

Unmasking of Deuterium Kinetic Isotope Effects on the Methane Monooxygenase Compound Q Reaction by Site-Directed Mutagenesis of Component B

Brian J. Brazeau, Bradley J. Wallar, and John D. Lipscomb*

Department of Biochemistry, Molecular Biology, and Biophysics and Center for Metals in Biocatalysis
University of Minnesota, Minneapolis, Minnesota 55455

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Methanotrophs such as *Methylosinus trichosporium* OB3b utilize methane as the sole source of carbon and energy.¹ The first step in the metabolism of methane is the O₂ coupled oxidation to methanol which is catalyzed by methane monooxygenase (MMO). The soluble MMO system consists of three components, a hydroxylase (MMOH), a reductase (MMOR), and a small effector protein termed MMOB.² The active site of the enzyme, located in MMOH, contains a binuclear iron cluster that is buried at least 12 Å below the surface of MMOH with no access channel to bulk solvent.³ MMOB has been shown to have an effector role in several aspects of MMO catalysis.⁴ In particular, our studies have shown that MMOB accelerates the rate of the reaction between O₂ and the diferrrous MMOH cluster by 1000-fold to initiate the catalytic cycle.⁵ A later intermediate in the cycle, termed Q, has been shown to be among the most powerful oxidants in biology.⁶ It contains a bis-μ-oxo-Fe(IV)₂ cluster⁷ that reacts directly with substrates. The yellow chromophore of Q allows its formation and subsequent reaction with substrates to be followed by stopped-flow techniques. The MMO system represents one of the few cases in chemistry and the only case in biology in which the oxygen insertion reaction into unactivated C–H bonds can be directly observed.

Our recent studies suggest that the reaction between Q and substrates (S) occurs in two steps:⁸



where k_{SB} is a function of the S binding on and off rate constants and k_{CH} is the C–H bond breaking rate constant.

The rate-determining step apparently depends on the size of the substrate and the strength of the C–H bond to be cleaved. This model has been used to account for the remarkable observation that the reaction of Q with methane has a primary deuterium KIE of about 50,⁹ while the same reaction with ethane

exhibits a KIE of unity. We hypothesize that for methane, C–H bond-breaking is rate-limiting,¹⁰ whereas the combination of faster cleavage of the weaker C–H bond of ethane and its larger size makes binding the rate-limiting step, thereby masking the isotope effect. Accordingly, a product isotope effect of 4.2 is observed for ethane,¹¹ showing that there is an isotope sensitive step in the reaction even though it is not rate-limiting for the single-turnover reaction with Q.

Although the O₂ gating effect of MMOB has been well documented, it was not known until recently that it also affects rates of intermediate conversion throughout the catalytic cycle. Site-directed mutagenesis of MMOB inspired by its NMR solution structure¹² has now been used to show that several steps of the catalytic cycle, including the Q reaction, are significantly affected by this component. One mutant N107G/S109A/S110A/T111A (MMOB_{quad})¹³ resulted in a form of MMOB that accelerates the rate of the Q decay reaction with large substrates (e.g., furan) and decreases the reaction rate with the smallest substrate, methane. On the basis of these results, we proposed that the most generalized function of MMOB is to open a channel into the MMOH active site that is tuned to the size of O₂ and CH₄ so that MMOH acts as a molecular sieve for this size molecule.

Considering these two studies together, it seems reasonable that if the failure to observe KIEs for large substrates is due to slow binding and if MMOB_{quad} accelerates binding, then larger substrates may exhibit an isotope effect when MMOB_{quad} is used in place of MMOB_{wt} during a single turnover. In this study, we have investigated reaction rates and the deuterium KIEs for the reactions between Q and methane, ethane, and propane. The results are in agreement with the model in which MMOB plays a role in controlling the rate of substrate binding to MMOH in the reactive Q intermediate state and may unexpectedly address the role of H tunneling in the methane C–H bond-breaking chemistry.

