane as their sole source of energy and carbon. The soluble MMO system comprises three protein components: a 251 kDa hydroxylase (MMOH), a reductase (MMOR) that transfers electrons from NADH to MMOH, and a cofactorless regulatory protein (MMOB). Reductive activation of dioxygen and subsequent methane oxidation take place at a carboxylate-bridged diiron cluster in the active site of MMOH.

The mechanism of dioxygen activation by the reduced, diiron(II) center (H_red) in soluble methane monooxygenase (sMMO) has received considerable attention in recent years. Studies performed with sMMO from both Methylococcus capsulatus (Bath) and Methylosinus trichosporium OB3b led to the spectroscopic characterization of several kinetically competent intermediates in this reaction (Scheme 1 and Refs. 3, 4). The first spectroscopically detected intermediate after addition of dioxygen to the reduced protein has been assigned as a (μ-1,2-peroxo)diiron(III) species (H_peroxo) based on comparison of its optical and Mössbauer spectroscopic characteristics with those of structurally characterized model complexes (5, 6). H_peroxo spontaneously converts into intermediate Q, a high valent diiron(IV) species with at least one bridging oxo ligand. This intermediate has been characterized by optical, Mössbauer, and EXAFS spectroscopy (3, 4, 6, 7); however, a suitable model complex is not yet available for structural comparison. The decay rate of intermediate Q exhibits a first order dependence on the concentration of hydrocarbon substrates (3, 4, 6, 8). This observation suggests that Q, or a subsequent unobserved intermediate, is responsible for methane oxidation.

Recent kinetic studies and theoretical calculations have suggested the presence of additional intermediates (6, 9, 10). For example, proton transfer steps have been observed in the formation and decay of H_peroxo—a result consistent with a protonated peroxo intermediate in the mechanism (9). Other kinetic studies provide indirect evidence for one or more intermediates preceding the formation of H_peroxo. These may include an enzyme-dioxygen Michaelis complex or a superoxo intermediate (6, 9–11).

Kinetic and equilibrium oxygen isotope effects have been determined recently for several proteins and enzymes that activate dioxygen (12–18). Such experiments directly probe reaction steps involved in the activation of dioxygen and can provide unique mechanistic information that may not be available by other means. In this study, oxygen-18 kinetic isotope effects determined for sMMO from M. capsulatus (Bath) together with steady-state kinetics and dioxygen binding studies yield further insights into dioxygen activation by this enzyme system.

**EXPERIMENTAL PROCEDURES**

General Considerations—Growth of M. capsulatus (Bath) cells and subsequent purification of MMOH were carried out as described previously (19, 20). Both MMOB and MMOR were obtained from recombinant expression systems in Escherichia coli as described elsewhere (21). Results from iron content (3.2–3.8 Fe/MMOH) and propylene activity (220–300 milliunits per mg MMOH dimer) assays were similar

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RESULTS AND DISCUSSION

Steady-State Oxidation of CH$_3$CN Catalyzed by sMMO—
Oxygen isotope effects have been examined recently for a series of dioxygen-binding proteins and dioxygen-activating enzymes (12–18). In these experiments, the proteins or enzymes discriminate between isotopologues in both the binding or consumption of dioxygen. Prior to performing similar experiments with methane monoxygenase, it was necessary to identify a suitable hydrocarbon substrate and to characterize kinetically its sMMO-catalyzed oxidation reaction.

The apparatus for measuring oxygen isotope effects necessitated the use of a liquid hydrocarbon substrate rather than methane, the physiological substrate. Therefore, CH$_3$CN was examined as a methane substitute under steady-state oxidation conditions, with MMOH/MMOB/MMOR = 1:1:0.2 at pH 7.0 in 25 mM MOPS (26). Fig. 1 shows the turnover of NADH at acetonitrile concentrations ranging from 0–3.0 mM. The plot displays the expected hyperbolic dependence of reaction velocity versus [CH$_3$CN]. The presence of a nonzero intercept on the ordinate, however, indicated that NADH was consumed even in the absence of hydrocarbon substrate. This activity corresponds to sMMO-catalyzed oxidase activity, generating water from dioxygen (Reaction 2).

