J|A|C|S
COMMUNICATIONS

Published on Web 07/09/2002

Reactions of Methane Monooxygenase Intermediate Q with Derivatized Methanes

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Received April 17, 2002

Soluble methane monooxygenase, sMMO, isolated from methanotrophic bacteria such as *Methylococcus capsulatus* (Bath), catalyzes the oxidation of methane to methanol by O_2. This multicomponent enzyme system comprises three proteins, a hydroxylase (MMOH) which contains a pair of carboxylate-bridged diiron centers involved in O_2 activation and substrate hydroxylation, a cofactorless coupling protein (MMOB), and a reductase (MMOR).[1,2] In addition to methane, sMMO oxidizes an assortment of hydrocarbon substrates that range in class and size from linear and branched alkanes to substituted derivatives such as acetonitrile.[3−8] The mechanism of hydrocarbon hydroxylation by sMMO has received increased attention in recent years. As a consequence, a di(μ-oxo)diiron(IV) intermediate, Q, in the catalytic cycle has been identified as the species primarily responsible for oxidizing methane and related alkanes.[9−12] Despite this and other notable advances,[1] details of the mechanism remain controversial. Studies to probe the reactivity of Q with methane revealed a large primary kinetic isotope effect, KIE, for its reaction with CH_4 versus CD_4.[12,13] Subsequent experiments carried out with native proteins from *Methylosinus trichosporium* OB3b and substrates larger than methane, namely, ethane and propane, afforded KIEs of unity.[11] We report here surprising and unanticipated results of reactions between Q and a series of methane derivatives, which provide new insight into hydrocarbon oxidation by Q.

The reactivity of Q with substrates of general formula CH_3-R, where R = CN, NO_2, and OH, and their deuterated analogues was probed by double-mixing stopped-flow spectrophotometry as reported previously.[12] For comparison, the reactivity of Q with methane and ethane was reinvestigated. This study yielded two key findings. First, we observe significant KIEs in reactions of Q with both acetonitrile and nitromethane, respectively. Second, we detect a substrate-binding step that occurs prior to oxidation, as revealed by the hyperbolic dependence of k_{obs} on substrate concentration, [S]. Although this type of binding step has previously been proposed,[11] no evidence for it has been reported.

Single-turnover kinetics experiments were performed on a Hi-Tech Scientific (Salisbury, UK) SF-61 DX2 stopped-flow spectrophotometer as described in detail elsewhere.[9,12] In brief, intermediate Q is generated by rapidly mixing fully reduced anaerobic hydroxylase, $MMOH_{red}$, in the presence of 2 equiv of MMOB with O_2-saturated buffer in the initial push of a double-mixing experiment. After a specified time delay that coincides with the maximization of Q, substrate-containing buffer is introduced in a second push to initiate both the reaction and data collection. Reactions were monitored by following the disappearance of Q at 420 nm, pH 7. Data collection and analysis were performed by KinetAsyst 3 software supplied by the instrument manufacturer. The data were fit to rate constants for single-exponential decay.

For methane and ethane, the optical signal of Q decayed with a first-order dependence on [S]. The second-order rate constants for each of these reactions, obtained from the slope of respective k_{obs} versus [S] plots (Figure S1), are presented in Table 1. A comparison of second-order rate constants obtained at 20 °C for the reaction of Q with CH_4 and CD_4 reveals a KIE, k_H/k_D, of 23.1. This value agrees with that of ~28 at 4 °C previously reported for this enzyme.[12] The presence of a KIE is consistent with a mechanism in which the rate-determining step involves C−H bond cleavage. Similar analysis of data from the reaction with ethane reveals the absence of a KIE, implying that C−H bond activation is no longer the rate-limiting process in this reaction. The latter result is equivalent to one first obtained in a similar study of the *M. trichosporium* OB3b enzyme.[11]

The second-order rate constants obtained for reactions of Q with methanol and, from the linear portions of k_{obs} versus [S] plots of acetonitrile and nitromethane, indicate that these reactions are considerably slower than the corresponding reaction with CH_4, despite weaker C−H bond strengths. It is clear from the magnitude of the KIEs that H-atom abstraction is the rate-limiting process in reactions with acetonitrile and nitromethane. For methanol, a KIE of unity was obtained, and k_{obs} exhibits a linear dependence on [S] over the range utilized (Figure S1). This situation contrasts with that for nitromethane and acetonitrile, where the experimental data were well fit to the kinetic model in eq 1.

$$Q + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} QS \xrightarrow{k_2} MMOH_{ox} + \text{product} \qquad (1)$$

The observed rate constant for Q decay is described in eq 2.

$$k_{obs} = \frac{k_2[S]}{k_{-1}/k_1 + [S]} \qquad (2)$$

Substrate binds reversibly to Q, forming a complex, QS, which in turn can react to afford oxidized product. At high [S], Q exists almost entirely as the enzyme−substrate complex. In this limit, k_{obs}, which is independent of [S], becomes equivalent to k_2. The parameter k_2 was obtained by fitting data in Figures 1 and S1 to eq 2; the results are provided in Table 1.