Transient kinetic experiments were performed and the data analyzed as previously described.¹⁴ When diferrrous MMOH was rapidly mixed with oxygenated MMOB_{wt} or MMOB_{quad}, the formation and decay of Q was readily observed at 430 nm (see, for example, Figure 1). The formation rate constant was unaffected by mutation of MMOB and the specific substrate used. However, the Q decay rate constant in the presence of methane, was much less when MMOB_{quad} was used as the effector (Table 1). In contrast, for the reaction between ethane or propane and Q, the decay rate constants were 2 or 2.5 times greater, respectively, in the presence of MMOB_{quad}.

Isotope effects on the Q decay reaction can also be directly determined as shown in Figure 1 for the reaction with ethane or *d*₆-ethane in the presence of MMOB_{quad}. It is evident that in the presence of *d*₆-ethane the decay rate constant is slower because more Q accumulates at the maximum and the maximum occurs at a later time. Because ethane and *d*₆-ethane give identical rates of Q decay when MMOB_{wt} is used,⁸ MMOB_{quad} causes the

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(14) Single-turnover reactions were performed using an Applied Photophysics SX.18MV stopped-flow spectrophotometer, and the data were analyzed as previously described (see refs 2, 6–8). MMOH was purified from *M. trichosporium* OB3b as described in ref 2. Both MMOB_{wt} and MMOB_{quad} were overexpressed in *E. coli* and purified as reported in refs 12a and 13. Substrate (Isotec or Cambridge) concentrations were determined on the basis of solubilities from Merck Index.

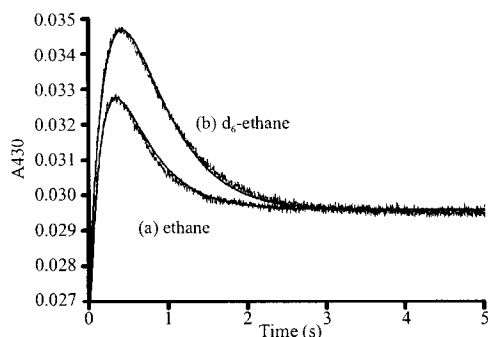


Figure 1. The **Q** time course with 15 μM MMOH, 30 μM MMOB_{quad} and 200 μM ethane at 4 °C, pH 7 (2-exp fit¹⁴ shown over the data).

Table 1. Second-Order Rate Constants and ²H KIEs for the Reaction between **Q** and Alkanes in the Presence of MMOB_{wt} or MMOB_{quad}^a

| substrate | k ($\text{mM}^{-1} \text{s}^{-1}$) | | KIE ($k_{\text{H}}/k_{\text{D}}$) | |
|--------------------------------|--|----------------------|-------------------------------------|----------------------|
| | MMOB _{wt} | MMOB _{quad} | MMOB _{wt} | MMOB _{quad} |
| methane | 14 ± 0.6 | 2.0 ± 0.02 | 42 | 6 |
| <i>d</i> ₄ -methane | 0.33 ± 0.02 | 0.33 ± 0.03 | | |
| ethane | 14 ± 1.0 | 30 ± 2.2 | 1 | 2 |
| <i>d</i> ₆ -ethane | 15 ± 1.0 | 15 ± 1.1 | | |
| propane | 0.95 ± 0.02 | 2.4 ± 0.2 | 1 | 1 |
| <i>d</i> ₈ -propane | 0.90 ± 0.03 | 2.6 ± 0.2 | | |

^a All rate constants were determined at 4 °C, pH 7.

apparent induction of a deuterium KIE for a reaction catalyzed by MMOH.

The KIEs for the reactions of **Q** with methane, ethane, and propane are summarized in Table 1. MMOB_{wt} elicits a large KIE for the **Q** reaction with methane, but none is observed for the reactions with ethane or propane. MMOB_{quad} elicits KIEs for the methane and ethane reactions but not the propane reaction. Interestingly, the KIE for methane is markedly reduced from 42 with MMOB_{wt} to 6 with MMOB_{quad}, a value consistent with classical transition-state theory.¹⁵ The KIE of 2.0 for the ethane reaction is about half the magnitude of the product isotope effect for the multiple turnover MMO system.¹⁰

Our results can be rationalized by considering the absolute rate constants together with the KIE values. The **Q** decay rate constants for the reaction with *d*₆-ethane in the presence of MMOB_{wt} or MMOB_{quad} are the same within error, indicating that the new KIE elicited by MMOB_{quad} arises from the 2-fold increase in **Q** decay rate constant for the reaction with ethane. In contrast, the reason for the decreased KIE for methane is a ~7-fold decrease in the rate constant for **Q** reaction with methane. We hypothesize that these cases provide insight into two different aspects of MMO catalysis.