To determine directly whether such oxidase activity occurs at saturating acetonitrile concentrations, the reaction was studied by monitoring the consumption of NADH, O$_2$, and CH$_3$CN. Consumption of NADH was followed optically by UV-visible spectroscopy, and dioxygen consumption was measured by using an oxygen electrode. Consumption of CH$_3$CN was determined by gas chromatographic analysis of the reaction mixture. The enzymatic activities measured by these different methods were identical within 10% for a given CH$_3$CN concentration above 3 mM, showing that NADH oxidation is fully coupled to oxidation of acetonitrile under these conditions.

Acetonitrile was oxidized quantitatively to glycolonitrile, HOCH$_2$CN, as determined by monitoring the oxidation of [13C$_2$]CH$_3$CN by $^{13}$C NMR spectroscopy. The simplest kinetic model that can explain the competition between oxygenase and oxidase activity is shown in Scheme 2, and the corresponding rate law is given by Equation 1.

$$V = k_0[E]_T + \frac{k_2[E]_T[CH_3CN]}{K_M + [CH_3CN]} \quad (Eq. 1)$$

Soluble MMO, represented by E, can bind CH$_3$CN and subsequently oxidize it to form HOCH$_2$CN with rate constant $k_2$. Alternatively, reducing equivalents from NADH can be used in the unproductive oxidase reaction, characterized by the kinetic rate constant $k_0$. At sufficiently high CH$_3$CN concentration, all enzyme will react with CH$_3$CN, and the reduction of dioxygen by NADH is fully coupled to the oxidation of CH$_3$CN. At intermediate CH$_3$CN concentrations, NADH is consumed in both the coupled and uncoupled reactions. In the absence of hydrocarbon, only the oxidase activity is observed. The kinetic pa-
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Determination of these values permitted calculation of the 18O natural abundance kinetic isotope effect (KIE) experiments were carried out for both sMMO-catalyzed dioxygen activity in the presence of CH3CN and oxygenase activity in the presence of 4.0 mM CH3CN. Experimental NADH and dioxygen, or the nature of enzyme component interactions, all of which are crucial features in the detailed mechanism of sMMO (27). For example, the kinetic constant $K_d$ does not necessarily represent the dissociation constant for CH3CN binding. Nevertheless, the model successfully accounts for the presence of uncoupled oxidase activity, including circumstances under which hydrocarbon substrates are available below kinetically saturating concentrations. In oxygen isotope effect experiments, all reactions that consume dioxygen contribute to the final isotope effect. Thus, it was important to identify conditions under which the sMMO dioxygenase and oxygenase reactions could be separated, to facilitate interpretation of the isotope effects.

**Oxygen Kinetic Isotope Effects**—Oxygen kinetic isotope effect (KIE) experiments were carried out for both sMMO-catalyzed dioxygen activity in the presence of CH3CN and oxygenase activity in the presence of 4.0 mM CH3CN. Experimental procedures were similar to those described previously for other dioxygen-activating enzymes (12, 13, 16, 17). The reactions employed natural abundance 18O-labeled dioxygen and examined the extent of discrimination between 18O- and 16O-containing dioxygen. For each experimental data point, it was necessary to determine the fraction conversion, $f$, namely the mole fraction of the initial dioxygen consumed in the reaction, as well as the dioxygen isotopic ratio before, $R_n$, and after, $R_p$, the reaction. Determination of these values permitted calculation of the 18O isotope effect according to Equation 2 (12).

$$18\text{O} V(K) = 1 + \ln(R_p/R_n) \ln(1 - f)$$ (Eq. 2)

The data obtained from these experiments are displayed in Fig. 2, which reveals that the isotope effects are invariant with respect to the fraction conversion and that the magnitudes are 1.0167 ± 0.0010 and 1.0152 ± 0.0007 in the absence and presence of CH3CN, respectively. Within experimental error, the oxygen isotope effects are identical for both the oxidase and oxygenase reactions.