Before discussing our results, we first consider a model proposed by Brazeau et al. to describe the reactions of Q with substrates.[14] The model postulates a two-step mechanism with substrate binding and C−H bond cleavage as the rate-limiting processes. Furthermore, they suggest that the role of MMOB is to control the rate of substrate entry into the active site of MMOH, which acts as a molecular sieve tuned to accommodate substrates of the size of CH_4 and O_2. Consequently, in reactions with substrates larger than methane, substrate binding becomes rate determining. Although this

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10.1021/ja0265759 CCC: $22.00

Table 1. Second-Order Rate Constants and KIEs for the Reaction of Intermediate Q with Substrates[a]

substrate	$k \times 10^{-2}$, $M^{-1}\,s^{-1}$	k_2, s^{-1}	KIE, k_H/k_D
CH_4	287 ± 9		23.1 ± 1.1
CD_4	12.4 ± 0.5		
C_2H_6	265 ± 11		1.00 ± 0.04
C_2D_6	266 ± 3		
CH_3CN		282 ± 10	46.4 ± 2.3
CD_3CN		6.06 ± 0.14	
CH_3NO_2		5.34 ± 0.02	8.1 ± 0.2
CD_3NO_2		0.66 ± 0.02	
CH_3OH	7.27 ± 0.06		1.01 ± 0.01
CD_3OH	7.19 ± 0.04		

[a] Experiments were performed at pH = 7 and 20 °C.

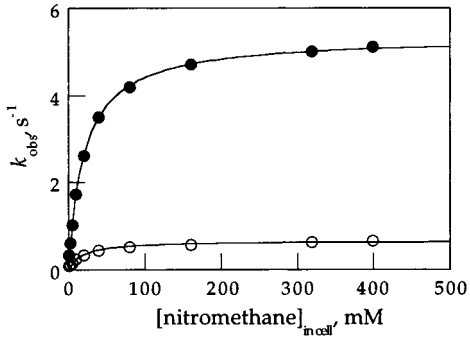

Figure 1. Plot of k_{obs} versus nitromethane concentration for the decay of Q at pH = 7 and 20 °C. Solid circles represent CH_3NO_2, and open circles represent CD_3NO_2.

model can account for results from the ethane experiment, it fails to explain the data from experiments with acetonitrile and nitromethane, both of which, although larger than methane, exhibit large KIEs in their reactions with Q.

So how do we rationalize our intriguing results? There are at least three possibilities for rate-limiting processes in the reaction of Q with substrate: (i) diffusion of substrate from solution to the active site, (ii) H-atom abstraction, and (iii) diffusion of product from the active site. Since we monitor Q consumption, process (iii) can be eliminated as a possible rate-limiting step, leaving two options. Quantum mechanical calculations establish that, for the reaction of Q with ethane, the activation barrier for H-atom abstraction is ~2−3 kcal/mol lower than that for methane.[15] We propose that lowering this barrier shifts the rate-determining step from C−H bond cleavage to diffusion of substrate to the active site, which is buried in the protein interior, thus accounting for the lack of a KIE. If this explanation is correct, then, on the basis of the magnitude of the absolute rate constants for the reactions of Q with CH_4, C_2H_6 and C_2D_6, we can conclude that for these hydrocarbons the activation energies for the two processes are fortuitously similar.[16]

Like ethane, the polar substrates have weaker C−H bond strengths than methane and would therefore be expected to have lower activation barriers for C−H bond activation. On the basis of the preceding argument for ethane, no KIEs are expected for reactions with these substrates. Instead, despite somewhat sluggish reactions, we see substantial KIEs with two of the three polar substrates. The explanation for these slow reactions requires an examination of the complete reaction pathway. For all three substrates, unlike methane and ethane, the free energy of aqueous solvation is negative. Thus, the energy of the solvated states lie below those of the gas phase, making it harder to extract the molecules from solution. The net result is a slower reaction rate because of the increase in the overall activation barrier for each reaction, as monitored by the kinetic activation parameters (Table S1). The KIEs for nitromethane and acetonitrile further require that the diffusional barrier for the polar substrates is lowered relative to that in ethane, thus rendering H-atom abstraction rate limiting. Methanol, however, does not exhibit a KIE, although one would expect its diffusional barrier to be similar to that of acetonitrile and nitromethane. We hypothesize that this result arises from a unique ability to form favorable electrostatic interactions through H-bond donation; that is, interactions of this kind presumably stabilize the transition state enough to lower its activation barrier below that of diffusion, which shifts the rate-limiting step from C−H bond cleavage.

In conclusion, the present results add significant insight into the reaction of MMOH intermediate Q with substrates. The substrates fall into two well-defined categories (Table 1), those for which the KIE is large and those for which it is essentially nonexistent. In the former group, the magnitude of the KIE varies between substrates but in all cases is greater than or equal to the baseline value computed from classical transition-state theory. A reasonable hypothesis is that the variations in the size of the KIE are due to differential tunneling effects, the details of which are being addressed in ongoing theoretical work. When H-atom abstraction is no longer the rate-determining step in hydroxylation, a plausible candidate is the diffusion of substrate from water into the protein interior. Phenomenologically, the barrier height for such a reaction for methane must be smaller than that for H-atom abstraction. For ethane, the experimental data reveal that the free-energy barrier is approximately equal to that for H-atom abstraction for methane—on the order of 18 kcal/mol—since the rate constants for the two substrates are nearly equal, although the entropy/enthalpy components are very different. For the polar substrates, the effects of favorable hydrogen-bonding interactions with water and the protein must be taken into account. Confirmation of the proposals stated above will, however, require extensive computations at an atomic level of detail.

Acknowledgment. This work was supported by NIH Grants GM32134 (S.J.L) and GM40526 (R.A.F). E.A.A. is a Martin Luther King Jr. visiting scholar at MIT.

Supporting Information Available: Figure S1 displaying information described in the text; Figure S2, Eyring plots for Q decay in the presence of each substrate; and Table S1 containing activation parameters for the decay of Q in the presence of substrates (PDF). This information is available free of charge via the Internet at http://pubs.acs.org.

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(16) The second-order rate constants for the "diffusion-limited" reaction with the ethanes are low compared to others reported for diffusion of substrates to surface-exposed enzyme active sites. A reviewer suggests that this higher than normal free-energy barrier to diffusion may be a consequence of protein fluctuations required to admit ethane into the active-site cavity and which methane is not expected to experience.

JA0265759