Using the model of a two-step mechanism for the reaction between **Q** and substrate, the observations of (i) a large deuterium KIE for the methane reaction, and (ii) no change in the rate of the *d*₄-methane reaction when MMOB_{quad} is used suggest that the C–D bond cleavage step is rate-limiting and that binding is relatively fast when either form of MMOB is present. The opposite case is seen for the propane and *d*₈-propane reactions, in which no isotope effect is observed and the reaction rate is faster when MMOB_{quad} is used. This suggests that binding is always rate-limiting, but MMOB_{quad} facilitates the binding of this larger substrate. The intermediate case is observed for the ethane reaction in which only MMOB_{quad} elicits a rate difference upon substrate deuteration. The larger rate constant observed for the reaction of ethane in the presence of MMOB_{quad} suggests that the reaction is

Table 2. The Rate-Limiting Step in the Reaction of **Q** with Substrates in the Presence of MMOB

| substrate | MMOB _{wt} | MMOB _{quad} |
|--------------------------------|--------------------|----------------------------------|
| methane | $k_{\text{C-H}}^a$ | $k_{\text{C-H}}$ |
| <i>d</i> ₄ -methane | $k_{\text{C-H}}$ | $k_{\text{C-H}}$ |
| ethane | k_{SB} | $k_{\text{SB}} + k_{\text{C-H}}$ |
| <i>d</i> ₆ -ethane | k_{SB} | $k_{\text{SB}} + k_{\text{C-H}}$ |
| propane | k_{SB} | k_{SB} |
| <i>d</i> ₈ -propane | k_{SB} | k_{SB} |

^a Fully rate-limiting only at $T > 17$ °C. See ref 8

limited by binding in the presence of MMOB_{wt} in accord with the lack of a KIE. The observed KIE in the presence of MMOB_{quad} suggests that bond breaking becomes at least partially rate-limiting for *d*₆-ethane and possibly ethane as well. If our hypothesis is correct, then the ethane binding rate constant is at least 30 $\text{mM}^{-1} \text{s}^{-1}$. Thus, the much smaller rate constant for propane binding is consistent with the notion that MMOH acts as a molecular sieve. The effects of MMOB_{quad} are summarized in Table 2. These results strongly support a mechanism in which MMOB controls the rate of substrate entry into the MMOH active site. When this rate is increased, the reaction rate becomes more dependent on the rate of C–H bond cleavage and thus on the C–H bond strength.

If methane reacts with **Q** in a two-step process and the model for MMOB regulation described here is completely correct, then methane should react at approximately the same rate in the presence of either form of MMOB, which is not the case. One potential difference in the methane reaction with **Q** is that H tunneling may play a role and give rise to the nonclassical deuterium KIE.⁹ Recent studies indicate that protein dynamics play a key role in tunneling reactions,¹⁶ strongly implying that protein motions can facilitate hydrogen atom transfer between the H-atom donor and acceptor, methane and **Q**, respectively, in this instance. Thus, one explanation for the decreased KIE for methane with MMOB_{quad} is that tunneling is eliminated due to a change in the active site dynamics related to the MMOH–MMOB interaction which alters the approach of methane to **Q**. Spectroscopic evidence has been reported for structural changes in MMOH induced by MMOB.^{3,17} Also, we have shown that the regioselectivity of substrate oxidation changes, depending on the presence of MMOB.¹⁸ It might be expected that classical H-atom abstraction by **Q** would be less sensitive to dynamics than the nonclassical tunneling reaction and thus would be much less affected by small changes elicited by the MMOB mutation. The MMOB_{quad} mutation may, in effect, cause methane to react with **Q** in the same way as other substrates, thereby significantly decreasing its reaction rate in accord with its greater C–H bond strength.

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