**Experimental Test for a Dioxygen-MMOH Michaelis Complex**—Rapid formation of a tight MMOH-O2 Michaelis complex has been implicated in single turnover studies that reveal no dioxygen concentration dependence on the decay of MMOHox (11). To facilitate interpretation of the oxygen isotope effects (see below), we sought direct evidence for such noncovalent binding of dioxygen to MMOH. We reasoned that dioxygen may bind in the active site or other hydrophobic cavities identified by x-ray crystallography for MMOH (28, 29). By using an oxygen electrode, we measured the change in solubility [O2] upon addition of an anaerobic solution of MMOHox to air-saturated buffer. No dioxygen uptake was observed for MMOH alone, MMOH + MMOB (1:1), or MMOH + MMOB + MMO (1:1:1); all samples yielded the same results obtained upon addition of anaerobic buffer with no protein. As a positive control, we performed the same experiment with deoxymethemerythrin, which binds dioxygen covalently with a $K_d$ of 15–30 μM (30). Nearly stoichiometric O2 uptake was observed, the decrease in [O2] corresponding to 0.8–0.9 equivalents of O2 per hemerythrin subunit. From this experiment we can establish a lower limit for dioxygen binding by MMOHox of $K_d$ ≥ 1 mM. Unfortunately, irreversible dioxygen reactivity prevents us from performing similar experiments with MMOB. We therefore cannot completely exclude the possibility that a change in the redox state of the metal center leads to a conformational change in a putative hydrophobic binding pocket and therefore to an alteration in the O2 affinity. Nonetheless, the result for MMOHox is important because it proves that such a putative nonmetal O2 binding site is not preformed in the isolated form of the enzyme and, we would argue, less likely to be present in the reduced form as well.

**Analysis of the Oxygen-18 Kinetic Isotope Effects: Constraints on the Identity of the First Irreversible Step in Dioxygen Activation**—Oxygen-18 kinetic isotope effects determined in the manner described above reflect reaction steps up to and including the first irreversible step involving dioxygen. The mechanism of sMMO has been studied previously under steady-state conditions to identify the relative sequence of substrate binding and reactivity and product release (31); however, specific insights into dioxygen activation steps have been obtained from pre-steady-state kinetics studies (3, 4, 6). Two kinetically competent intermediates, Hperoxo and Q, have been characterized spectroscopically (Scheme 1), and there is kinetic and computational evidence for additional species (6, 9–11). Possible identities of additional intermediates include an enzyme-dioxygen Michaelis complex (11), a superoxo species (10), an isomer of Hperoxo (6), and/or a protonated peroxo species (9). Inclusion of each of these proposed intermediates yields the mechanism in Scheme 3. The structure for Hperoxo is inferred from spectroscopically similar, structurally characterized model complexes (5, 6). Spectroscopic evidence strongly supports the presence of at least one oxo bridge in Q. Experimental studies have not yet established which, if any, of these steps is irreversible.

The experimental data outlined above allow us to evaluate certain features of this proposed mechanism. Formation of a tight MMOH-O2 Michaelis complex has been implicated in several single turnover studies that reveal no dioxygen concentration dependence on the formation or decay of transient inter-

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mediates (3, 4, 6, 11). In one case, it was proposed that formation of the Michaelis complex is effectively irreversible, that is in Scheme 3 $k_{-1} \gg k_{1}$ (11). Because noncovalent dioxygen binding in a hydrophobic cavity to generate H$_{red}$O$_2$ should have little effect on the O–O vibrational frequency, this step should exhibit no significant isotope effect. Consequently, this proposal is inconsistent with the present observation of a substantial oxygen-18 kinetic isotope effect.

Alternatively, the mechanism may involve pre-equilibrium, tight binding of dioxygen to form H$_{red}$O$_2$ followed by an irreversible kinetic step, that is $K_1 \gg k_{-1}$ and $k_{-1} \gg k_{2}$ (3, 4, 6, 11). We obtained no evidence for tight dioxygen binding with H$_{ox}$. It is possible that H$_{red}$ has a much higher affinity for O$_2$ than H$_{ox}$, but it would have to bind dioxygen noncovalently with affinities approaching that of covalent binding by hemerythrin to account for the experimental observations in the single turnover experiments. Even if such a noncovalent H$_{red}$O$_2$ complex is formed, its irreversible formation would not contribute to the oxygen KIE.

The magnitude of kinetic isotope effects can also provide insights into the nature of the dioxygen-activation steps (12, 16, 18). Equilibrium isotope effects (EIE) have been calculated for the reduction of dioxygen to various species based on known vibrational frequencies for dioxygen and the corresponding products (Table I, entries 1–5). Equilibrium isotope effects have also been measured for several reversible dioxygen-binding iron proteins, including myoglobin, hemoglobin, and hemerythrin (Table I, entries 6–8, and Ref. 17). Equilibrium and kinetic isotope effects are not directly comparable, however, because EIE reflect changes in bond order for oxygen between two equilibrium states, whereas KIE reflect changes in bond order between reactant and the transition state. From Table I it can be inferred that a reduction in bond order of 0.5 corresponds to a discrimination of 3% between $^{18}$O–$^{16}$O and $^{18}$O–$^{18}$O. When dioxygen reduction is accompanied by protonation or metal coordination, these effects tend to be attenuated (e.g., compare entry 2 with entries 4 and 6). The KIE of 1.015 found in the present study indicates that the bond order changes substantially in the first irreversible step, but it is not possible to identify this step based solely on the magnitude of the KIE. Nonetheless, it is interesting to note that the KIE found here is consistent with a rate-limiting outer-sphere electron transfer from iron to O$_2$, followed by rapid combination of the superoxide species to afford H$_{superoxO}$. Formation of a superoxide species has also been proposed to be the first irreversible step in the reductive activation in a number of other oxygen-activating enzymes (12, 15, 16). Recent density functional theory (DFT) calculations have identified a possible superoxo intermediate species with an energy comparable with that of H$_{superoxO}$ (10). Further work is required to characterize the early dioxygen activation intermediates, clarify the relationship between kinetic and equilibrium isotope effects, and gain additional insights into the nature of the first irreversible step in the reaction.

As described above, the isotope effects for oxidase and oxygenase activity are identical (Fig. 2). This result is similar to the isotope effects in tyrosine hydroxylase, which were not affected by changes in substrate (12). In contrast, the oxygen isotope effects measured for dopamine β-monooxygenase were sensitive to the substrate identity, suggesting that substrate plays a role in the first irreversible step (18). The identical isotope effects for the oxidase and oxygenase activities suggest that the initial dioxygen activation steps are the same for both reactions. This activated oxygen species can then react either with substrate to form a hydroxylated product or can be further reduced to yield water. Like CH$_4$ and most other hydrocarbon substrates, CH$_3$CN probably reacts with intermediate Q (6). Oxidase activity should therefore also result from reduction of intermediate Q, with MMOR being the reducing agent, because oxygenation of CH$_3$CN competes with this activity. If oxidase activity were the consequence of reduction of an earlier intermediate, no competition by CH$_3$CN would be expected. Consequently, the first irreversible step in dioxygen activation must precede the decay of Q (Scheme 3, step 6).

In summary, several new insights have been obtained about the mechanism of dioxygen activation by the reduced, diiron(II) center in sMMO from M. capsulatus (Bath). Oxygen kinetic isotope effect measurements, obtained under steady-state turnover conditions, reveal identical values for both oxidase and oxygenase reactions catalyzed by sMMO, and thus provides evidence for a common pathway of oxygen activation. The isotope effects and dioxygen binding studies do not support either the formation of an irreversible Michaelis complex between MMOH and dioxygen or a rapid reversible binding of O$_2$. Although the origin of the isotope effect is not fully understood, one plausible scenario is the irreversible formation a superoxide adduct that subsequently converts to H$_{superoxO}$. The oxygen KIE determined in this study will be valuable in calibrating computational studies on the oxygen activation pathway (10).

REFERENCES

1. Valentine, A. M., and Lippard, S. J. (1997) J. Chem. Soc. Dalton Trans. 3925–3931
2. Wallar, B. J., and Lipscomb, J. D. (1996) Chem. Rev. 96, 2625–2657
3. Lee, S.-K., Nesheim, J. C., and Lipscomb, J. D. (1993) J. Biol. Chem. 268,
